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ABSTRACTS

European Muscle Conference 2015 Abstracts

Young Scientist Session

Oral presentations

Differential expression of heme oxygenase-1 in satellite cells and muscle of *mdx* mice: effect on microRNAs

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Introduction: Muscle injury during progression of Duchenne muscular dystrophy (DMD) is associated with chronic inflammatory response which impairs muscle regeneration. Heme oxygenase-1 (*Hmox1*) is crucial anti-inflammatory, cytoprotective enzyme degrading heme to ferrous ions, carbon monoxide and biliverdin. Surprisingly, *Hmox1* role so far has not been elaborated in DMD, despite the fact that inflammation and heme released from injured muscle are strong inducers of *Hmox1*. Interestingly, recently we found that *Hmox1* influences murine skeletal myoblasts differentiation, attenuating the expression of myogenic regulatory factors and myomirs (miR-1, miR-133a, miR-133b, miR-206), while increasing mir-146a (Kozakowska et al., *Antioxid Redox Signal*, 2012). Therefore, we decided to elucidate the role of *Hmox1* in mice model of DMD.
Results: In gastrocnemius muscle of male *mdx* mice (C57BL/10ScSn-Dmdmdx/J; Jackson Lab) the *Hmox1* expression started to be significantly induced from 8 week of life and persisted till one year in comparison to wild-type controls. In contrast, the expression of miR-1 and miR-133a/133b were downregulated. However, miR-206, was significantly higher already at 2 weeks old *mdx* mice than in WT animals and steadily increased by even 6–7 fold at 12, 24 and 52 weeks. Inflammation in *mdx* mice is associated with upregulation of NF-κB, what was observed in 6 weeks old mdx mice. At this time point also miR-146a, a target of NF-κB, was upregulated and persisted in older mice. In hind limb muscles of 8 and 52 weeks old *mdx* mice lower number of satellite cells (mSCs; CD34 + α7integrin +) was detected. Surprisingly, these cells had reduced expression of *Hmox1* with concomitant upregulation of miR-206. However, FACS analysis suggest that the main cell population expressing *Hmox1* in muscles are myeloid cells.
To further investigate the role of *Hmox1* in muscular dystrophy the 6 weeks old WT and *mdx* mice were treated for two weeks with SnPPIX (10 mg/kg, every other day), the inhibitor of *Hmox1* activity. Increased level of lactate dehydrogenase (LDH) and creatine kinase (CK) in plasma of *mdx* mice treated with SnPPIX indicate higher muscle injury when *Hmox1* was inhibited.
Additionally, the double KO mice (*mdx* × *Hmox1* −/−) generated in our laboratory were weaker than WT or *mdx* mice as determined by treadmill test. Up-regulated level of LDH and CK in plasma of double KO mice once again indicate higher muscle injury when there is no active *Hmox1*. Moreover in gastrocnemius muscle of 12 weeks old double KO mice there was elevated expression of miR-146a, indicating for the pronounced inflammation.
Conclusions: Obtained data suggest, that *Hmox1* induction is strongly associated to the muscle inflammation, evidenced by miR-146a induction, by NF-κB upregulation and downregulation of miR-1 and miR-133 myomirs. Interestingly, reduced expression of *Hmox1* in satellite cells of *mdx* mice may be associated with miR-206 upregulation, potentially leading to earlier satellite cells exhaustion. However, it still remains to be established whether presence of *Hmox1* in the muscles is playing a protective or detrimental role in DMD.
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Whole exome sequencing strategy in studies on limb girdle muscular dystrophy and myofibrillar myopathy

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Limb-girdle muscular dystrophy (LGMD) is a heterogeneous group of genetic disorders, characterized by proximal distribution of muscle weakness. Myofibrillar myopathy (MFM) affects additionally distal

muscles and although has more specific morphologic features on muscle biopsy, it may be considered a form of LGMD. So far over 30 different *loci* associated with the diseases have been identified. Novel genes are still to be discovered as up to 50 % clinical cases are left without genetic diagnosis.

To make differential diagnosis fast and reliable, as well as to find novel genes involved in the LGMD and MFM we have adapted Whole Exome Sequencing (WES) approach. However finding the causal mutations among thousands of private variants identified using WES poses a big challenge. In our workflow variants filtering and prioritization was based on the mutation effect, dbSNP minor allele frequency and in-house database comparison. Further interpretation of filtered variants was based on putative influence on biochemical and signaling pathways, predicted pathogenicity, and genotype-phenotype correlation.

So far we have analyzed DNA samples from 34 LGMD patients, and in 21 cases we found mutations in the genes already associated with LGMD: *CAPN3*, *DYSF*, *FKRP*, *ANO5*, *PLEC1*, *LMNA* and *SGCA*. Two patients had more than one known gene affected (*CAPN3* + *DYSF* and *LMNA* + *PLEC1*). Additional variants in genes related MFM and other muscle diseases were also found (*TTN*, *DES*, *COL6A2*, *COL6A3*, *LDB3/ZASP*, *RYR1*, *LMNA*, *LARGE*, *NEB*, *FLNC*). In some cases those might be causative mutations, in some those might modify the clinical phenotype.

Genetic diagnosis was possible more often in LGMD subgroups clinically determined as dysferlinopathies and calpainopathies with 15 out of 20 patients harboring pathological variants in known genes. In the group of 14 patients with less typical phenotype causative mutations were found only in three cases.

The study shows that exome sequencing can be applied as a single genetic test to reliably characterize the comprehensive spectrum of genetic variants. At present it can provides a genetic diagnosis in 50 % of patients with LGMD/MFM. We expect that further bioinformatics analyses can lead to discovery of new genes involved in the disease.

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Session 1: Acto-Myosin Interactions

Oral presentations

Unusual myosin lever arms optimize trafficking in different actin environments

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Both myosin VI and myosin X have been proposed to have unusual components of their lever arms, as compared to myosin V, including stable, single alpha helical (SAH) domains. Furthermore the nature of where and how dimerization takes place in these myosin VI and X, and thus how the lever arms are terminated and positioned, has been the subject of debate. For myosin VI, we deleted a stable, single α-helix (SAH) domain that has been proposed to function as part of a lever arm to amplify movements, without impact on in vitro movement or in vivo functions. A myosin VI construct that used this SAH domain as part of its lever arm was able to take large steps in vitro, but did not rescue in vivo functions. It was necessary for myosin VI to internally dimerize at a position proximal to the SAH domain, triggering unfolding of a three-helix bundle and calmodulin binding in order to step normally in vitro and rescue endocytosis and Golgi morphology in myosin VI-null fibroblasts. In the case of myosin X, a new structure provides additional evidence that the a component of what has been identified as an SAH domain does indeed extend the lever arm, while the distal part of this region forms an anti-parallel coiled coil, as has been previously proposed. Creating zippered dimers that permit this geometry reveals that myosin X can take larger steps on bundles than on single actin filaments, suggesting that the combination of unusual lever arm and anti-parallel geometry may be necessary for optimizing myosin X movement on actin bundles.

The aim of this work is to incorporate cooperativity into Huxley-type cross-bridge model in thermodynamically consistent way.

Design of a high throughput screening: disrupt the super relaxed state of myosin to cure obesity and diabetes

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Myosin is an abundant ATPase protein. It is estimated that 10 % of muscle tissues weight is myosin. Due to its abundance, myosin can be a good target to raise basal metabolic rate in animals.

A new low-ATP-consumption state of myosin has recently been proposed (1). This new state has been called the "Super Relaxed State (SRX)" of Myosin. Structural evidences of the SRX state are recently been published as a close complex formed by the two myosin heads (2). It is characterized by an ATPase time constant in the order of 300 s versus the 15 s for the so called "Disordered Relax State" (DRX)(3). The idea is that behind that large number of "dormant" ATPases, there is the key to raise basal metabolism in a physiological way. The amount of myosin in the SRX state is estimated to be approximately 60 % of the total. The equilibrium between SRX state myosins and DRX state myosins is cooperativeness-driven. Controlling this equilibrium may lead to an increase in basal metabolism that would consume additional energy up to 1000 kcal/day. We studied the effect of several Regulatory Light Chain mutants on the SRX state and we applied this information on the development of a high throughput screening. We are searching a molecule that is able to inhibit the SRX state in skeletal muscle fibers. We screened 2000 molecules of an FDA approved library and we found a potential leading compound.

References:

1. Cooke et al. (2011) *J Mol Biol.*
2. Padrón et al. (2008) *J Mol Biol.*
3. Stweart et al. (2010) *Proc Natl Acad Sci.*

Posters

The effect of the E139del mutation in TPM2 gene on movement of β-tropomyosin strands and the response of actin in the thin filaments during the ATPase cycle

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We have investigated how the congenital myopathy-causing mutation E139del in β-tropomyosin (TM) affects TM's position on actin filament and the spatial arrangement of actin subunits during the ATPase cycle in the absence and presence of troponin. We labelled recombinant wild-type and mutant E139del TMs with 5-IAF and F-actin with FITC-phalloidin and incorporated them into ghost muscle fibres. The reconstructed thin filaments of the fibres were decorated by myosin S1 and polarized fluorescence was measured at different stages of the ATPase cycle. It was found that the position of wild-type TM was correlated with the conformational state of actin at all the intermediate stages of the ATPase cycle. At transition from the weak-binding to the strong-binding actomyosin states actin monomers turned to the filament periphery and the TM strands moved towards the inner domains of actin subunits. The E139del mutation kept the TM strands near the inner domains of actin but decreased the number of the switched on actin monomers throughout the cycle. Troponin ($\pm$ Ca^{2+}) dramatically changed the position of the wild-type and mutant TMs during the ATPase cycle. At low Ca^{2+} actin monomers turned to the filament center, whereas TM strands moved towards the outer domains of actin. On the contrary, at high Ca^{2+}, actin monomers turned to the filament periphery and the TM strands moved towards the inner domains of actin. The E139del mutation stabilized the TM strands near the inner actin domains both at low and high Ca^{2+} and increased the proportion of the switched on and off actin monomers at high Ca^{2+} and at low Ca^{2+}, respectively. Thus troponin keeps its ability to switch actin monomers from the off to the on state in thin filaments containing the E139del mutant TM. The aberrant movement of the E139del TM causes abnormal response of the contractile system that may result in muscle weakness observed in patients with the E139del mutation.

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Strained myosin-actin cross bridges with bound phosphate reattach extremely rapidly

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It is generally thought that unloaded shortening followed by rapid stretch during the *ktr* procedure detaches all cross bridges so that the recovery of force following the end of stretch starts from a baseline of zero force [1, 2]. However, in practice, there is frequently an appreciable force at the end of the stretch. This most likely represents cross bridges that have reattached at some point during the shortening or stretch. We have noticed that the force at the end of the stretch is greater in the presence of inorganic phosphate (Pi) and present here results for a range of Pi concentrations.

Permeabilized rat soleus fibres were prepared as previously described [3] and activated with pCa 4.5, at 15 °C, either with or without added Pi, keeping the ionic strength constant by adjusting the content of KCl. The *ktr* consisted of a 20 % release followed, after 15 ms, by a rapid stretch back to the original length (lo).

Isometric force declined with increasing concentrations of Pi but at the same time the force at the end of the stretch progressively increased. The time course of the subsequent recovery of force was almost identical in the absence and presence of Pi and the maximum velocity of shortening derived from the force–velocity relationship was similar, if not slightly faster, in the presence of Pi.

It is most unlikely that any cross bridges still attached after the unloaded shortening would survive a stretch of 20 % lo, implying that attachment and turnover must have occurred several times during the stretch, a time of no more than 1–2 ms. The presence of Pi shifts the cross bridge states towards an increasing proportion of relatively low force bridges with bound Pi (AM.ADP.Pi) and the fact that the force after a stretch increases with increasing Pi concentration implicates this type of cross bridge intermediate. Pi does not speed the recovery of force after the end of the stretch so a high proportion of AM.ADP.Pi cross bridges in the isometric state does not increase turnover. Consequently, it appears that the very rapid attachment and turnover of cross bridges during stretch is a function of M.ADP.Pi released from strained AM.ADP.Pi cross bridges.

References:

1. Brenner B, Eisenberg E (1986) *Proc Natl Acad Sci USA* **83**: 3542–3546.
2. Metzger JM, Moss RL (1990) *J Physiol* **428**: 751–764.
3. Gilliver SF et al. (2011) *Exp Physiol* **96**: 171–178.

Novel non-fluorescent, non-phototoxic and non-cytotoxic blebbistatin derivatives

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Blebbistatin, the most popular myosin II inhibitor, is a well-characterized tool for investigating the biological roles of myosin II isoforms. Despite the widespread use of blebbistatin, its application is substantially hindered by its adverse characteristics. On one hand, it has serious side effects as its blue-light sensitivity causes phototoxicity and photoconversion of the molecule, and even in the absence of light it is cytotoxic at commonly applied concentrations. On the other hand its strong fluorescence make imaging experiments increasingly difficult because blebbistatin forms insoluble highly fluorescent aggregates adhering to several types of surfaces and affecting the viability of model organisms. Recently we discovered that a nitro-substitution at C15 position greatly improves the characteristics of blebbistatin, as the substitution increases its stability, reduces its fluorescence and eliminates toxic side effects without affecting the myosin II inhibitory properties. The non-fluorescent, non-phototoxic, non-cytotoxic para-Nitroblebbistatin has already become a successful replacement for blebbistatin with identical inhibitory properties, tested by independent researchers on several model organisms.

The Arg167His and Lys168Glu mutations in α-tropomyosin (Tpm1.1) disrupt the regulation of actin-myosin interaction during the ATPase cycle

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Previous analyses showed that mutations in TPM1 gene, which resulted in two substitutions: Arg167His and Lys168Glu, in α-tropomyosin (Tpm1.1), caused severe dysfunctions in Ca-dependent activation of actin-myosin ATPase. The goal of the present study was to investigate the effects of the mutations on the orientation of troponin-tropomyosin complex on actin filament at different stages of the actomyosin ATPase cycle.

The recombinant wild-type and mutant Tpm1.1, myosin subfragment-1 (S1) and F-actin were labeled respectively with 5-IAF, 1,5-IAEDANS and FITC-phalloidin. The fluorescently labeled proteins were incorporated into ghost muscle fibres and used for the analysis of polarized fluorescence. The data allowed us to estimate the extend of Ca^{2+}-induced shift in the position of the wild-type and mutant Tpm1.1 on the filament, the fraction of actin monomers switched into the 'on' state and the fraction of myosin S1 bound in rigor.

At low Ca^{2+} the substitution Arg167Gly shifted tropomyosin strands towards inner domain of actin and the fraction of the actin subunits in the 'on' state was increased as compared to the wild-type tropomyosin. In the presence of Ca^{2+} this mutation caused a shift of tropomyosin towards outer domain of actin and did not change the fraction of the actin subunits in 'on' state. This tendency was maintained throughout the ATPase cycle, resulting in inhibition of strong-binding state of actomyosin. This indicates that the mutations Arg167Gly in Tpm1.1 affected troponin's ability to switch off actin monomers at low Ca^{2+} and to activate the cross-bridges at high Ca^{2+}.

In contrast, tropomyosin carrying the substitution Lys168Glu was shifted towards the outer actin domain at both low and high Ca^{2+}. The fraction of the actin subunits in 'on' state did not change at high Ca^{2+}, while the amount of the myosin heads strongly bound to F-actin decreased at high Ca^{2+}, but increased at low Ca^{2+}, showing that troponin lost its ability to fully activate the cross-bridges at high Ca^{2+} and to deactivate them at low Ca^{2+}. The data indicate that both mutations disrupt the regulatory functions of troponin.

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Fluorides as nucleating factors for actin polymerization

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Actin specifically cleaved between Gly42 and Val 43 within the DNase-I-binding loop with ECP32/grimelysin or protealysin cannot polymerize unless it contains Mg^{2+} as a tightly bound cation (Khaitlina et al., 1993; Tsaplina et al., 2009). However, polymerization of the Mg-bound cleaved actin is slow because the nucleation step of its polymerization is strongly inhibited. Previously we have shown that AlF_4 promotes nucleation of protealysin cleaved (Pln) Mg-G-actin even in the absence of 0.1 M KCl (Tsaplina et al., 2012). AlF_4 is unstable in solution; therefore it is usually formed upon incubation of actin solution with NaF followed by addition of $AlCl_3$. Here we show that NaF also accelerates polymerization of Pln-actin and compare the effects of NaF on the properties of Pln-actin with those produced by AlF_4.

Pln-Ca-actin was obtained by the cleavage of rabbit skeletal muscle actin with recombinant protealysin and transformed into Mg-actin by incubation with 0.2 mM EGTA/0.1 mM $MgCl_2$. Similarly to the effect of AlF_4, the presence of 5 mM NaF abolished the long lag phase in the time course of Pln-actin polymerization suggesting stabilization of nuclei formed at this initial step of polymerization. However, in contrast to the effect of AlF_4, NaF accelerated polymerization of Pln-Mg-actin only in the presence of 0.1 M KCl. Also in contrast to AlF_4, NaF did not inhibit the enhanced steady-state ATPase activity of F-actin assembled from the cleaved monomers and did not increase the amount of protein recovered in the pellet upon Pln-F-actin ultracentrifugation. To determine whether binding of AlF_4 and NaF is accompanied by conformational changes within actin subdomain 2 we have probed accessibility of actin subdomain 2 to proteolysis with trypsin and protealysin. Both AlF_4 and NaF inhibited proteolysis of G-Mg-actin with trypsin at Lys 68 within the nucleotide cleft indicating the cleft narrowing, although the inhibitory effect of NaF was much smaller than that of AlF_4. AlF_4 also diminished accessibility of the cleavage site Gly42-Val43 within the DNase-I-binding loop to protealysin, whereas NaF did not produce any effect. On the other hand, both AlF_4 and NaF inhibited the latter cleavage in F-Mg-actin. These data suggest that, similarly to AlF_4, NaF promotes association of Mg-containing actin monomers due to conformational changes within the nucleotide cleft that tighten the intermonomer contacts involving the DNase-I-binding loop.

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The role of isoforms of the thin filament proteins in atrial contractility studied with the in vitro motility assay

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Contractile function of the heart depends on the pattern of contractile and regulatory proteins in cardiomyocytes. Changing of the conditions of heart functioning is accompanied by a change in the isoform composition of cardiomyocytes proteins.

We studied the role of tropomyosin (TM) and α-actin isoforms in functioning of atrial myocardium. For this effect of TM with different content of α- and β-chains on Ca^{2+}-regulation of acto-myosin interaction was investigated using in vitro motility assay with cardiac (*ca*A) and skeletal (*sk*A) α-actin isoforms.

We studied a dependence of the sliding velocity of the thin filaments on Ca^{2+} concentration and parameters of the Hill equation of '*p*Ca-velocity' relationship. αβ-TM and *sk*A were prepared from *m. psoas* of the rabbits. Troponin, *ca*A, αα-TM and atrial myosin were extracted from rabbit hearts. TM extracted from rabbit myocardium was an α-chain homodimer. TM from rabbit skeletal muscle contained 40 % β-chain. Myosin from atria had α-heavy chain and atrial light chains. Regulated thin filaments were reconstructed from F-actin, troponin and TM according to Gordon et al. [1].

We found that α/β-chain ratio of TM did not affect maximal velocity of the regulated thin filaments. Ca^{2+} sensitivity of the '*p*Ca-velocity' relationship (pCa_{50}) did not depend on the α/β-chain ratio of TM with *sk*A (6.83 and 6.80 for αα-TM and αβ-TM, respectively). However, with *ca*A Ca^{2+} sensitivity was higher for αα-TM as compared to αβ-TM (7.35 and 6.76, respectively). Earlier we have shown

that β-chain of TM increased calcium sensitivity of the '*p*Ca–velocity' relationship for V3 isomyosin with both *sk*A and *ca*A [2], similar to the results previously obtained for cardiac muscle [3]. It however decreased calcium sensitivity of the relationship for V1 isomyosin with *ca*A [2]. Therefore, β-chain of TM decreased calcium sensitivity of the '*p*Ca–velocity' relationship for myosin with α-heavy chain.

Thus, isoforms of α-actin, TM and myosin heavy chains affect calcium regulation of acto-myosin interaction in myocardium that may play a role in maintenance of the effective functioning of the heart muscle both during ontogenesis and in pathological conditions.

References:

1. Gordon A et al. (1997) *Biophys J* **72**: 1295–1307.
2. Kopylova G et al. (2015) *J Muscle Res Cell Motil* **36**: 108.
3. Muthuchamy M et al. (1998) *J Mol Cell Cardiol.* **30**: 1545–1557.

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Modelling the oscillatory behaviour of Actomyosin complex in muscle contraction

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Muscle contraction is generated by cyclical interactions between actin and myosin, which can generate force and/or displacement through the formation of the actomyosin complex. The actomyosin complex is described in detail in its stable configurations, usually indicated as one "pre-power stroke" and one or more "post power stroke" states, but wether in these configurations it is rigidly linked to the conformation observed in cryomicroscopy or it has some degree of thermal oscillations comparable to the length scale of the power stroke, is not well know and is usually not taken into consideration in mathematical modelling.

In this work we analyse, by means of mathematical models, the implications that this intimate property of the actomyosin complex can have on muscle contraction at the single cross bridge, the single fiber and the whole organ levels. We define a comprehensive model of the cross bridge cycle for three different hypotheses on the minima of the actomyosin energy landscape: an infinitely steep minima with a high energy barrier in the post power stroke state, as proposed in [1], without the high energy barrier as considered in [2] and a finite curvature in the minima as proposed in [3].

These three scenarios are included in a realistic sarcomere/fiber model and, at an higher level, into a whole heart simulator developed at the University of Tokyo (UT Heart). We observed that the wider minima allow the lever arm to explore all the possible states in the energy landscape dynamically during the life of the attached state, while the steeper minima generate fewer power stroke and repriming events within the single attachment-detachment event. As a result, it is shown that the dynamical oscillations generate a more efficient contraction mechanism, with a higher force sustained by a lower number of attached cross-bridges. This features, observed at the single fiber level, is also preserved in the macroscopic level, generating a higher efficiency in the simulation of the cardiac function.

References:

1. Huxley A, Simmons R (1971) Proposed mechanism of force generation in striated muscle. *Nature* 533–538.
2. Smith D, Sleep J (2006) Strain-dependent kinetics of the myosin working stroke, and how they could be probed wit optical-trap experiments. *Biophys J* 3359–3369.
3. Marcucci L, Truskinovsky L (2010) Mechanics of the power stroke in myosin II. *Physical Rev E.*

Mutations in the central part of tropomyosin affect the flexibility of thin filament

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Although tropomyosin (Tpm) mainly contains residues characteristic of the 'canonical' heptad repeats which stabilize the coiled-coil dimer of two α-helices, it has several non-canonical residues which are thought to partially destabilize the dimer. Recently, it has been shown that substitutions of two non-canonical residues, G126 and D137, located in the central part of the Tpm molecule by canonical ones change properties and the regulatory function of the thin filaments containing corresponding Tpm mutant (Sumida J et al., 2008, *J Biol Chem* **283**: 6728–6734; Nevzorov I et al., 2011, *J Biol Chem* **286**: 15766–15772; Shchepkin D et al., 2013, *Acta Naturae* **5**: 126–129; Matyushenko A et al., 2014, *FEBS J* **281**: 2004–2016).

We used a two-beam optical trap to test the effect of the stabilizing mutations in the central part of the Tpm molecule on the bending stiffness of reconstructed thin filaments (consisting of F-actin, Tpm and troponin complex). The following samples were used: F-actin and Tpm mutants on the base of recombinant human Tpm1.1 isoform 1, C190A, D137L/C190A, G126R/C190A, and G126R/D137L/C190A (Matyushenko A et al., 2014, *FEBS J* **281**: 2004–2016). As two continuous Tpm chains reinforce the actin filament, one would expect the total bending stiffness of the reconstructed filament to change in a measurable way upon changes in the Tpm bending stiffness.

A measuring dumbbell-like probe was formed by a filament segment attached to two beads 1 mm in diameter hold by the two optical traps. One trap was static and held a bead used as a force transducer while an acousto-optical deflector moved the beam holding the second bead, causing stretch of the dumbbell. The distance between the beads was measured using image analysis of micrographs. An exact solution to the problem of bending of an elastic beam attached to two beads and subjected to a stretch was used for analysis of the displacement-force data.

We showed that substitution of non-canonical residues in the central part of Tpm with canonical ones, G126R and D137L, and especially their combination, caused an increase in the bending stiffness of the reconstructed thin filaments. The data confirm that the effect of these mutations on the regulation of the actin-myosin

interaction may be caused by an increase in the tropomyosin bending stiffness.

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The essential role of activation loop in effective muscle contraction: physiological, structural and mechanistic insights

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We found that the 'activation loop', a conserved actin-binding region of myosin interacts with the N-terminal segment of actin. This interaction accelerates the movement of the relay, stimulating myosin's ATPase activity, which we demonstrate by in vitro and in vivo approaches using transgenic *Caenorhabditis elegans* strains. The interaction between the activation loop and actin results in efficient force generation, but it is not essential for the unloaded motility. In order to reveal the structural background of actin activation we modelled in silico the weak binding actomyosin in the initial powerstroke state by docking an actin trimer to prepowerstroke myosin. After the docking, during the relaxation by a 100-ns long unrestrained molecular dynamics we found that actin binding induced an extra primed myosin state. In this extra primed state the myosin lever turned by 18° further 'up' and the switch 2 loop is further closed compared to the crystal structure of prepowerstroke myosin. The existence of the extra primed myosin state was also confirmed in single molecule measurements with interferometric scattering microscopy (iSCAT).

Does tropomyosin slide or roll over F-actin during the activation of muscle fibers?

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Data from both electron microscopy and Molecular Dynamics simulations (Li et al., 2010; Li et al., 2011) have led to a model in which αα-tropomyosin (Tpm1.1st (a.b.b.a)) is pre-shaped (i.e. bent) to present one specific face to the F-actin filament. It revealed that tropomyosin is rather stiff, which precludes much curvature variance in situ. These observations suggested that tropomyosin, rather than rolling during regulatory transitions, slides across the actin filament while always presenting the same face to actin, i.e. so that the same residues along tropomyosin survey the surface of F-actin (Lehman et al., 2013). This conclusion was confirmed by a cryo-electron microscope structure of a myosin-activated thin-filament (Behrmann et al., 2012) and by energy landscape analysis (Orzechowski et al., 2014). However, another recent cryo-EM structure (von der Ecken et al., 2014) implies that tropomyosin, during the transition between its positions on F-actin, may undergo a significant amount of pseudo-rotation (i.e. a rolling around its own local double-helical axis which modifies the face presented to F-actin), thus reviving the sliding-versus-rolling debate. This possibility raises questions regarding the extent of backbone and side-chain reorganization required by such a pseudo-rotation. Therefore, we are assessing the energetics of changes in tropomyosin conformation associated with pseudo-rotation. Moreover, we are quantifying the shape complementarity of tropomyosin crystal structures to the F-actin-tropomyosin model, including αα- and ββ-smooth muscle tropomyosin in our analysis (since their curvature is almost identical to that of the striated muscle isoform, despite considerable sequence variation, Rao et al., 2012). The relatively low r.m.s. deviation between coordinates of the crystal structures and the model supports the view that tropomyosin remains bent to face F-actin in a specific way and does not significantly pseudo-rotate during muscle activation.

Structural and functional properties of tropomyosin with amino acid substitutions M127A/I130A and M141A/Q144A in the middle part of its molecule

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We applied different methods to investigate how amino acid substitutions M127A/I130A and M141A/Q144A in the middle part of tropomyosin (Tm) molecule affect the structure and functional properties of Tm. According to predictions, these substitutions, i.e. replacement of canonical residues M127, I130, M141, and Q144 in the hydrophobic core of the Tm molecule by non-canonical Ala, should destabilize the coiled-coil in the middle of Tm. Therefore we expected that these substitutions might have a significant influence on structural and functional properties of Tm. We applied differential scanning calorimetry to investigate the effects of these substitutions on the thermal unfolding of Tm. The results showed that substitutions M127A/I130A and M141A/Q144A have no appreciable influence on the domain structure of Tm. This can be explained as follows: the middle part of Tm is already strongly destabilized by two non-canonical residues, D137 and G126, and therefore it seems unlikely that substitutions M127A/I130A and M141A/Q144A may cause any additional destabilization of this part of the molecule. On the other hand, we have found using the in vitro motility assay that these substitutions strongly affect functional properties of Tm. It has been shown that substitutions M127A/I130A and especially M141A/Q144A significantly enhance maximal sliding velocity of regulated thin filaments containing Tm and troponin at high Ca^{2+} concentrations and, moreover, they increase Ca^{2+}-sensitivity of the actin-myosin interaction underlying this sliding. Surprisingly, these effects were very similar to those observed earlier for Tm mutants carrying stabilizing substitutions D137L, G126R, and D137L/G126R in the middle part of the Tm molecule, which strongly increased the thermal stability of Tm (Matyushenko A et al., 2014, *FEBS J* **281**: 2004–2016). These results indicate that functional effects of stabilizing or destabilizing substitutions in the middle of Tm can be caused not only by changes in the Tm structure, but also by their influence on the actin-myosin

interaction, especially in the presence of troponin and Ca^{2+}. These functional effects might be explained by the influence of stabilizing or destabilizing substitutions in the middle part of Tm on the interactions between this part of actin-bound Tm and certain sites of the myosin head.

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Ribbon-shaped structure of vertebrate smooth muscle myosin filament is not assembled from tropomyosin but from caldesmon. A long overdue correction!

Apolinary Sobieszek

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In my early studies on smooth muscle, I described a crude myosin fraction (CMF) in which self-assembly of myosin filaments was observed. For the first time, the 14.4 nm periodicity could be seen on these filaments stemming from the regular arrangement of myosin heads on the filament surface (Sobieszek A, 1972, *J Mol Biol* **70**: 741–744). In my model presented in 1975, the filaments were assembled from the bipolar myosin dimers arranged on a six-stranded helix of repeat 72 nm, residue translation 28.8 nm, and pitch 3×144 nm. These parameters are consistent with lengths of the myosin molecules, the "smooth edges" and the short bipolar filaments (Sobieszek A, 1977, *The Biochemistry of Smooth Muscle*, Stephens NL, ed., pp 413–443, University Park Press, Baltimore, MD).

In the CMF we also observed formation of long ribbon-shaped aggregates exhibiting the 5.6 nm periodicity characteristic of the actin filament and/or tropomyosin paracrystals (Sobieszek A, Small JV, 1973, *Phil Trans R Soc Lond B* **265**: 203–212). This latter periodicity became obscured, apparently as results of interaction of the ribbons with the myosin component of this fraction; we therefore concluded that these ribbons were made of tropomyosin and they might be related to the myosin ribbons observed in the studies of the whole smooth muscle using electron microscopy (EM) (Lowy J, Small JV, 1970, *Nature* **227**: 46–51; Small JV, Squire JM, 1972, *J Mol Biol* **67**: 117–149). Subsequently, Small concluded that the ribbons observed in the EM sections represented an artificial side-by side aggregation of the myosin filament, but the observation of the ribbons in the CMF was not addressed (Small JV, 1977, *J Cell Sci.* **24**: 327–349). Thus, the origin and composition of the ribbons seen in CMF have remained unresolved until now. After reexamination of SDS/PAGE data from earlier studies, together with my current knowledge on caldesmon (CaD) and tropomyosin (TM), I came to the conclusion that the ribbons of CMF were not made of TM but CaD. This explains the observed 5.6 nm actin filament periodicity and their apparent interaction with myosin. A conclusion long overdue in view of the fact that CaD, a major component of the smooth muscle thin filament, was discovered in 1984 (Bretscher A, 1984, *J Biol Chem* **259**: 12873–12880) and its interaction with myosin has been described in numerous studies since then. The interesting aspect of this conclusion is that, to the best of my knowledge, no regular CaD assemblies have been demonstrated so far. The implication in the regulation of actin-myosin interaction in smooth muscle will be discussed in light of the new insight.

Comparison of maximum power of intact and skinned muscles from the wild rabbit (*Oryctolagus cuniculus*, Linnaeus)

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Is the power output of skinned fibre fragments from a hind limb muscle of wild rabbit representative of the power-generating locomotory musculature? To approach this question we have (i) compared power output of bundles of intact fibres with that of single skinned fibres from *peroneus longus* (PL) and *extensor digiti-V* (ED-V), and (ii) compared power output of single skinned fibres from the small PL and ED-V muscles with that of fibres from larger muscles that provide most of the power for locomotion. Maximum power was measured in force-clamp experiments at 25 °C. We found that intact and skinned fibres from PL and ED-V produced the same maximum power/$F_{IM} \cdot L_o$, $0.645(s^{-1}) \pm 0.037$ ($N = 16$) and 0.589 ± 0.019 ($N = 141$) respectively. Skinned fibre powers for PL and ED-V were the same as skinned fibre power in the larger locomotory muscles. More than 90 % of the fibres in both the small and large limb muscles are fast-twitch, type 2 fibres, as shown by MHC immunofluorescence. Amongst the type 2 fibres from both small and large muscles, maximum skinned fibre power increases in the order $2a < 2x < 2b$ ($0.447 \pm 0.039\ s^{-1}$ ($N = 10$), $0.599 \pm 0.031\ s^{-1}$ ($N = 17$), $0.750 \pm 0.046\ s^{-1}$ ($N = 17$)). The good match between skinned fibre and intact muscle power at 25 °C, together with the similar patterns of type-2 fibre mechanics for small and large muscles, indicates that the mechanical properties of fibres taken with needle biopsy are likely to be representative of the locomotory muscle mass.

Docking troponin T onto tropomyosin

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The troponin T tail converges on the "core-domain" of troponin where Ca^{2+}-binding affects troponin I and C conformation. High resolution structures (Takeda et al., 2003; Vinogradova et al., 2005) have been obtained for the core-domain, while corresponding lower resolution EM-reconstruction has provided the general outlines and orientation of the entire troponin complex on native thin filaments (Yang et al., 2014). However, the resolution of the reconstruction is insufficient to define residue–residue interactions along actin-tropomyosin, leaving some uncertainty about the polarity of elongated troponin T (TnT) along thin filaments (Paul et al., 2009). Previous crosslinking work indicated that tropomyosin residue 174 may interact with TnT between residue positions 171 and 174 in the rabbit TNNT3 isoform TnT2FA sequence (Mudalige et al., 2010). Here we expanded on this work, by developing an in silico docking protocol (based on Kozakov et al., 2010) in order to localize TnT on all-atoms models of the actin-tropomyosin filament (Orzechowski et al., 2014). Strong electrostatic contact is observed for a series of highly

conserved residues at the TnT2 N-terminus (between K166 and R174, numbered as in TnT2FA ID-P02641-5) across the tropomyosin coiled-coil from residues S174 to E184. Interestingly, this region of tropomyosin contains mutation hotspots that cause hypertrophic cardiomyopathies (D175 N and E180G). In addition, the docking shows strong anti-parallel residue-specific electrostatic binding complementarity between tropomyosin (residues 230–275) and the TnT tail (residues 73–122), which includes sequences in the phylogenetically conserved region of TnT1. Surprisingly, residues Q250 in Drosophila and E250 in vertebrate tropomyosins have different chemical polarities; however, tropomyosin-TnT sequence alignment shows a corresponding site-specific polarity reversal in Drosophila and vertebrate TnT residues. Interestingly, sites on tropomyosin isoforms from troponin-free smooth muscles which might otherwise bind to TnT are not conserved (Brown & Cohen, 2005). Apparently, here evolutionary pressures either relaxed or dictated new tropomyosin sequences needed for interactions with other tissue-specific actin-associated proteins.

Session 2: Muscle Development and Repair

Oral presentations

BMP signaling controls satellite cell dependent postnatal muscle growth

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Postnatal/juvenile muscle growth is achieved by both an increase in myofiber size and the addition of further myonuclei, whereas myofiber number does not increase further. Satellite cells are the resident muscle stem cells which proliferate in growing muscle to supply new myonuclei. Little is known of how satellite cell function is controlled during the postnatal/juvenile growth phase to permit correct muscle mass development. We have previously shown that BMP (bone morphogenetic proteins) signaling regulates embryonic myogenesis by determining the entry of embryonic muscle precursors into muscle differentiation. Here we demonstrate that this pathway also defines postnatal/juvenile muscle growth. We found that juvenile satellite cells express P-Smad1/5/8 and contain transcripts of BMP signaling components such as BMP4, BMP6 and BMPR1A, showing that BMP signaling is active in these cells. Abrogating BMP signaling in satellite cells in juvenile Rosa26-Lox-Stop-Lox-Smad6-IRES-GFP;$Pax7^{CreERT2/+}$ mice decreased the pool of satellite cells and muscle fibres contained less myonuclei and were smaller than those from control mice. We show that blockade of BMP signaling decreased satellite cell proliferation and diminished the myonuclear recruitment during myofiber growth as the underlying cellular mechanism and this severely retarded muscle growth. In addition, failure of satellite cell proliferation during the postnatal/juvenile growth phase strongly reduced the final satellite cell reservoir in mature muscle. In conclusion, these results show that correct BMP signaling in satellite cells is required for satellite cell dependent myofiber growth and for the generation of the adult satellite cell pool. In the future it will be of large interest to determine whether the BMP signaling pathway is altered in childhood neuromuscular disorders and whether this inflicts satellite cell dependent muscle growth.

A role for Klf5 in myoblasts as a differentiation switch from proliferation to fusion

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Kruppel-like factor 5 (Klf5) is a ubiquitously expressed zinc finger transcription factor and the function stringently depends on the context as well as cell and tissue type. Klf5 is shown to be involved in several cellular processes such as the maintenance of the undifferentiated state in blastocysts, cell cycle regulation, apoptosis, differentiation, and migration.

A base-line expression of Klf5 is also present in steady state skeletal muscle and in vitro proliferating myoblasts. This basal expression of Klf5 is induced along with acute injury regeneration in vivo and myoblast fusion in vitro. We have previously shown that Klf5 localizes to the myonuclei along with fusion and its SUMOyation drives this traffic. Transfection experiments using non-SUMOylatable constructs verify the disruption of this trafficking. Overexpression of Klf5 in proliferating myoblasts induces a cell cycle arrest and the opposite is observed with a dominant-negative construct that accelerates the cell cycle. These observations are further confirmed by conditional silencing of Klf5 in proliferating myoblasts. Silencing of Klf5 accelerates motility and lessens the attachment of dividing myoblasts.

We further investigated the role of Klf5 in myoblast differentiation. Silencing Klf5 strikingly inhibited the fusion which was also confirmed by transfection of dominant negative constructs. The silencing of Klf5, prohibited the upregulation of several markers of fusion, differentiation and myotube maturation such as beta catenin, desmin, NCAM and M-Cad. Silencing of Klf5 following fusion did not exhibit any impact on myotube maturation pinpointing its essential role in fusion. Klf5 silenced myoblasts also failed to fuse with their non-silenced GFP + counterparts, verifying an essential role for the induction of fusion.

Klf5 silencing repressed the induction of fusion markers as well as myogenin, the terminal regulator of differentiation. This differentiation block could be counteracted by the forced expression of MyoD. These observations pinpoint Klf5 downstream of MyoD and upstream of myogenin. In order to verify this role, we transdifferentiated 3T3-L1 preadipocytes to muscle by forced expression of MyoD. These cells exhibited an upregulation of Klf5 along with fusion in a dose dependent manner.

These results attribute a novel role for Klf5 as a functional switch for the cell cycle arrest and induction of differentiation in myoblasts. Our current efforts are concentrated to elucidate the partners and targets of Klf5 along this process.

Roles of ADAM8 in elimination of injured muscle fibers prior to skeletal muscle regeneration

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When skeletal muscle suffers injury, skeletal muscle-specific stem cells are activated and differentiate to form new muscle fibers that replace damaged ones. This regeneration process of skeletal muscle recapitulates myogenesis during development to some extent. Activation of myogenic transcription factors, Myf5/MyoD and myogenin, for example, is essential both for myogenesis during development and for myogenic activation and differentiation of muscle stem cells during regeneration in common. Regenerative myogenesis, however, includes processes different from developmental myogenesis; adult muscle stem cells generate muscle fibers solely at injured sites, and therefore, injured muscle fibers need to be eliminated before regenerative myogenesis starts.

Inflammatory cells, including neutrophils and macrophages, play roles in the elimination of damaged, necrotic muscle fibers. These cells infiltrate into injured muscle, remove necrotic tissue or cell debris, and stimulate stem cell growth and differentiation. Evidence suggests that proper inflammatory reactions are necessary for muscle regeneration. In this study, we show that efficient elimination of injured muscle fibers during regeneration requires ADAM8, a member of a disintegrin and metalloprotease (ADAM) family. Skeletal muscle of dystrophin-null mice, an animal model for Duchenne Muscular Dystrophy, deteriorates by the lack of ADAM8, which is characterized by increased area of muscle degeneration and increased number of necrotic and calcified muscle fibers. Upon cardiotoxin-induced skeletal muscle injury, neutrophils invade into muscle fibers through the basement membrane and cluster in wild type mice, but not in ADAM8-deficient mice although neutrophils of the latter infiltrate into interstitial tissues similarly to those of wild type mice. Neutrophils lose their adhesiveness to blood vessels after infiltration, which includes an ectodomain shedding of P-Selectin Glycoprotein Ligand-1 (PSGL-1) on their surface. Expression of PSGL-1 on the surface of neutrophils remain higher in ADAM8-deficient than in wild type mice. These results suggest that ADAM8 mediates a conversion of migratory behaviors of neutrophils by the removal of their adhesiveness to blood vessels after infiltration into injured muscle.

Posters

Ultrastructural colocalization of titin and myosin during sarcomere assembly in regenerating vertebrate skeletal muscle after intoxication with notexin

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The giant filamentous protein titin spans from the Z-disk to the M-band in the sarcomere of striated muscle cells, where it functions as a molecular spring during stretching and relaxation. During myofibrillogenesis, titin is thought to act as a molecular template for the assembly of thick filaments, because super-repeats of titin domains in the C-zone of the A-band show a ~43 nm periodicity coinciding with the ~43 nm spacing of myosin heads and MyBP-C.

In this study we used immunogold electron microscopy in conjunction with Diaminobenzidine (DAB) chromogen labelling and three-dimensional reconstruction of electron micrographs to visualize titin and myosin localization at different stages of myofibrillogenesis in regenerating rat *soleus* muscle after notexin–induced myofibril breakdown. DAB-labeling of antibodies against the heavy chain of muscle–myosin II allowed us to visualize individual thick filaments before incorporation into myofibrils. Beginning at day two after intoxication we observed single thick filaments in the cytosol colocalized with titin. To identify different regions of titin, we used an I-band antibody (T12), an antibody for the A–I-junction (MIR) and an antibody against the C-terminus of titin (TTN9). The anti-MIR and anti-TTN9 titin epitopes generally colocalized with the thick filaments, whereas the anti-T12 epitope, although showing close proximity to the thick filaments, did not colocalize with them. However, the anti-T12 epitope colocalized with Z-bodies. On day 2–3 after intoxication, single thick filaments and titin aligned and together with the Z-bodies formed premyofibrils. Shortly after, the Z-bodies fused to Z-discs and contracted sarcomeres appeared. Titin epitopes were observed at their expected 'final' positions in the sarcomere, but also extra-sarcomeric, though very near the myofibrils. Four days after intoxication we found mostly mature myofibrils with a clear striation pattern. Most titin was seen incorporated into the sarcomere, but some titin was still present around the myofibrils. Our results support a model in which titin acts as a molecular scaffold protein for the assembly and the integration of thick filaments into the sarcomere during skeletal muscle regeneration.

Can transient miRNA overexpression induce myogenic differentiation of embryonic stem cells?

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Embryonic stem cells (ESCs) are pluripotent and may differentiate into any cell type building adult organism. But, efficiency of their differentiation into specific lineages, like mesodermal or myogenic, is very low. Several various approaches, including lentiviral overexpression of various miRNA have been reported to induce ESCs differentiation. For example, overexpression of miR1 induces ESCs differentiation into cardiomyocytes. We examined how transient miRNA overexpression can be used for directed ESCs differentiation. ESCs transfected with exogenous miRNAs were differentiated in outgrowths of embryoid bodies, i.e. ESCs aggregates which serve as a model for ectoderm, endoderm, and mesoderm formation, cultured under conditions supporting myogenic differentiation. Surprisingly transient miRNA overexpression turned out to be relatively stable. High levels of overexpressed miRNA were detectable even 9 days after ESCs transfection (in embryoid bodies cultured for 7 days). Next, miRNA affected ESCs differentiation, directing them towards myogenic lineages. Importantly, miRNA overexpression decreased expression of pluripotency factors involved in pluripotency e.g. Oct4 and resulted in increased expression of myogenic markers, e.g. myosin heavy chains. Thus transient miRNA overexpression have a potential to direct differentiation of stem cells.

Role of Cullin-3 in skeletal muscle development

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Muscle mass maintenance is tightly regulated by an equilibrium between muscle growth and atrophy, reflecting the balance between protein synthesis and degradation at the cellular level. Muscle proteins are mainly degraded through the ubiquitin–proteasome system requiring the tagging of substrates via a well-defined enzymatic cascade [1, 2]. First, the E1-Ubiquitin Activating Enzyme covalently attaches to ubiquitin through an ATP driven step. Once activated, ubiquitin is transferred to E2-Ubiquitin Conjugating Enzymes. The last step requires the concerted action of E2-Ubiquitin Conjugating Enzymes and E3-Ubiquitin Ligases, which transfer the ubiquitin from the E2-enzyme onto the substrate. In mammals, the largest family of E3 ligases corresponds to the Cullin-RING ligase family. RING (*Really Interested New Genes)* domains proteins associate with one member of the Cullin family (Cullin-1, 2, 3, 4a, b, 5, 7, 9) in order to form an active E3-ligase complex. Each Cullin protein is able to bind to a fixed subset of substrate adaptors, thereby achieving specificity for a range of cellular substrates. Apart few muscle-specific substrate adaptors of Cullin-1 that have been shown to exhibit key roles during muscle atrophy [3], little is known about the regulation of protein degradation during muscle development, and the muscle specific roles of other Cullins. Interestingly, Cullin-3 localizes to myofibrils and is involved in muscle protein breakdown [4]. While intensively studied in the context of cancer, its role in muscle is mainly unexplored. Moreover, association of mutations in genes coding for Cullin-3 substrate adaptors with myopathies has been described. Indeed, we hypothesized that Cullin-3 may play a greater role than expected for skeletal muscle development and function.

Using in vitro and in vivo techniques, we show the absolute necessity of Cullin activity for myogenesis and demonstrate the essential role of Cullin-3 in skeletal muscles for post-natal life. Physiological and histological analyses suggest that absence of Cullin-3 in skeletal muscles lead to respiratory defect. These data strongly support an important role for Cullin-3 in skeletal muscle development. Undergoing proteomic analyses of skeletal muscle will give us insights regarding deregulated signaling pathways in absence of Cullin-3 during muscle development.

References:

1. Mitch WE et al. (1996) *New Engl J Med* **335**: 1897–1905.
2. Bonaldo P, Sandri M (2013) *Dis Model Mech* **6**: 25:39.
3. Bodine SC et al. (2001) *Science* **294**: 1704–1708.
4. Lange S et al. (2012) *Mol Biol Cell* **23**: 2490–2504.

The effect of interleukin-8 on microRNA expression in rat primary skeletal myoblasts

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Cross-talk between different cell types during tissue development occurs through secreted factors, including common inflammation-related members, involved in autocrine/paracrine interactions. MicroRNAs, a novel class of small, noncoding RNAs, modulate gene expression post-transcriptionally, and play important roles in many aspects of muscle function. The aim of the study was to examine microRNA profile in rat skeletal primary myoblasts subjected to differentiation in the presence of interleukin-8. IL-8 (1 ng/ml) did not affect myotube formation, the early (MyoD, myogenin) and late (myosin heavy chain) markers of myogenesis on the 11th day of differentiation, however it slightly decreased cell viability, assessed in MTT test. Microarray analysis revealed up-regulation of 5 miRNAs: miR-154-3p (fold change: +110.8), miR-541-5p (+71.8), let-7a-1-3p (+30.9), miR-376c-3p (+1.84) and miR-338-3p (+1.6), in IL-8-treated myocytes. Using Target Scan database we found target genes for all miRNAs with expression modified by experimental treatment and we performed ontological classification of these genes in terms of biological process and molecular function using PANTHER database. Genes targeted by miRNAs modified by IL-8 in rat myocytes were involved in several cellular processes (GO:0006468, $n = 70$), in particular metabolic process (GO:0008152), cell communication (GO:0007154), developmental processes (GO:0032502), biological regulation (GO:0065007), DNA-dependent transcription (GO:0006350), cell cycle (GO:0007049), cellular component organization (GO:0016043), cell adhesion (GO:0007155) and mesoderm development (GO:0007498). An ontology group highly enriched by potential targets of experimentally modified miRNAs was also transport (GO:0006810, $n = 37$). Predicting targets for miRNAs altered by IL-8 encoded proteins, which manifest transporter activity (GO:0005215, $n = 21$), kinase activity, GO:0016301, $n = 12$), and structural molecule activity (GO: 0005198, $n = 21$), in particular, structural constituent of cytoskeleton, GO:0005200, $n = 16$). In conclusions: (1) IL-8 does not directly alter myogenic program, however it can regulate miRNA expression in rat primary myoblasts; (2) IL-8 potentially regulates numerous cellular processes, such as metabolism, cell communication, adhesion, transcription, transport, and cytoskeleton organization, important in skeletal muscle cell development and function. Elucidation of cellular mechanisms of these effects requires further investigation.

Phosphorylation of titin at the Z-disk/I-band junction as a potential mediator of sarcomere assembly

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Introduction: The Z-disk/I-band junction of the giant protein titin connects the inextensible Z-disk with the extensible I-band segment. The Z/I titin region is encoded by Ttn exons 27/28 and contains four Ig-domains (Z7-I1) interspersed by three linker regions. Within these linker regions, we identified three conserved phosphorylation sites in mouse titin (UniProtKB identifier, A2ASS6): S1805, S2078 and S2080. We hypothesized that the phosphorylation at Z/I titin could be important for sarcomere assembly and occur early in myocyte development.

Methods and Results: Using heart tissue from stable isotope labeling by amino acids in cell culture (SILAC) mouse mixed with wild-type (WT) or calcium/calmodulin dependent protein kinase-II (CaMKII) δ/γ double-knockout (DKO) mouse heart, we found S1805 as a novel CaMKII-dependent phosphorylation site. Phosphorylation at this site

was decreased in DKO hearts by 87 %. In addition, in vivo phosphoproteomics also identified serines S2078 and S2080 at the Z/I titin junction as constitutively phosphorylated sites. We used synthetic human titin peptides to test for phosphorylation by various kinases. Phosphorylation and autoradiography on these titin fragments revealed that CaMKII and G-protein-coupled receptor kinase-4 (GRK4) phosphorylated both S1805 and S2080, whereas cyclin-dependent kinase-1 (CDK1), extracellular signal-regulated kinase-2 (ERK2), p38 mitogen-activated kinase-δ (p38δ) and casein kinase-2 (CK2) phosphorylated S2078. Interestingly, S2080 was found to be phosphorylated in fetal rat hearts (e18) and in early stages of C2C12-myotube differentiation. Moreover, yeast-two-hybrid screens "fished" various potential interactors of titin at the Z-disk/I-band junction, which might be regulated by phosphorylation.
Conclusion: Phosphorylation of titin at the Z/I-junction is mediated by various protein kinases, which could play an important role in sarcomere assembly during early stages of myofibrillogenesis.

The influence of Sdf-1-treated cells transplantation on skeletal muscle regeneration

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Skeletal muscle regeneration depends on the presence of satellite cells. These quiescent cells connected with muscle fiber are activated in response to muscle injury. Once activated they resume the cell cycle, start to proliferate, and then differentiate into myoblasts that fuse to form new myofibers. However, under certain circumstances, for example muscular dystrophies, the process of muscle reconstruction is diminished. Among the therapies considered as a cure for muscle diseases are cells transplantations. Except satellite cells few population of other stem cells are characterized by myogenic potential, e.g. mesenchymal stem cells isolated from Wharton's jelly or embryonic stem cells. Proper homing of exo- or endogenous stem cells is crucial for the success of such therapies. Among the factors controlling this process is SDF-1 (stromal derived factor-1) that was shown to be responsible for stem cells mobilization to injured muscles. The aim of this project was to analyse the role of SDF-1 in mobilization of transplanted cells to injured skeletal muscles. The effective migration of stem cells to and within injured muscle allows efficient tissue engraftment by transplanted cells. The process of skeletal muscle regeneration was investigated after Sdf-1 treatment of skeletal mouse muscles, primary myoblasts isolated from them, as well as mouse embryonic stem cells or human mesenchymal stem cells transplantation. To follow transplanted cells localization we used myoblasts expressing β-galactosidase or embryonic stem cells constitutively expressing histone H2B-GFP. Human mesenchymal stem cells were immunolocalized. Moreover, before the transplantation the cells were either pre-treated with Sdf-1 or transfected with siRNA silencing either CXCR4 or CXCR7, i.e. two known Sdf-1 receptors. The results of the experiments revealed that Sdf-1 treatment improved the efficacy of the regeneration, enhances the migration of the transplanted myoblasts or embryonic stem cells in vivo in CXCR4 dependent manner. Next, the transplanted cells followed myogenic program and participated in formation of new myofibers. Human mesenchymal stem cells very rarely were observed in regenerating muscles. However, after transplantation of these cells improvement of regeneration was observed. Summarizing, Sdf-1 treatment improves skeletal muscle regeneration by mobilizing endogenous stem cells.

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Expression profiling of Wnt signaling factors in the nurse cell harboring *Trichinella* spp

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Infection of mammalian skeletal muscles with larvae of parasitic nematode *Trichinella* spp. leads to formation of an intracellular niche for the larva, called a nurse cell (NC). The NC results from a fusion of infected degenerating myotube with mis-differentiating muscle satellite cells. Its stage of growth arrest is accompanied by senescent phenotype. The NC-larva complex becomes surrounded by collagen capsule and circulatory rete. As such, it is maintained throughout the life span of the host. NC pathology pertains to disturbances in muscle regeneration process.

Wnt signaling is primarily known to regulate basic developmental processes, such as cell proliferation, differentiation, polarity and migration. Wnt ligands are also regulators of postnatal tissue homeostasis by influencing stem cell fate decisions. Muscle satellite cells are stem cells. In accord, Wnt signaling was demonstrated to promote muscle regeneration. System analysis of NC transcriptome indicated involvement of Wnt signaling in maintenance of its phenotype. In the present study the expression of genes participating in Wnt signaling in NC was studied by RT^2 Profiler PCR Arrays (Qiagen) on a model of mouse trichinellosis.

NC was found to express Wnt growth factors that may exert autocrine stimulation, with Wnt5b, Wnt11 and Wnt16 being expressed at the highest level. Expression of Fzd receptors 1 through 8 indicated putative activation in NC of canonical and non-canonical Wnt signal transduction cascades. β-catenin-dependent canonical signaling route may lead to upregulation of cyclins D, c-Myc, VEGF, Fra1 and Jun, all factors involved in determination of hypermitogenic stimulation, growth arrest, angiogenesis and inflammatory functions found displayed by NC. Wnt/Ca^{2+} non-canonical signaling route may lead to upregulation of PKC family members which may in turn activate transmembrane NADPH oxidase complex, further contributing to NC inflammatory properties. The other atypical Wnt signaling pathway, Wnt/PCP (Planar Cell Polarity), may lead to JNK and subsequent AP1 transcription factor activation, which may induce in NC expression of chemoattractants and pro-inflammatory cytokines. Thus, based on expression pattern of Wnt signaling factors in NC, Wnt signaling appears as an important determinant of NC main biological functions, including growth arrest in response to hypermitogenic stimulation, as well as secretory phenotype.

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Drosophila as a disease model for myosin myopathies

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Myosin heavy chain (MyHC) is a molecular motor that converts chemical energy into mechanical force, and is indispensable for body movement and heart contractility. Hereditary myosin myopathies have emerged as an important group of muscle diseases with variable clinical and morphological expression depending on the mutated isoform and type and location of the mutation. Myosin storage myopathy (MSM) and Laing distal myopathy are the result of usually dominant mutations in the gene for slow/β cardiac MyHC (*MYH7*). Protein aggregation is part of the features in some of these myopathies.

We have recently established models for myosin myopathies in the fruit fly *Drosophila melanogaster*. We have focused on myosin myopathies associated with the mutations located in the middle and distal part of the slow/*β*-cardiac MyHC rod region (LMM) associated with several distinct morphological and clinical phenotypes depending on the location of the mutated residue. Mutations in the distal end of the tail of slow/β cardiac MyHC are associated with MSM with protein aggregates and mutations in the mid region of the cardiac MyHC rod associated with Laing distal myopathy, often without protein aggregates.

Because of conservation of the muscle structure and the high prevalence of genetic tools available for use, *Drosophila* is a good model to study muscle myopathies. The recently developed MiMIC system to replace the single endogenous *myosin heavy chain* gene (*mhc)* with mutated forms in flies and tools to image sarcomeric components in living animals have been used.

We investigate the effect of the mutations during the development of the larval body wall muscles, where sarcomere formation and structure can be visualized using live imaging, immunohistochemistry and confocal microscopy. Here we present the current status of the project aiming to establish *Drosophila* as a disease model to study the pathology and mechanism behind MyHC muscle myopathies.

Cellular compartments responsible for the augmentation of donor satellite cell engraftment within irradiated dystrophic skeletal muscle

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We have shown that donor mouse satellite cells contribute to significantly more muscle regeneration if the dystrophic host mouse muscle has been irradiated prior to intra-muscular cell transplantation [1]. Irradiation creates a window of opportunity to graft donor satellite cells with great efficiency. However, this effect is dose, dose-rate, and time dependent. Regarding dosage, higher radiation doses (25 Gy) enable donor satellite cell engraftment only hours after irradiation, whilst the effect is completely lost after 72 h; on the other hand, a lower dose (18 Gy) allows efficient engraftment between 3 and 72 h after irradiation but this effect is lost 4 weeks later [1]. The obvious explanation, a simple case of niche depletion and replacement, may not fully explain the observed effect, as at the time of donor cell transplantation there is still a population of endogenous satellite cells present. Furthermore, almost complete ablation of host satellite cells severely hinders grafting of donor cells [1]. Thus, some form of active modulation of the satellite cell niche, triggered by irradiation of the host muscle, must be involved.

Research dating back to co-transplantation experiments of irradiated and non-irradiated tumour cells in the 1950s indicate that after a group of cells are lethally damaged by radiation they secrete pro-mitotic signals to adjacent undamaged cells, triggering their proliferation. This is believed to have evolved to allow adjacent cells to replace those lost due to injury. We therefore hypothesized that in dystrophic muscle, lethally irradiated cells secrete pro-mitotic factors that create a permissible environment for cell engraftment. As these cells are progressively cleared from the tissue the source of pro-mitotic factors is lost, and with it the permissible environment for cell grafting. Data will be presented from co-transplantation experiments in which various cellular components from irradiated skeletal muscle have been grafted into non-irradiated muscles admixed with donor satellite cells, to attempt to elucidate which compartment within skeletal muscle is mediating the augmentation in grafting efficiency of donor satellite cells.

References:

1. Boldrin L et al. (2012) *Stem Cells* **30**: 1971–1984.

Differentiation potential of Pax7 −/− pluripotent stem cells in in vitro and in vivo studies

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Skeletal muscles are characterized by the ability to regenerate after injury caused by excessive exercise, freezing, ischemia or disease. However, in the case of sarcopenia, muscular dystrophy, and some other muscle diseases, endogenous pool of myogenic precursors, i.e. satellite cells is insufficient to effectively repair damaged tissue. Transplantation of stem cells that could replenish satellite cells population and support muscle regeneration could be considered as possible therapy of such diseases. Thus, such cells needs to be able to undergo myogenic differentiation. Among the factors crucial for the specification of skeletal muscle precursor cells during myogenesis and maintaining of satellite cells within adult muscle is Pax7. Moreover, this transcription factor is an anti-apoptotic agent which regulates satellite cells pool and their self-renewal. In the current study we investigated the role of Pax7 in myogenic differentiation of pluripotent stem cells. As a tool we used wild type and Pax7 −/− embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs).

In in vitro studies we used 5-azacytidine, i.e. hemimethylating agent, to induce myogenic differentiation of Pax7 +/+ and Pax7 −/− ESCs or iPSCs. 5-azacytidine replaces cytosine during DNA replication. Its impact at the epigenetic modifications was shown to result from interference with the function of DNA methylases. We found that 5-azacytidine treatment of pluripotent stem cells induces their differentiation leading to the formation of myotube-like structures. Next, we revealed that in vitro differentiating Pax7 −/− ESCs downregulated regulator of pluripotency, i.e. Nanog while upregulated Flk1 or Pdgfrα, i.e. mesoderm markers, more efficiently than wild type cells. Moreover, 5-azacytidine-treated Pax7 +/+ and Pax7 −/− ESCs and iPSCs express myogenic transcription factors, e.g. MyoD, Myf5, and myogenin.

In in vivo studies fluorescently marked Pax7 +/+ and Pax7 −/− -ESCs and iPSCs were transplanted into cardiotoxin injured mouse *gastrocnemius* muscles. Cells of both genotypes, i.e. Pax7 +/+ and Pax7 −/−, were able to undergo myogenic differentiation in vivo, but

only ESCs participated in the formation of new muscle fibers. Pax7 −/− ESCs more efficiently colonized damaged skeletal muscles than Pax7 +/+ cells. We conclude that myogenic differentiation of Pax7 −/− pluripotent cells occurs more readily than of Pax7 +/+ cells.
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The functional role of MEGF10, a putative transmembrane receptor of the multiple epidermal growth factor family, during regulation of satellite cell myogenesis

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Skeletal muscle stem cells (satellite cells) are essential for myofibre regeneration. Multiple epidermal growth factor-like protein 10 (MEGF10) is expressed in satellite cells and plays a role in regulating proliferation and differentiation. MEGF10 is a single-pass transmembrane protein consisting of an EMI domain and 17 atypical EGF-like domains in the extracellular region. The EMI domain is predicted to interact with phosphatidyl serine. The smaller, intracellular region of MEGF10 contains NPXY and YXXL motifs, predicted to bind PTB-binding and SH2 domains. Truncating mutations in MEGF10 cause severe early-onset myopathy, areflexia, respiratory distress and dysphagia (EMARDD), whereas missense mutations cause a milder variant with evidence of minicores on muscle biopsy (Logan CV *et al.*, 2011, *Nature Genetics* **43**: 1189–1192; Boyden SE et al., 2012, *Neurogenetics* **13**: 115–124). This autosomal recessive congenital myopathy is characterized by reduced muscle fibre size, resulting in severe muscle weakness. The pathogenic mechanisms, by which loss-of-function of MEGF10 causes these phenotypes, remain unclear.

The expression profile for MEGF10 in satellite cells attached to single myofibres in culture is distinct to that of isolated cultured satellite cells, suggesting that the myofibre niche is important in regulating expression. Mammalian cell expression and purification of the MEGF10 extracellular domain and a truncated form lacking the N-terminal EMI domain indicate that both are heavily post-translationally modified and facilitate muscle cell attachment to a non-adherent surface. These data suggest that MEGF10 facilitates cell–cell and/or cell-substrate adhesion. We predict that pathogenic missense mutations in the EGF-like domains will abrogate MEGF10 adhesion.

EMARDD is a very rare form of muscle myopathy, currently identified in only seven families, with no curative treatment available. Insights into the role of MEGF10 during muscle regeneration by satellite cells may identify novel therapeutic targets for a broad range of skeletal muscle myopathies and dystrophies. This could advance therapies for patients with muscular dystrophies and myopathies, particularly where current treatments ameliorate the symptoms rather than targeting the disease.

The role of TGFβ1 pathway inhibition in skeletal muscle regeneration and myoblasts differentiation

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Skeletal muscle is characterized by an ability to regenerate in response to injury or disease. The significant role in muscle regeneration is played by muscle-specific stem cells, i.e. satellite cells. In the response to the injury satellite cells become activated, proliferate, fuse to myotubes, and reconstruct destroyed muscle fibers. Next, the extracellular components contribute to successful muscle repair. However, in case of some muscle types regeneration might be affected by the excessive development of fibrosis. Repair process differs depending on muscle type. Thus, regeneration of so called fast muscles, such as EDL proceeds properly leading to the reconstruction of functional tissue. However, the slow muscle, i.e. Soleus, regeneration is disturbed by the fibrosis. Muscle repair is controlled by various factors: cytokines, growth factors, such as TGFβ1, and also enzymes impacting at extracellular matrix (ECM). Among these enzymes are matrix metalloproteases (MMP). Two of them, i.e. MMP-2 and MMP-9, were shown to be characteristic for skeletal muscles and involved in its regeneration. Next, also TGFβ1—a multifunctional cytokine—was shown to be involved in the regulation of fibrosis development. TGFβ family members are well known as stimulators of ECM production and modulators of the expression of ECM degrading enzymes and proteinase inhibitors. Thus, it initiates a fibrotic cascade in skeletal muscle regeneration. TGFβ1 mediates MMP-2 and MMP-9 activation. The interplay between TGFβ1 and MMP-2, MMP-9 in slow and fast twitch muscle regeneration has not been established, yet. In our current study we tested how the inhibition of TGFβ1 pathway impacts at MMP-2 and MMP-9 activity in regenerating Soleus and EDL muscles. We also studied its role in in vitro differentiating myoblasts isolated from these muscles. Next, we examined the effect of TGFβ1 pathway inhibition on muscle regeneration. An impairment of TGFβ1 pathway was achieved using injection of anti-TGFβ receptor I (TβRI) antibodies into injured Soleus or EDL muscles or by treating in vitro cultured myoblasts isolated from both muscles with siRNA complementary to TβRI mRNA or suramin, i.e. TβRI inhibitor. This approach allowed us to uncover differences between both types of muscle in the role of TGFβ1 pathway in the regeneration. Thus, our research may bring the understanding of mechanisms underlying the differences in muscle regeneration between slow and fast twitch muscles.

Generation of targeted insertion in the Klf5 gene of mouse myoblasts (C2C12 cells) using CRISPR/Cas9 system

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Klf5 is a zinc finger transcription factor that is expressed in early embryonic stem cells as well as adult somatic epithelial tissue. The function of Klf5 is diverging in a context dependent manner in cells and tissues. During development, Klf5 has a role in the maintenance of undifferentiated state in embryonic stem cells. Moreover, Klf5 is also acting on cellular processes such as cell migration, apoptosis, inflammation, angiogenesis and differentiation. Our previous studies showed a novel role for Klf5 as a regulator of proliferation and differentiation in myoblasts. Detecting Klf5 at the protein level harbors several technical obstacles. Commercially available antibodies exhibit low affinity, low specificity and fail to recognize post-translationally modified forms that are directly relevant to the function. These obstacles prevent further functional protein studies, such as western blots, protein co-immunoprecipitation and chromatin immunoprecipitation (ChIP) assays. Since novel genome editing tools provide an opportunity to achieve robust genetic modifications, we employed CRISPR/Cas system to establish a stable cell line which carry V5

epitope tag into the N-terminal of Klf5 gene. Insertion into the target side of Klf5 gene via CRISPR-Cas9 system provided an opportunity to overwhelm the above mentioned obstacles. Our previous efforts using transgene constructs confirmed that an N-terminal epitope tag would not interfere with the KLF5 function. C2C12 myoblasts are accepted to be easy-to-clone cells with relatively rapid expansion capacity. However, several technical obstacles complicate the efficiency of genome editing in C2C12 myoblasts. Initially, low efficiency of transfection is limiting the success of these applications. Furthermore, likewise embryonic stem cells or other somatic stem cell, the ratio of homologous recombination is much lower compared to several other cell lines. PCR based methods for the screening of positive colonies encompasses risks of false positives due to the retention of DNA elements delivered for homologous recombination. Apparently, protein based methods for the screening and validation of engineered positive clones are mandatory for reliable evaluation. Here we confirm a successful targeted insertion of a desired tag sequence into the exon 1 of the Klf5 gene both at the DNA and protein levels. These cells will be used as tools for the follow-up protein studies of the observations presented in another abstract (Akpulat U et al.). These studies will aid for the elucidation of the role of Klf5 in skeletal muscle differentiation and repair.

The role of heme oxygenase-1 in cardiotoxin-induced skeletal muscle injury

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Heme oxygenase-1 (HO-1) is a heme-degrading enzyme of well-known anti-inflammatory and cytoprotective properties. Recently, we have found that it also strongly influences murine skeletal myoblast differentiation, inhibiting the expression and activity of MyoD and myomirs (miR-1, miR-133a, miR-133b, miR-206), while increasing mir-146a (Kozakowska et al., *Antioxid Redox Signal* 2012). The aim of the present study is to investigate the role of HO-1 in skeletal muscle regeneration.

Gastrocnemius muscles of HO-1 +/+ and HO-1 −/− 3-month-old mice were injected with cardiotoxin (CTX, 25 μl of 20 μM) to induce sterile muscle injury. Lack of HO-1 was associated with higher muscle degeneration (evidenced by higher levels of lactate dehydrogenase and creatine kinase in sera) and inflammation (assessed by semi-quantitative histopathological analysis of gastrocnemius muscle sections and associated with elevated protein level of IL-6, IL-1β, MIP-1 and MIG in skeletal muscle tissue on the 1st and 3rd day after injury). Additionally, in HO-1 +/+ mice the expression of HO-1 was strongly induced at day 1 after injection, followed by steady decrease to the normal level at 28 day after injury. Whereas in muscles of HO-1 −/− mice delayed expression of MyoD, accompanied by inhibited miR-206 and induced miR-146 expression was observed. On the other hand regeneration in HO-1 deficient animals may be induced by elevated areteriogenesis, as suggested by increased number of arterioles in HO-1 −/− muscles after injury. Regeneration rate was however similar between in HO-1 +/+ and HO-1 −/− mice, although regenerated myofibers of HO-1 −/− animals had larger cross-section area.

Flow-cytometry analysis revealed higher number of muscle satellite cells (mSC; CD45-CD31-Sca1-α7i + CD34 +) in 3- and 6-month-old HO-1 −/− mice, and also at end-stage of muscle regeneration (28 days after CTX-induced muscle injury). They are, however, predominately in G0 phase of cell cycle, whereas the presence of HO-1 enables cell cycle entry, increasing the number of mSC in G1 phase. Accordingly, upon activation mSC express HO-1 at significantly higher level and the number of activated mSC (CD45-CD31-Sca1-α7i + CD34-) is diminished 3 days after injury in HO-1 −/− mice.

In conclusion, lack of HO-1 results in increased muscle degeneration and inflammation at early stages of muscle injury, accompanied by increased arteriogenesis in latter phases. Presence of HO-1 results in predisposition of quiescent mSC to become activated and facilitates their entry in cell cycle.

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MicroRNA expression patterns in post-natal mouse skeletal muscle development

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Introduction: MicroRNA (miRNAs) are small non-coding RNA molecules that act as transcriptional regulators, primarily by repressing the expression of their specific gene targets. In mature organisms, miRNAs play a major role in the maintenance of skeletal muscle homeostasis and facilitate the adaptation of muscle structure, function and metabolism in response to internal and external stress signals. In this study, we investigated the expression patterns and putative roles of miRNAs during the early post-natal development stages of the mouse quadriceps muscle.

Methods: Twenty C57BL/6 mice were sacrificed at the age of 2 days (n = 4), 2 weeks (n = 5), 4 weeks (n = 5) and 12 weeks (n = 6) and RNA was extracted from the muscle. MiRNA expression was assessed using the TaqMan Array Rodent MicroRNA A + B Cards Set v3.0. The Expression Suite software was used to extract and normalize the data using the global normalization function. Linear trend in Ct values was assessed using a robust regression model; Kruskal–Wallis' test was used to compare time Ct values for miRNAs with no significant trend. Predicted Ct values from a robust quadratic model were used to classify miRNAs into clusters of Ct profiles using the Wald's distance. For each cluster, top cellular functions and miRNA-mRNA target interactions were determined using the online software package Ingenuity System Interactive Pathway Systems.

Results: Out of the 768 miRNA measured, 354 (46 %) were significantly expressed in muscle in at least one of the time points (Ct32). Thirty of these miRNAs (8 %) were expressed at one time point only. Collective miRNA expression levels significantly decreased (43 %) or increased (16 %) with time ($p < 0.05$). Out of 10 miRNAs clusters, 8 were selected for further analysis based on the significance levels of the linear and the Kruskal–Wallis' tests. The most highly ranked cellular functions likely to be regulated by each miRNA cluster included cellular development, cellular growth and proliferation, cell death and survival, DNA replication and protein synthesis.

Conclusion: This study provides an overview of the role and regulation of miRNAs in neo-natal and post-natal mouse skeletal muscle samples. Our results collectively suggest that miRNAs play an essential regulatory role during the very-early muscle development

stages, and that their expression levels tend to decrease as the muscle grows and matures.

MicroRNA profile in rat primary skeletal myoblasts exposed to interleukin-15

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The purpose of the study was to examine microRNA profile in rat skeletal primary myoblasts subjected to differentiation in the presence of interleukin-15. IL-15 (1 ng/ml) did not affect myotube formation, the early (MyoD, myogenin) and late (myosin heavy chain) markers of myogenesis on the 11th day of differentiation, however it increased 2-times the desmine transcript. Microarray analysis revealed down-regulation of 9 miRNAs, i.e. miR-322-3p, miR-542-3p, miR-7a-5p, miR-455-3p, miR-192-5p, miR-127-5p, miR-423-5p, miR-433-3p and miR-125b-2-3p, in IL-15-treated myocytes. The Target Scan and the PANTHER databases were used to find target genes for these miRNAs and to perform ontological classification in terms of biological process and molecular function.

Genes targeted by miRNAs modified by IL-15 in rat myocytes were primarily involved in metabolic process (GO:0008152, n = 123), in particular, in cellular protein modification process (GO:0006464, n = 18), regulation of catalytic activity (GO:0050790, n = 17), lipid metabolic process (GO:0006629, n = 16), proteolysis (GO:0006508, n = 15), cellular amino acid metabolic process (GO:0006520, n = 12), protein phosphorylation (GO:0006468, n = 11), generation of precursor metabolites and energy (GO:0006091, n = 9) and regulation of transcription (GO:0006366, n = 8). A lot of targets were associated with cellular process (GO:0009987, n = 81), regulating developmental process (GO:0032502, n = 39), cellular component organization (GO:0016043, n = 24) and cell cycle (GO:0007049, n = 21). An ontology group highly enriched by potential targets was transport (GO:0006810, n = 40), in particular protein transport (GO:0015031, n = 22), and cell communication (GO:0007154, n = 46). Gene ontology classification with regard to molecular function revealed two, highly enriched and partially overlapping category, i.e. catalytic activity (GO:0003824, n = 74) and protein binding (GO:0005515, n = 43). Predicting targets for IL-15-modified miRNAs encoded proteins manifesting transporter activity (GO:0005215, n = 20) and structural constituent of cytoskeleton (GO:0005200, n = 17). In conclusions: (1) IL-15 does not directly alter myogenic program, however it can regulate miRNA expression in rat primary myoblasts; (2) IL-15 potentially affects important cellular processes, such as metabolism, transport, cell communication and cytoskeleton organization in primary myoblasts, however further research is required to clarify the mechanisms of its action.

Kirrel1, a mammalian homolog of the Drosophila muscle cell fusion regulator Kirre, is alternatively spliced and is temporally expressed during murine muscle regeneration

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Kirrel1 is a mammalian homolog of a Drosophilian type 1 transmembrane protein, Kirre, known to be crucial for myoblast fusion events during embryonic muscle development in Drosophila. Myoblast fusion with each other or with damaged muscle fibres is a key process in muscle regeneration. Extensive research on myoblast fusion in Drosophila indicates it is a highly complex process with multiple genes involved. The genes controlling adult muscle progenitor cell (satellite cell) fusion events in mammals are less defined. Recently Sohn et al. [1] found a Kirrel1 mRNA transcript in mouse skeletal muscle. During in vitro myogenesis, C2C12 cells express two murine Kirrel1 splice variants [2].

We hypothesise that Kirrel1 mRNA expression will be temporally upregulated during skeletal muscle regeneration, following the time course of cell fusion events in vivo.

Adult male mice (3–6 months) were used. We induced a moderate crush injury to the gastrocnemius muscle as previously described [3]. Muscle was harvested on days 7, 12 and 14 post-injury from the injured and contra-lateral limbs.

H&E staining confirmed presence of small newly formed fibres at day 7. Total Kirrel1 mRNA expression was highest 7 days post-injury ($\sim$5.5 fold). At later time points days 12 and 14 post injury, total Kirrel1 mRNA was still $\sim$2 fold higher. Sequenced PCR amplicons highlighted presence of three alternatively spliced mRNA variants of Kirrel1 in regenerating mouse muscle, which is a novel finding. Kirrel1 A and B share significant sequence identity, but differ in both the 5′ and 3′ ends, with Kirrel1 B truncated at the 3′ end. The alternative splicing of Kirrel1 B at its 3′ end would result in shortening of its intracellular domain. Both Kirrel1 A and B mRNA variants were upregulated post-injury.

These results, combined with findings in Drosophila, suggest that Kirrel1 may be an important component of the muscle regeneration process in mammals. Improved understanding of Kirrel1 during muscle regeneration may aid in reducing the rehabilitation time frame from muscle injuries.

References:

1. Sohn RL et al. (2003) *PNAS* **106**: 9274–9279.
2. Durcan PJ et al. (2014) *Physiol Rep* **14**: 11.
3. Myburgh KH et al. (2012) *P Nutr Biochem* **23**: 1072–1079.

AEBP1/ACLP is upregulated in differentiation, injury repair and fibrotic degeneration of skeletal muscle

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AEBP1/ACLP is an ambiguous gene with several attributed functions in development and postnatal life. Among these several cellular events, adipogenic differentiation, cell adhesion, pattern development and fibrosis are the well-understood. The protein products of this gene are obscure. AEBP1 is the short isoform that acts as a transcriptional repressor by targeting the AP2 promoter and ACLP, which is the long isoform that harbors a leader sequence that directs the peptide to the extracellular compartment. The latter is known to be associated with development of the connective tissue, injury repair and fibrosis in certain pathological conditions.

AEBP1/ACLP displays a moderate expression in skeletal muscle where the role is not known. In order to elucidate this role, we have investigated the spatial and temporal expression of AEBP1/ACLP in defined models of skeletal muscle differentiation, injury repair and fibrosis at the mRNA and protein levels.

AEBP1/ACLP expression is present in steady state dividing myoblasts. This basal expression is upregulated (up to 4 folds) upon the induction of differentiation in both C2C12 cells and primary rat myoblasts. This in vitro observation models skeletal muscle differentiation. Considering that differentiation and post-natal injury repair share several common aspects, we also investigated the expression of AEBP1/ACLP in acute injury-repair model. AEBP1/ACLP expression is present in steady state skeletal muscle. In the course of cardiotoxin induced injury, its expression peaks up to 5 folds in the 6th day of regeneration. This time point concomitantly corresponds to the fusion of the myofibers. Since AEBP1/ACLP is also known to be associated with fibrotic events in chronic pathological conditions, we also have investigated its expression in skeletal muscle fibrosis. Tenotomy induced skeletal muscle degeneration mimics endomysial and perimysial fibrosis without inflammatory events. AEBP1/ACLP expression is upregulated up to 10 folds in early time-point samples. AEBP1/ACLP expression is localized to several cells that are residing in the endomysial and perimysial space during acute in jury repair and chronic fibrosis.

These results depict a novel role for AEBP1/ACLP in extracellular remodeling of the skeletal muscle during injury repair as well as fibrotic degeneration. Our current efforts are focused on pinpointing the source of this expression as well as the precise function.

Systemic myokine responses following eccentric exercise in humans

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Introduction: Muscle adaptation which occurs following exercise-induced muscle damage has been associated with an inflammatory response before the completion of muscle repair or regeneration (Philippou et al., 2012). Cytokines and other peptides produced and released by muscle fibers (myokines) exert autocrine/paracrine, or endocrine effects, and there is a growing interest in their potential involvement in those processes (Henriksen et al., 2012). The purpose of this study was to investigate systemic myokine responses for several days after eccentric exercise in humans.

Methods: Nine healthy men volunteers (age: 25.7 ± 5.1 years, height: 180.5 ± 5.0 cm, mass: 77.2 ± 8.0 kg; mean ± SD) performed 50 maximal eccentric muscle actions using the knee extensor muscles of both legs. Blood samples were withdrawn before and at 6 h, 2 days and 5 days post-exercise. Serum levels of myokines interleukin (IL)-6, irisin and follistatin, as well as of sclerostin, were measured by ELISA using commercially available kits. Changes in all variables were analyzed using one-way ANOVA and relationships between them were examined using Pearson's correlation coefficient (r). The level of statistical significance was set at $p < 0.05$.

Results: Significantly increased levels of circulating IL-6 were observed 6 h and 2 days after the exercise-induced muscle damage, while irisin levels exhibited a gradual decrease post exercise, reaching significance on day 5 ($p < 0.05$). Significant positive correlations were revealed between the post-exercise percent changes in IL-6 and follistatin over time ($r = 0.80–0.95$, $p < 0.01$).

Conclusion: The findings of the present study suggest that eccentric exercise may trigger a systemic myokine response, potentially as part of the inflammatory and/or repair process following exercise-induced muscle damage, where the myokines IL-6, irisin as well as follistatin might be especially involved.

References:

Henriksen T et al. (2012) *Recent Pat Biotechnol* **6**: 167–171.
Philippou A et al. (2012) *Adv Clin Chem* **58**: 49–87.

Involvement of HSPB8 in zebrafish muscle development

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Small heat shock proteins (HSPBs) belongs to the family of heat shock proteins (HSPs). In the zebrafish (*Danio rerio*) 13 HSPB have been identified among them HSPB1/HSPB7/HSPB12 exist in skeletal muscle. In our research, we characterized HSPB8 in zebrafish embryos. Both the transcript *HSPB8* and protein HSPB8 are present in muscles of *D. rerio* developing embryos and larvae. In our studies, we used hybridization in situ and immunocytochemistry techniques.

Zebrafish embryos reveal increased HSPB8 gene expression under heat shock conditions. This suggests that HSPB8 can play a role as chaperone protein in zebrafish. Immunocytochemical analysis show that in normal conditions HSPB8 localizes in Z and M line whereas after heat shock it is distributed through whole sarcomere. This fact suggests that HSPB8 chaperones may also play stabilization of muscle structures.

In order check HSPB8 involvement in the zebrafish development, we have prepared knock down experiments by the used morpholino oligomers. We have used two different type of morpholino that block transcription by bind specific morpholino oligomers to HSPB8 transcript. Morphants showed abnormalities in the tail length and muscle structure. Our results suggest HSPB8 may influence on the proper development of skeleton muscles in zebrafish.

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New muscular models to study molecular mechanisms involved in sarcopenia: extraocular and interosseous muscles of sheep

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Sarcopenia is defined as a decline in skeletal muscle mass and function (in terms of strength and power) that occurs with several pathological conditions and aging. The disappearance of muscle fibres and their replacement with adipous or fibrous tissue is one among the factors contributing to sarcopenia.

In this study we aimed to assess whether the conversion of interosseous muscle into the proximal sesamoidean ligament (PSL) can be considered as a model of sarcopenia. Actually, as described previously (Mascarello & Rowlerson, 1995, *J Anat* **186**: 75–86) in sheep, the muscle component of the PSL, which is well developed at birth, undergoes a rapid and progressive involution postnatally and is completely replaced by fibrous tissue within 6 months. The disappearance of muscle fibres is accompanied by an invasion of mononucleated cells.

Samples of sheep PSL, lateral rectus (LR, an extraocular muscle), massetere (M, representative "slow" muscle) and semimembranosus (SM, typical "fast" muscle) muscles at 1 day, at 30 days and 6 month were collected and analyzed by:

– SDS/PAGE and WB with specific antibody in order to separate and identify MyHCs (myosin heavy chain) isoforms expressed at different age;

– Real Time PCR to evaluate and compare the expression of different MyHC genes, myogenic factor, fibrogenic markers;

– Immunohistochemical analysis of sections of frozen tissues to characterize cellular population and observe if cellular death may be due to necrosis or apoptosis;

– Electron microscopy in order to detect satellite cells.

SDS page and western blot analysis showed that fast and slow isoforms were always expressed in EOMs while, in PLS, the prevalent postnatal isoform was the slow; at 1 month of life it was the only detectable and was reduced to 10 % at 6 month. As expected, M and SM showed a progressive shift into slow and fast phenotype during development. Real Time PCR analysis confirmed these data: myogenic factors were almost absent at 6 months in PSL whereas they were highly expressed in EOM and in other skeletal muscles tested, as M and SM. In contrast, TGFß was progressively more expressed postnatally in PSL muscle. Finally, electron microscopy showed a marked decrease in the abundance of satellite cells in PLS.

The present data support the conclusion that sheep PSL might be used as a "model" to study sarcopenia and the future aim is to understand the molecular mechanisms involved in its progression.

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Adenosine-mediated modulation of ACh-evoked currents induced in *Xenopus* oocytes following embryonic skeletal muscle membrane microtransplantation

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The microtransplantation technique is a very simple and useful approach to study the functional properties of ion channels and receptors present in membranes isolated from different tissues, in particular post-mortem human tissues. When the membranes carrying their original receptors and channels are injected into *Xenopus* oocytes, they fuse with the oolemma together with lipids and any associated native proteins. In this way, they are easily analysed electrophysiologically.

Ion channels can be modulated by several kinases whose activity is linked with G-coupled protein receptors (GCPRs). For example, purinergic adenosine receptors (P1Rs) are GCPRs expressed in almost all cells, including skeletal muscle, and we have recently reported that these receptors, in particular the A_{2B} subtype, modulate the activity of nicotinic acetylcholine receptors (nAChRs) expressed in mouse myotubes cultured in vitro. In this study we aimed to investigate whether this modulatory effect was maintained once these receptors are microtransplanted into *Xenopus* oocytes.

Embryonic skeletal muscle cell membranes isolated from mouse myotubes were injected into oocytes and the two-electrode voltage-clamp technique was used to record the acetylcholine (ACh)-mediated currents (1 mM). The AChR desensitization, measured as a current decay $T_{0.1}$ and $T_{0.5}$ (time necessary for the current to decay by 10 and 50 % from its peak value) was measured and compared to that obtained in the presence of adenosine deaminase, to remove the endogenous adenosine, or in the presence of specific P1-receptor ligands. We found that the P1R ligands affected the ACh-current decay but this effect was mediated by the A_1 subtype, rather than the A_{2B} subtype. Additional experiments will be performed to investigate if the A_1 receptor is endogenously expressed in the oocytes.

Our results demonstrate that the interplay between the P1Rs and the embryonic nAChR expressed in myotubes is different when the receptors are transplanted into the oocyte membrane. Adenosine is involved in many disorders and P1Rs represent a target for the development of new drugs. Therefore, it is important to understand whether the microtransplantation technique could be a suitable approach for studying the adenosine-mediated effects on ion channels.

Towards directed differentiation of pluripotent stem cells into skeletal myoblasts

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Pluripotent stem cells are a potential source of various cell types for regenerative medicine. Despite accumulating knowledge there is currently no efficient and reproducible protocol for the generation of skeletal muscle myoblasts. Such cells could be used in cell therapy for skeletal muscle diseases such as muscular dystrophies (Grabowska et al., 2012). Formation of myoblasts during mammalian embryo development is a stepwise process controlled by numerous signalling cues. Among them Sonic hedgehog (Shh) and Wnt family proteins play the key role as they induce expression of MRFs (Myogenic Regulatory Factors) in myogenic precursor cells. As a result precursor cells convert into myoblasts which differentiate and fuse with each other forming multinucleated myotubes and, eventually, myofibers (Tajbakhsh, 2009). Interleukin-4 (IL-4) plays a pivotal role in myoblasts fusion as it recruits myoblasts into growing myotubes (Horsley et al., 2003).

The main aim of our studies is to establish a protocol for directed and efficient differentiation of pluripotent stem cells (such as embryonic stem cells, ESCs) into skeletal myoblasts. We focused at the potential role of the main regulators of embryonic myogenesis—Shh, Wnt-11, and IL-4—in this process. First, we determined the level of endogenous expression of these factors in undifferentiated

and differentiating ESCs at mRNA and protein level. Next, we checked whether ESCs synthesize receptors for Shh, Wnt11 and IL-4. Presence of appropriate receptors suggests that ESCs are able to respond to aforementioned proteins presence in the culture medium. Finally, we estimated the influence of exogenous Shh, Wnt-11 and IL-4 on ESCs morphology, proliferation and differentiation. We determined the levels of expression of ectodermal (Pax6), endodermal (GATA-4), and mesodermal (Brachyury, Mesogenin) markers as well as myogenic markers such as Pax3, Pax7 and MRFs. We also assessed the influence of Shh, Wnt11 and IL-4 on ESC ability to fuse with skeletal myoblasts.

We proved that Shh, Wnt-11 and IL-4 have influenced ESCs differentiation but in different ways. While Shh promoted neural differentiation of ESCs, Wnt11 and IL-4 enhances expression of mesodermal markers and MRFs.

A novel functional rice against skeletal muscle atrophy caused by unloading

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Skeletal muscle subjected to unloading conditions is vulnerable to be atrophied. We reported that ubiquitin ligase Cbl-b plays a role in unloading-mediated skeletal muscle atrophy: Cbl-b ubiquitinates and leads to degradation of IRS-1, an important IGF-1 signaling intermediate molecule, resulting in muscle mass loss. We also reported that intramuscular injection of a pentapeptide, DGpYMP, mimetics phosphorylated site sequence of IRS-1, significantly inhibited denervation-induced loss of skeletal muscle mass. In the present study, we examined the effects of oral administration of Cblin-like peptide (QDGYMPW) transgenic rice on denervation-induced muscle mass loss. We first generated transgenic rice seeds in which 15-tandem repeated Cblin-like sequence were fused to storage protein glutelin gene. Cblin-like peptide was released from recombinant protein by digestion with chymotrypsin or pancreatin in vitro. In addition, the digestive Cblin-like products inhibited Cbl-b-mediated IRS-1 ubiquitination. Moreover, dietary Cblin-like peptide transgenic rice seeds inhibited skeletal muscle atrophy through a decrease in IRS-1 degradation in vivo. Furthermore, Cblin-like peptide was detected in portal blood of mice of post-prandial state. Our present results suggest that Cblin-like peptide transgenic rice seems a dietary therapy to prevent skeletal muscle atrophy.

The myosin chaperone UNC-45 is organized in tandem modules to support myofilament formation

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The activity and assembly of various myosin subtypes is coordinated by conserved UCS (UNC-45/CRO1/She4p) domain proteins. One founding member of the UCS family is the *Caenorhabditis elegans* UNC-45 protein important for the organization of striated muscle filaments. Our recent structural and biochemical results demonstrated that UNC-45 forms a protein chain with defined periodicity of myosin interaction domains. Intriguingly, the UNC-45 chain serves as docking platform for myosin molecules, which promotes ordered spacing and incorporation of myosin into contractile muscle sarcomeres. The physiological relevance of this observation was demonstrated in *C. elegans* by transgenic expression of UNC-45 chain formation mutants, which provokes defects in muscle structure and size. Collaborating with the molecular chaperones, Hsp70 and Hsp90, chain formation of UNC-45 links myosin folding with myofilament assembly.

Session 3: Skeletal Muscle Diseases

Oral presentations

Understanding the role of KBTBD13 in striated muscle function and in nemaline myopathy

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Nemaline myopathy (NM) is a clinically and genetically heterogeneous disease that is characterized by muscle weakness and the presence of rod-like structures (i.e. nemaline bodies) in muscle fibers. It is among the most common non-dystrophic congenital myopathies. A recent gene implicated in NM is *KBTBD13*—a member of the kelch protein family (disease referred as NM-*KBTBD13*). Only little is known regarding the functions of KBTBD13, which might include a though it is shown to work as an adaptor to Cullin-3 ligase, thus implicating its role in the ubiquitin–proteasome degradation system (UPS).

In the current study, we used a C2C12 cellular model, which forms myotubes on differentiation, to study the functioning of KBTBD13 and its role in NM. We used a mycDDK-KBTBD13 plasmid to transfect the cells and various biochemical assays as read out. We observed that mRNA levels of KBTBD13 go up with differentiation of C2C12 cells. To elucidate the cellular localization of KBTBD13, we performed immunocytochemistry. Our preliminary results show a cytosolic localization in undifferentiated cells whereas a typical perinuclear staining of KBTBD13 occurs upon differentiation. This might indicate that KBTBD13 localizes with the Golgi apparatus or the endoplasmic reticulum. Furthermore, we treated the cells with MG132, a proteasomal inhibitor, in the presence or absence of KBTBD13. The expression of KBTBD13 lead to a slight increase in the ubiquitination pool, indicating a role in the stabilization of ubiquitination of targeted substrates. The direct interacting partners of KBTBD13 are still unknown, and therefore in future work we aim to perform proteomics analyses on C2C12 cells that overexpress KBTBD13. We are also generating disease associated mutations in KBTBD13 (Arg248Ser, Lys390Asn, Arg408Cys) to more closely

study the disease phenotype associated with NEM6. A better understanding of the molecular mechanism underlying the function of KBTBD13 will enable the identification of targets in NM-*KBTBD13*.

POLG mutations are a frequent cause of mitochondrial disease in a group of Polish patients with mitochondrial DNA deletions

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Here we present the result of nuclear genes analysis in the group of 23 patients of the Department of Neurology, Medical University of Warsaw and their family members. All the patients were diagnosed with mitochondrial disease (mitochondrial myopathy—4, mitochondrial encephalopathy—6, MERRF—2, PEO—4, PEO + —4, KSS—2, SANDO—2). For most of them RRFs (18 patients) or other myopathic changes (3 patients) in muscle biopsy were observed. The screening for common mtDNA mutations (m.3243A > G, m.8344A > G, m.8993T > G, m.8993 T > C) gave negative results. mtDNA deletion screening revealed multiple deletions in 13 patients, two mtDNA deletions were observed in 5 patients and one deletion in 5 patients. *POLG* gene encoding catalytic subunit of the only DNA polymerase in human mitochondria was analyzed at first. While multiple mtDNA deletions are a clear indication for *POLG* analysis, we decided to broaden the study group including patients with two mtDNA deletions and patients with one deletion and family history suggesting autosomal dominant or recessive trait of inheritance (3). In two cases the decision was made only on a clinical basis. The whole *POLG* coding region was sequenced in 23 patients. Two previously described mutations of recessive character were found in 2 subjects (one p.Thr251Ile, p.Pro587Leu homozygote, one homozygote p.Trp748Ser one compound heterozygote p.Thr251Ile, p.Pro587Leu/p.Lys1191Asn). In three cases the patients were found to be compound heterozygotes with one known mutation and one potentially pathogenic unknown variant (p.Trp748Ser/p.Ser998Pro, p.Arg309Leu/p.Gln968Glu, p.Arg290Cys/p.Arg309Cys) and in one case only one potentially pathogenic unknown variant (p.Ala518Thr) was found. For all undescribed and one poorly characterized *POLG* variants further bioinformatic and population analysis and family segregation studies were performed to support the theory of their pathogenic character. Their pathogenic character was also confirmed in yeast model.

For patients negative for *POLG* mutation the analysis of the other nuclear genes involved in mtDNA stability like *POLG2*, *c10orf2*, *SLC25A4* or *SPG7* was performed.

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Differential expression and functional activity of proteasomes in idiopathic inflammatory myopathies

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Idiopathic inflammatory myopathies (IIM) are heterogeneous group of muscle diseases characterized by infiltration of immune cells into skeletal muscle causing muscles inflammation and damage. The upregulation of MHC-I molecule in IIM indicates increased antigen presentation and involvement of proteasome system. Previously, we found mRNA of IFN-γ inducible proteasome (immunoproteasome) subunits (β1i and β5i) significantly increased in IIM muscle biopsies showing its possible role in diseases pathogenesis (Ghannam et al., 2014, *PLoS ONE* **9**: e104048). Here, we investigated the functional role of immunoproteasome subunit in the pathogenesis of IIM.

Western blot analysis of muscle biopsies from Inclusion body myositis (IBM) (n = 3), Immune-mediate necrotizing myopathy (IMNM) (n = 3), Dermatomyositis (DM) (n = 3) patients and healthy controls (n = 2) showed upregulation of immunoproteasome subunits in all diseased condition but not in healthy one. Dual immunofluorescence detected immunosubunits β1i and β5i only in the infiltrated muscle fiber in all (n = 6 for each cases) studied disease conditions, whereas healthy muscle fiber showed no staining of β1i and β5i. Muscle fiber expressing MHC-I showed increased expression of immunosubunits. Among the infiltrating cells, staining of immunosubunits was lowest in CD8 + T cells. In contrast, CD68 + and CD14 + cells showed strong staining of these subunits. Similarly, in vitro study in human myoblast showed increased immunosubunit expression under the influence of IFN-γ in correlation with the increased cell surface expression of MHC-I. Immunoproteasome ability to produce high affinity antigen for MHC-I is determined mainly by its chymotrypsin-like (CTL) catalytic activity (Schwarz et al. (2000) The Journal of Immunology 164: 6147–6157). Here, we found that myoblast is able to significantly increase its CTL activity upon IFN-γ exposure. Furthermore, in well-established experimental autoimmune myositis (EAM) mice model, along with enhanced chymotrypsin-like (CTL) activity of muscle proteasome, immunoblot showed a significant increase in immunosubunit expression in muscle of EAM compared to wild type. Treatment with Rapamycin caused significant decreased in CTL activity of proteasome as well as improvement of myositis in EAM. These results suggest direct involvement of IFN-γ induced immunosubunits in the pathogenesis of myopathies through enhanced upregulation of MHC-I.

Posters

Treatment of *mdx* mice with novel RyR1 stabilizers ameliorate muscular dystrophy progression

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In the *mdx* mouse model of Duchenne muscular dystrophy, the sarcoplasmic reticulum ryanodine receptor (RyR1) has been found to be abnormally nitrosylated leading to calstabin1 depletion and subsequent calcium leak through the channel. RyR1 modulators enhance RyR1-

Calstabin1 binding preventing calcium leak, reducing biochemical and histological evidence of muscle damage and improving muscle function. In this work we have studied the effect of A6 and A7, two novel RyR1 modulators that showed low in vitro cytotoxicity in mouse and human myotube cultures. The compounds were administered to 1 month-old male *mdx* mice for 5 weeks in drinking water. During this period mice were exercised weekly in a treadmill and forelimb strength was assessed using a grip strength meter. At the end of the experiment, muscles were dissected for histological and molecular analysis. In addition, we analyzed intracellular calcium levels in isolated fibres from flexor digitorum brevis muscles. We found that both treatments reduced histological evidence of muscle damage, normalized expression of several genes upregulated in *mdx* muscles and improved muscle function. Furthermore, we observed that these treatments significantly reduced resting intracellular calcium levels. In conclusion, our results show that A6 and A7 are effective in reducing intracellular calcium levels and muscular damage in *mdx* mice. In addition, they consolidate RyR1-calstabin complex as a useful therapeutic target for drug development against muscular dystrophies.

Structural and biochemical characterisation of human skeletal muscle mutations in tropomyosin at the critical residues R90, R167 and R244

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Tropomyosin is an actin and troponin binding protein that plays a fundamental role in the cooperative-allosteric regulation of muscle contraction. It has a 7-fold repeated motif of about 40 amino acids that corresponds to the 7 actins that a single tropomyosin molecule interacts with. Recent reports pinpointed actin binding determinants within tropomyosin in the absence of troponin-Ca^{2+}. They suggested a critical ionic interaction of tropomyosin R90, R167 and R244 with actin. This tropomyosin residue together with actin Asp 25 form a contact site repeated with every actin monomer covered by tropomyosin (Involving residues K6, K48, R90, K128, R167, K205 and R244). Interestingly mutations in these sites have been reported in patients with skeletal muscle myopathy. We aimed to investigate the role of three tropomyosin residues (R90, R167 and R244) in the allosteric transitions within thin filaments. Circular dichroism, co-sedimentation with actin and ATPase assays demonstrated that these mutations had little or no effect on the folding or the thermal stability of tropomyosin. ATPase assays, fluorescence spectroscopy and transient kinetics were used to assess the functional effect of these mutations on the Ca^{2+} dependent inhibition and activation of the acto-myosin ATPase, the size of the cooperative unit, the transition between the blocked and closed state and the affinity and kinetics of Ca^{2+} interaction with troponin and thin filaments. Preliminary data indicate that mutations in these residues have distinct effects on the tropomyosin function and suggest that they are not functionally equivalent.

STORM superresolution microscopy to measure Z-disk and A-band width in human diaphragm muscle

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Diaphragm weakness in mechanically ventilated patients prolongs ventilator dependency, increases morbidity and is associated with long-term functional limitations. We hypothesize that diaphragmatic weakness is partly explained by ventilation-induced structural changes in the sarcomere.

To date, no light microscopy techniques are available to determine structural changes in the sarcomere with nanoscale precision. Therefore, we are developing STochastic Optical Reconstruction *Microscopy* (*STORM*) for imaging of muscle fibers with a spatial resolution of $\sim$20 nm. To validate the technique, we compared data obtained by STORM with those obtained by electron microscopy and initially focused on measuring A-band and Z-disk width.

Diaphragm muscle biopsies were obtained from mechanically ventilated critically ill patients (ICU; $n = 3$) and control subjects (CON; $n = 4$). One part of the tissue was processed for EM, whereas from the other part single myofibrils were isolated. The myofibrils were stained for myosin (type I and II) and α-actinin and imaged using STORM. Z-disk and A-band widths were determined from both EM and STORM images.

There was a trend towards increased Z-disk width in ICU (STORM: 100 ± 6 *vs.* 119 ± 8; EM: 89 ± 1.7 *vs.* 98 ± 11 nm, CON *vs.* ICU). In contrast, A-band width tended to be smaller in ICU than in CON (STORM: 1.54 ± 0.14 *vs.* 1.47 ± 0.08; EM: 1.45 ± 0.05 *vs.* 1.30 ± 0.02, CON *vs.* ICU). Note that both Z-disk and A-band widths were slightly larger when analyzed by STORM than by EM, which might be caused by the lack of tissue shrinking in STORM.

In conclusion, STORM is a promising light microscopy technique to localize individual sarcomeric proteins with nanoscale precision.

Various effect of c.821G>A (Arg274Gln) and c.821G>T (Arg274Trp) mutations in mitofusin 2 gene on clinical manifestation in CMT2A patients and protein structure and function

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Charcot-Marie-Tooth disease type 2A (CMT2A) is caused by mutations in the gene *MFN2*. Mfn2 is one of two mitofusin GTPases involved in mitochondrial fusion, maintenance of organelle integrity and mtDNA content, connection with endoplasmic reticulum and suppression of injury-mediated proliferation of vascular smooth muscle cells. It is not known how mutations in *MFN2* cause axonal degeneration in CMT2A disease. While amino acids substitutions in Mfn2 lead to structural changes that might affect cellular functions and localization thus various mutations can cause various clinical manifestations. Previously, the mutation p.Arg274Gln (c.821G > A) was reported in patient Charcot-Marie-Tooth (CMT) disease with a typical clinical course (Zuchner et al., 2004, *Nature Genetics* **36**: 449–451). Now, we detected another mutation located in the same codon, p.Arg274Trp (c.821G > T) in the patient with early-onset

CMT with a moderate clinical course (involvement of proximal muscles) coexisting with a mild mental retardation. The questions rise how these two mutations affect mitofusin 2 structure and how these changes affect protein functions and cause different clinical outcome. To solve these problems, two parallel methodological approaches were used. First, Polish patient's skin fibroblasts were cultured under standard and glucose-restricted conditions to follow mtDNA/nDNA ratio during 72 h of culture. Second, structure of human Mfn2 obtained with homology modelling based on bacterial dynamin-like protein (BDLP) was elaborated to study the influence of amino acids substitutions on protein structure.

Real-time PCR ddCt analysis of control and patient's fibroblasts mtDNA/nDNA ratio showed that initially, after 24 h of culture, there was no difference between the analyzed cells. However, in the successive time periods, the decrease in the mtDNA/nDNA ratio in the fibroblasts harboring p.Arg274Trp mutation was observed, reaching 45 % in the regular medium and 63 % in the glucose-restricted medium at 72 h time point, while there were no such changes in control cells.

Molecular dynamics revealed dislocation of alpha-helix containing substituted amino acid due to changes in intermolecular interaction types in mutated Mfn2. Simulations, lasting 20 ns without any restraints, were carried out using NAMD2 software with force-field CHARMM27. We also report that discussed residue is located closely to the GTP-binding pocket and might influence ligand binding and hydrolysis.

It might be concluded that both mtDNA/nDNA ratio and in silico modelling seem to be good methods to assist in assessment of pathogenicity of given mutation in *MFN2*.

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Contractile dysfunction in permeabilized muscle fibers of nemaline myopathy patients with the Dutch founder mutation in the *KBTBD13* gene

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Background: Nemaline myopathy (NM) is among the most common non-dystrophic congenital myopathies and is characterized by muscle weakness. Recently, a novel implicated gene was discovered—*KBTBD13*. The majority of NM patients with *KBTBD13* mutations (NM-*KBTBD13*) carry the Dutch founder mutation (c.1222C > T, p.Arg408Cys missense mutation). NM-*KBTBD13* patients have muscle weakness, a typical slowness of movement and experience muscle stiffness. Here, we investigated the contractile performance of permeabilized muscle fibers of NM-*KBTBD13* patients to obtain insight into the effect of this mutation on myofilament function.

Methods: Single muscle fibers were isolated from biopsies of six NM-*KBTBD13* patients and six control subjects (CTRL). Fibers were permeabilized, mounted in a single fiber setup and activated using exogenous calcium. Tension was determined by dividing force by the fiber's cross-sectional area. Furthermore, we measured the calcium-sensitivity of force generation, cross-bridge cycling kinetics and the passive stiffness of the muscle fiber. Data is expressed as mean ± SEM.

Results and Discussion: In line with the observed muscle weakness in NM-*KBTBD13* patients, the maximal active tension was lower in NM-*KBTBD13* fibers (70 ± 8 mN/mm^2 in NM-*KBTBD13 vs.* 122 ± 4 mN/mm^2 in CTRL). Also, cross-bridge cycling kinetics were slower in NM-*KBTBD13* fibers (5.0 ± 0.3 s^{-1} in NM-*KBTBD13 vs.* 8.2 ± 0.9 s^{-1} in CTRL). Hence, slower cross-bridge cycling kinetics could play a role in the slowness of movement. In addition, the calcium-sensitivity of force generation was higher in NM-*KBTBD13* (pCa_{50}: 5.93 ± 0.08 in NM-*KBTBD13 vs.* 5.75 ± 0.02 in CTRL), which might contribute to the impaired relaxation kinetics in NM-*KBTBD13* patients. Both the increased calcium-sensitivity of force generation and the higher passive stiffness in NM-*KBTBD13* at a sarcomere length of 3.2 μm (109 ± 8 mN/mm^2 in NM-*KBTBD13 vs.* 66 ± 14 mN/mm^2 in CTRL) can contribute to the muscle stiffness that NM-*KBTBD13* patients experience.

Conclusion: Here, we studied the contractile performance of single muscle fibers isolated from biopsies of NM-*KBTBD13* patients. The data indicate that changes observed at the myofilament level might contribute to the clinical phenotype of NM-*KBTBD13*.

X-MET as an in vitro model to study pathological disorders associated with skeletal muscle

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X-MET, ex vivo Muscle Engineered Tissue, is an in vitro bioengineered three-dimensional vascularized skeletal muscle that closely mimics the complex morphological and functional properties of skeletal muscle tissue (Carosio et al., 2013). In this work we aimed to develop and improve the generation of a 3D structure as a model to study pathological disorders associated with skeletal muscle such as muscular dystrophy and ALS. We analyzed whether pathological conditions could affect structural organization and myogenic program of X-MET obtained from two transgenic mice: SOD1^{G93A} mice, the mouse model of ALS and *mdx* mice, the mouse model of DMD. Preliminary results demonstrated that X-MET obtained from pathologic mice showed some aspects of the pathology: the absence of dystrophin protein expression in X-MET from *mdx* mice lead to alteration of the connective layer that surround myofibers and affect the myogenic program, as in skeletal muscle of *mdx* mice; while the expression of SOD1^{G93A} induces in the myotube of X-MET an atrophic phenotype as in skeletal muscle fibers of SOD1^{G93A}mice.

References:

Carosio S et al. (2013) *Scientific Rep* **3**: 1420. doi: 10.1038/srep01420.

Cyclophilin D, a target for counteracting skeletal muscle dysfunction in mitochondrial myopathy

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Introduction: Mitochondrial myopathies are genetically heterogeneous metabolic disorders, originating from the dysfunction of one or more mitochondrial metabolic pathways [1]. Muscle weakness and exercise intolerance are hallmark symptoms in mitochondrial disorders. To date, no effective treatment exists. We previously reported that an excessive mitochondrial Ca^{2+} uptake in isolated muscle fibers, that could be inhibited by the cyclophilin D (CypD) inhibitor, cyclosporine A (CsA), may play a central role in the disease process [2]. In this study, we report the effects of a chronic administration of CsA in a mouse model of mitochondrial myopathy.
Methods: The muscle-specific *Tfam* knock-out (KO) mice were treated with CsA (120 μg/jour) for 4 weeks (from 12 to 16 weeks of age). Maximal force production was assessed on whole EDL muscles and single FBD fibers. Cytosolic [Ca^{2+}] was also measured with the fluorescent Ca^{2+} indicator indo-1. RT-PCR and western blot were used to assess the expression of genes and proteins related to mitochondrial and cytosolic Ca^{2+} handling. Muscles biopsies were obtained from control individuals and subjects presenting mitochondrial disease.
Results: CypD protein levels were increased in both mice and patients with mitochondrial myopathy (+60 and +200 %, respectively). CsA treatment: extended lifespan of Tfam KO mice by 4 weeks; restored the free cytosolic Ca^{2+}; prevented skeletal muscle weakness and the decrease in CASQ1 protein expression. COX1 protein expression was still lower in *Tfam* KO mice after treatment as compared to untreated mice.
Conclusion: CsA treatment improved *Tfam* KO skeletal muscle function by improving muscle fiber Ca^{2+} handling. The dominating problem in this model of mitochondrial myopathy may be the progressive muscle weakness rather than the energy deficiency. Overall, our results indicate that CsA treatment can be an effective therapeutic strategy to prevent muscle weakness in mitochondrial myopathies.

Nebulin deficiency in adult muscle causes sarcomere defects and muscle-type dependent changes in trophicity—novel insights in nemaline myopathy

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Nebulin is a giant filamentous protein that is coextensive with the actin filaments of the skeletal muscle sarcomere. Nebulin mutations are the main cause of nemaline myopathy (NEM), with typical NEM adult patients having low expression of nebulin, yet the roles of nebulin in adult muscle remain poorly understood. To establish nebulin's functional roles in adult muscle we performed structural and functional studies on a novel conditional nebulin KO (Neb cKO) mouse model in which nebulin deletion was driven by the muscle creatine kinase (MCK) promotor. Neb cKO mice are born with high nebulin levels in their skeletal muscle but within weeks after birth nebulin expression rapidly falls to barely detectable levels Surprisingly, a large fraction of the mice survive to adulthood with low nebulin levels (< 5 % of control), contain nemaline rods, and undergo fiber-type switching towards oxidative types. Nebulin deficiency causes a large deficit in specific-force and mechanistic studies provide evidence that a reduced fraction of force-generating crossbridges and shortened thin filaments contribute to the force deficit. Muscles rich in glycolytic fibers upregulate proteolysis pathways (MuRF-1, *Fbxo30*/MUSA1, Gadd45a) and undergo hypotrophy with smaller cross-sectional areas, worsening their force deficit. Muscles rich in oxidative fibers do not have smaller weights and can even hypertrophy, offsetting their specific-force deficit. These studies reveal nebulin as critically important for force development and trophicity in adult muscle. The Neb cKO phenocopies important aspects of nemaline myopathy (muscle weakness, oxidative fiber type predominance, variable trophicity effects, nemaline rods, etc.) and will be highly useful to test therapeutic approaches to ameliorate muscle weakness, with novel targets identified by our work.

Muscle gene transfer mediated by mesoangioblasts and the PiggyBac transposon system

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Duchenne muscular dystrophy (DMD) is one of the most prevalent muscular dystrophies, for which no permanent cure exists as yet. It is an X-linked disease in which dysfunctional dystrophin leads to muscle wasting. Transplantation of myogenic stem cells genetically modified to express a functional dystrophin would be an attractive therapeutic option. However, the success of DMD cell therapy has been modest due to several factors; one of them being the difficulty to achieve efficient gene transfer, owing to the large size of the dystrophin gene, another being suitable progenitor cells. The present study investigates the potential use of transposable vectors (PiggyBac) to express dystrophin in mesoangioblasts. Mesoangioblasts are muscle progenitor cells in the embryonic dorsal aorta and in the adipose tissue of mice and humans, with a reported ability to fuse with myofibers. The PiggyBac transposon system allows efficient transgene expression by transposition between the vector and the chromosome using a 'cut and paste' mechanism. The goal of this study is to achieve persistent dystrophin expression in mouse muscles, by introducing the transgene into mesoangioblasts using the PiggyBac system followed by their transplantation in mouse muscles. Intramuscular transplantation of wild type mesoangioblasts containing the integrating transposons in mouse tibialis anterior muscles led to widespread GFP expression in myofibers in vivo. Transposition in mesoangioblasts allowed persistent expression of GFP for over six months after transplantation. At six months, 20 ± 9 % of the muscle cross-section areas showed GFP positive myofibers. These findings provided a proof-of-principle that the PiggyBac transposable vector system may have potential to achieve persistent expression in a significant proportion of the transplanted muscles. To further assess this,

we isolated mesoangioblasts from mdx (dystrophic) mice and introduced a microdystrophin coding sequence with the aid of the PiggyBac transposable vector. We could achieve expression of microdystrophin in mdx mouse muscles, despite the larger size of the transposable vector. Currently, we are using this approach to assess the functionality of treated murine muscles and potential therapeutic effects.

Calcification in dystrophic mouse diaphragm

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The study was performed on diaphragm of α-sarcoglycan-null mice. The sarcoglycans (α-, β-, γ-, and δ-sarcoglycan) are transmembrane glycoproteins associated to the major complex formed by dystrophin at the cell membrane of striated muscles. The dystrophin complex links the intracellular cytoskeleton to the extracellular matrix and holds signal transduction properties. Defects of α-sarcoglycan gene perturb the stability and function of dystrophin complex and cause type 2D limb girdle muscular dystrophy, a severe autosomal-recessive human disorder. The α-sarcoglycan-null mouse develops, as in the human disorder, progressive muscular dystrophy and endomysial fibrosis. The present study investigated the ultrastructural, histological and immuno-histochemical characteristics of α-sarcoglycan-null diaphragm. The diaphragm muscle displays widely scattered dystrophic calcification, usually in apparently healthy muscle fibres and in areas with negligible signs of inflammation. Ultrastructural analysis shows within muscle fibres groups of laminated bodies, most likely of mitochondrial origin. The recurring presence of hydroxyapatite crystals identifies these structures as calcified bodies. These bodies are usually dispersed among myofibrils and cell organelles of surviving muscle fibres, or when more advanced are grouped forming plaques. Areas of dystrophic calcification demonstrate the presence of cells with features typical of osteoblasts, osteoclasts and chondrocytes, associated with the expression of chondrogenic and osteogenic specific protein markers. This observation suggests that dystrophic calcification is associated to a focal bone/cartilage-like transformation in the α-sarcoglycan-null mouse diaphragm.

Role of CCAAT enhancer binding proteins (C/EBP) family in muscle differentiation and development of *rhabdomyosarcoma*

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Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma of childhood. It is believed to be of muscle origin due to its morphological similarities to striated muscles and expression of proteins from myogenic regulatory factors (MRFs) family. Noteworthy, RMS was reported to exhibit traces of osteogenic and adipogenic differentiation in place of proper myogenesis. Also, recent studies identified family of transcription factors involved in choosing the fate between three mentioned tissues. Those are CCAAT enhancer binding proteins (C/EBPs) among which α, β, γ, δ and ζ isoforms should be mentioned. C/EBPs were shown to be crucial players governing proper myogenesis. Out of mentioned isoforms, β and δ are of special interest, as abnormalities in expression of these two isoforms were shown to have deleterious effects in case of RMS development.

Our recent studies have pointed out for relationship between aggressiveness of RMS and ratio of C/EBP β and γ. In more aggressive, alveolar subtype of RMS β isoform is markedly elevated, compared to more benign embryonal RMS, whereas promyogenic γ isoform is expressed more abundantly in embryonal RMS. Concomitantly, within the cell three variants of C/EBP β might be found: LAP, LAP* and LIP, among which LAP and LAP* are dominant-positive and LIP is dominant-negative form. Interestingly, in case of RMS, by forced overexpression of both C/EBP β LIP (but not LAP and LAP*) and C/EBP δ, it is possible to promote muscular differentiation of RMS.

Of note, we have linked both C/EBP β and γ with heme oxygenase-1 (HO-1), another marker of poor prognosis in RMS. HO-1 degrades heme to ferrous ions, biliverdin and carbon monoxide, the latter decreasing C/EBPδ binding to promoter of myoD, a master regulatory switch for myogenesis. Moreover, forced overexpression of enzymatically active, but not inactive HO-1 elevated the level of anti-myogenic C/EBP β, followed by decreased ability of forming myotube-like structures in RMS.

In summary, in this study we report differential expression of various of C/EBPs in RMS cell lines and suggest the balanced expression of C/EBP isoforms as important factor in process of myogenesis.

No recycling = no growth? Insights from the *ky/ky* mouse

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The adaptability of skeletal muscle to mechanical forces is neatly captured with the axiom "use it or lose it". Though physical stresses are important triggers for muscle maintenance and hypertrophy, the exact mechanisms by which muscle detects and translates mechanical stress remain ill defined.

The *ky/ky* mouse, lacking the sarcomeric Z-disc protein KY, shows reduced muscle mass and strength. An additional phenotype is the inability to undergo compensatory hypertrophy in surgical overloading experiments, indicating that KY is necessary for the translation of increased tension into growth signalling [1]. As yet, a definitive molecular function for KY remains unclear. However, the absence of KY appears to perturb the recycling of the cytoskeletal crosslinker Filamin C [2] in the recently proposed mechanism of Chaperone Assisted Selective Autophagy [3] (CASA) with the CASA complex apparently locked within the cytoskeleton. This prevents clearance via autophagy and allows aggregate formation, particularly in the tonically active soleus which experiences consistently high tension. It is proposed that inefficiencies in CASA may result in sustained, elevated pro-autophagy signalling, thereby antagonising protein synthesis. Therefore, muscles are made incapable of properly growing in response to increased tension during organism growth and in compensatory overload experiments.

A myoblast KY knockout cell line is currently in development using CRISPR/Cas9 genome editing technology, with the aim of exploring potential impairment in tension-induced signalling in depth.

References:

1. Blanco G et al. (2001) *Hum Mol Genet* **1**: 9–16.
2. Beatham J et al. (2004) *Hum Mol Genet* **15**: 2863–2874.
3. Arndt V et al. (2010) *Curr Biol* **20**: 143–148.

Effect of uremia on myosin head cross-bridge kinetics, sarcomere length and diameter of skeletal muscle fibres

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Chronic Kidney Disease is characterised by functional abnormalities of skeletal muscle, such as muscle weakness and easy fatigability. These abnormalities are strongly associated with an increase of waste products in the blood (uremia). Chronic uremia can lead to reduced functional capacity and premature death. The mechanisms underlying muscle dysfunction due to chronic uremia are unclear. We investigated the effects of uremia on myosin head cross-bridge kinetics, sarcomere length and diameter of fibres from rabbit psoas muscle.

Renal insufficiency was induced surgically (removal of right kidney and partial nephrectomy of left one) in New Zealand female rabbits. Surgery and euthanasia protocols were approved by the University of Thessaly ethics committee. Psoas muscle samples were excised from control (sham-operated) and uremic animals at 3 months post-surgery. After 24-h permeabilization treatment fibres were stored in 50 % glycerol solution at −20 °C until mechanical assessment.

Single skinned fibres were investigated in solutions containing 5 mM ATP, 10 mM phosphocreatine and 20 U/ml creatine kinase at pH 7 and 22 °C. After attachment, the maximal and minimal diameter of the fibre and the resting sarcomere length were measured in relaxation solution at a near slack position. Subsequently, fibres were maximally activated under isometric conditions by increasing the free Ca^2 concentration to 10^{-5} M. When force reached a plateau, step-like stretches of 0.3 % fibre length were performed to induce isometric force transients. The time to peak of stretch-induced delayed force increase (t) was evaluated as a measure of cross-bridge kinetics.

Our results (mean ± SD) comprised 42 fibres from four uremic animals and 21 fibres from two control animals. Fibres of the uremic animals exhibited larger t values (uremic: 67 ± 18 ms, control: 57 ± 16 ms; P0.05). Furthermore, fibres of the uremic animals exhibited larger resting sarcomere lengths (uremic: 2.25 ± 0.33 μm, control: 2.05 ± 0.17 μm; P0.01) and smaller mean diameters (uremic: 70 ± 19 μm, control: 79 ± 13 μm; P0.05).

In conclusion, our results suggest that uremia can induce a slowing of myosin head cross-bridge kinetics and remodelling changes concerning fibre diameters (atrophy) and sarcomere structure. The larger sarcomere lengths in fibres of uremic animals could be due to a decrease of forces restoring the sarcomere length at resting conditions.

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Systemic and muscle specific redox and biochemical status in chronic kidney disease

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Chronic kidney disease is accompanied by several skeletal muscle abnormalities, in part linked to hypokinesis and in part to uremic toxicity. Skeletal muscle's weakness and wasting, alterations in its structure and metabolic imbalances, are components of functional and morphological alterations which are collectively associated with uremic myopathy. Complex mechanisms that stimulate muscle dysfunction have been proposed, and oxidative stress may be implicated. Our aim was to evaluate both systemic wide effects of uremia looking at blood and skeletal muscle in a rabbit model of renal insufficiency. We were harvested and homogenized psoas and soleus muscle samples from nephrectomised NZ rabbits according to institutional ethics approval. Blood samples were collected and separated in serum, plasma and red cell lysate. All samples were stored at −80 °C until analyzed for Glutathione Reduced (GSH), Glutathione Oxidized (GSSG), Glutathione Reductase Activity (GR), Catalase Activity (CAT), Protein Carbonyls (PC), Thiobarbituric Acid Reactive (TBARS), Total Antioxidant Capacity (TAC), Glucose, Cholesterol, Triglycerides and Uric Acid. PC concentration was significantly higher in uremic psoas (1.08 nmol/mg protein) compared to control psoas (0.54 nmol/mg protein), and significantly higher in uremic soleus (2.21 nmol/mg protein) compared to control psoas (0.70 nmol/mg protein), ($p < 0.05$). Moreover GR activity tended to be higher in uremic psoas compared to control ($p = 0.053$). Regarding blood analysis, CAT activity in control group was significantly higher compared to uremic group (p0.05). TBARS concentration tended to be higher in the uremic group compared to control ($p = 0.05$). A correlation between glucose blood and psoas levels rho = 0.79, $p < 0.05$ and between PC psoas and soleus levels rho = 0.86, $p < 0.05$ was found. Our findings reveal a generalized increase in oxidative stress which can make it a pathological element of interest in uremia, possibly explaining in part the observed muscle dysfunction in renal patients. Interestingly blood levels of most redox and metabolic indices examined did not reflect muscle concentrations. This should be taken into account when interpreting the literature. Further efforts should be made in order to achieve a better understanding of its causes and to identify new tools effective in its clinical management.

Sporadic inclusion body myositis and other myopathies with TDP-43 aggregates. Clinical, morphological, radiological and genetic study

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Sporadic inclusion body myositis (sIBM) is one of the most common acquired myopathies in patients above the age of 50 years. Both proximal and distal muscles' weakness is observed, and the quadriceps, foot extensors and deep finger flexors are most severely affected. Effective therapy is not known and progressive course finally leads to immobilization. Both degenerative and inflammatory

processes are involved in its pathogenesis, and recently TDP-43 aggregates was suggested that might be a hallmark of the disease.

We report of eight patients with sporadic inclusion body myositis and four control patients with other myopathies—three with limb-girdle muscular dystrophy (LGMD) and one with genetically confirmed proximal myotonic myopathy (myotonic dystrophy 2, DM2). Patients were assessed clinically and electrophysiologically. Skeletal muscle biopsy was performed in all patients and samples were evaluated on light and electron microscopy. In 6 patients with inclusion body myositis and in 3 from control group the immunostaining for the β-amyloid, TDP-43, tau protein and α-synuclein was performed. Additionally, proximal one thirds of the thighs and lower legs were scanned by 64-row computed tomography. Causative mutations in *TARDBP*, *VCP*, *HNRNPA1*, *HNRNPA2B1* genes and analysis of *C9ORF72* hexanucleotide repeat expansion were evaluated.

"Rimmed vacuoles" considered characteristic for sIBM were noted in all samples and TDP-43 aggregates were present in muscles in all examined cases. Moreover, accumulation of other proteins like β-amyloid, α-synuclein and tau protein, was present in the majority of examined samples. Computed tomography results showed that the atrophy was most pronounced within the quadriceps femoris and gracilis muscles in the thighs, and within the medial head of the gastrocnemius muscle and the tibialis anterior muscle in the lower legs. All studies of known genes involved in sIBM pathogenesis gave negative results in our study.

Morphological and immunohistochemical assessments proved, that none changes are disease specific. However, muscle biopsy remains important for excluding other possible diagnoses of neuromuscular disorders.

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Autoregulation of MBNL1 protein function

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Muscleblind (MBNL) proteins belong to a family of RNA-binding proteins with crucial roles in alternative splicing and polyadenylation regulation, mRNA stability and cellular localization. During tissue differentiation, expression level of MBNLs increases, while functional downregulation of their activity is a key contributor to the pathomechanism of a dominantly inherited muscle disorder—myotonic dystrophy (DM). MBNLs recognize their RNA targets using four zinc-finger (ZnF) domains arranged in two tandems. Deep sequencing of RNA molecules cross-linked to immunoprecipitated protein particles (CLIP-seq) revealed that MBNL1 binds to the 5′-most region of *MBNL1* exon 1 (e1) encoding both the major part of 5′UTR and N-terminal region of MBNL1 protein, which indicated a possible autoregulative function of MBNLs. Based on the literature data, we put forward four hypotheses with regard to the possible role of MBNL1 binding to its own precursor or mature mRNA. Particularly, we show that binding to the precursor mRNA induces excision of e1 from the mature transcript. This regulates cellular steady state-levels of MBNL1 protein by generating an e1-deficient mRNA, which encodes truncated, highly inoperative and unstable MBNL1 isoform lacking the first two ZnFs. We also demonstrate that *MBNL1* e1 splicing not only depends on MBNLs themselves but also on the *MBNL1* transcription start, which varies between tissues. Taken together, we reveal a novel, multi-layered mechanism that controls MBNL1 protein level through e1 splicing.

Deficiency of miR-378a impairs revascularisation after hind limb ischemia

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MiR-378a is a microRNA highly expressed in skeletal muscles and plays a role in muscle differentiation and cell metabolism. Recently it was found to stimulate also tumor angiogenesis. However, no data exists on the involvement of miR-378a in regenerative revascularization of ischemic tissues. To test such a hypothesis we differentiated murine myoblasts in vitro and used the murine model of hind limb ischemia (HLI).

We showed that miR-378a expression is upregulated during myoblast differentiation in vitro. In the murine model of HLI we showed that the blood flow measured using laser Doppler is inhibited while the number of necrotic toes is higher in miR-378a-deficient(−/−) animals *vs*. wild type (WT) mice subjected to femoral artery ligation. A decrease in expression of both miR-378a strands shortly after induction of HLI in WT mice was observed. This was associated with an enhanced inflammatory response in ischemic muscles of miR-378a −/− mice as evidenced by enhanced level of IL-1β, TNF-α, IL-5, IL-6, VCAM-1, KC and MCP-1, and slightly augmented leukocyte infiltration. Blood count showed lower levels of monocytes circulating in the blood of miR-378a −/− mice. Moreover, FACS analysis revealed higher percentage of macrophages, granulocytes and dendritic cells in ischemic muscles of miR-378a −/− mice compared to WT. VEGF and angiopoietin-2 levels were elevated in the absence of miR-378a after HLI, while vessel-stabilizing angiopoietin-1 expression decreased in those animals. In addition, we showed that proangiogenic cells isolated from the bone marrow of miR-378a −/− mice and selectively grown on fibronectin coated plates reveal decreased angiogenic potential in vitro. Although no differences between genotypes were found in the number of capillaries in the ischemic muscles, we showed a slight increase in the number of arteries in ischemic muscles of miR-378a −/− mice.

Surprisingly, miR-378a overexpression neither local or systemic by adeno-associated vectors did not improve reperfusion in miR-378a −/− mice, but did slightly enhance reperfusion in WT mice. This suggests that certain basal level of miR-378a expression is needed for its biological effects.

Taken together, our results indicate a proangiogenic role for miR-378a in the ischemic tissue, which could be mediated through its effect on the inflammatory response.

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The prevalence of microrearrangements in the *CLCN1* gene underlying congenital myotonia in the cohort of Polish patients

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Thomsen/Becker myotonia congenita (MC) is the most common skeletal muscle channelopathy resulting from mutations in the *CLCN1* (7q34) gene, which encodes the skeletal muscle chloride channel ClC-1. Up to date more than 250 different mutations, mostly missense or nonsense, are known and the majority are associated with autosomal recessive Becker myotonia. Sequence defects causing both recessive and dominant MC, are scattered throughout the gene, although clustering in dominant Thomsen myotonia was documented.

Due to the sparse data on heterogeneous genetic background and diversed phenotype, investigation on Thomsen/Becker myotonia in population of Polish patients was undertaken.

The molecular testing using the Salsa MLPA P350 designed to detect deletions/duplications in the *CLCN1* and *KCNJ2* genes was performed for 68 unrelated probands, clinically and electrophysiologically evaluated as affected with CM, in whom myotonic dystrophies (DM1, DM2) were excluded.

The MLPA analysis indicated heterozygous deletions in 6 individuals and 2 cases of homozygous deletions within exon 13. Moreover one individual was found to carry the heterozygous deletion in exon 13 and probable point mutation in exon 5. The following Sanger sequencing verification confirmed 14 bp deletion in exon 13 c.1437_1450 in all identified by MLPA carriers, and two already known neighbouring point mutations in exon 5 c.[568G > T; 569G > C] changing codon p.Gly190Ser in one patient mentioned above.

Moreover in one subject the pilot study based on Next Generation Sequencing (NGS) with the use of Illumina TruSightOne revealed the SNP polymorphism in exon 18 and the recessive point mutation c.501C > G in exon 4.

For now in three patients the diagnosis of recessive Becker myotonia has been established: in two homozygous carriers of 14 bp deletion in exon 13 and in one individual with the same heterozygous deletion and point mutation in exon 5 (4.4 %). The known recurrent 14 bp deletion in exon 13 was found relatively frequently in the studied cohort of Polish patients (in 7 patients out of 68 probands—10.3 %).

For the remaining probands the NGS sequencing with the use of custom myotonia panel is planned.

Metabolic profile of dystrophic *mdx* mouse cardiac and diaphragm muscles at later stages of disease: effects of omega-3 therapy

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Duchenne muscular dystrophy (DMD) is a recessive X-linked muscle disease characterized by progressive and irreversible degeneration of the muscles. The *mdx* mouse is the classical model for DMD, with the cardiac and diaphragm (DIA) muscles being severely affected at later stages of the disease. Corticoids can delay the progression of the disease but show severe side effects. We previously demonstrated in the *mdx* that omega-3 therapy ameliorated DIA dystrophy, at earlier stages of the disease. In the present investigation, we used ^{1}H magnetic resonance spectroscopy (MRS) to study the effects of omega-3 on the metabolic profile of cardiac and DIA muscles from *mdx*, at later stages (13 months) of the disease. *Mdx* mice (8 months old) received omega-3 oil (commercially available fish oil; FDC Vitamins; Omega-3), for 5 months. Normal (C57BL/10) and untreated-*mdx* mice received mineral oil. Metabolic data was compared to histopathological (fibrosis and inflammatory areas) and molecular (tumor necrosis factor, TNF-alpha and tumor growth factor, TGF-beta) analysis. Among the 42 metabolites identified from the MRS spectra, 26 were analyzed by the principal components analysis (PCA) program. Metabolites related to energetic metabolism (valine, pyruvate, glycerol, lactate, creatine), inflammation (aspartate) and oxidative stress (oxypurinol) were altered in the *mdx* compared to normal mice, in DIA and/or in the heart. Taurine, lactate, creatine, oxypurinol, glycerol, valine and pyruvate were decreased in *mdx* DIA compared to normal samples. In *mdx* heart, glycerol and formate were decreased in contrast to aspartate, valine, creatine-phosphate and glycolate that were increased, compared to normal heart. Oxypurinol was significantly increased in normal DIA (167 ± 69) compared to normal heart (37 ± 14.3). AMP was significantly decreased in normal DIA (4 ± 0.7) compared to normal heart (12 ± 4.7). In the cardiac muscle, omega-3 decreased valine and aspartate (36 % decrease). In the dystrophic DIA, which showed higher levels of fibrosis than *mdx* heart, omega-3 reduced metabolites related to inflammation (aspartate) and oxidative stress (oxypurinol). We demonstrated that omega-3 can distinctly affect metabolic pathways in dystrophic heart and diaphragm, at later stages of dystrophy.

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Targeting pkcθ in mdx to ameliorate muscular dystrophy

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Protein kinase C θ (PKCθ) is a member of the PKCs family highly expressed in both immune and skeletal muscle cells; given its crucial role in adaptive, but also innate, immunity, it is being proposed as a valuable pharmacological target for immune disorders. We previously showed that lack of PKCθ in *mdx*, the mouse model for Duchenne muscular dystrophy (DMD), in the bi-genetic *mdx*/θ −/− mutant, improved muscle healing and regeneration, preventing massive inflammation. We show here that the observed phenotype is primarily due to lack of PKCθ in hematopoietic cells, as revealed by BM transplantation experiments. Indeed, our preliminary results suggest that lack of PKCθ is associated with a significant reduction in the frequency of CD45 + leukocytes (CD3 + lymphocytes and F4/80 + CD11b + macrophages) in dystrophic muscle during the early stages of the disease. This reduction in inflammatory cell infiltration was associated with a reduction in muscle fiber damage and increased regeneration potential. Moreover, we show that pharmacological inhibition of PKCθ activity in young *mdx* mice leads to a reduction of muscle damage and inflammation, and improves muscle performance. Together, these results suggest that PKCθ can be proposed as a pharmacological target to ameliorate the progression of muscular dystrophy, reducing the activation of the inflammatory pathways.

Histone deacetylase 4 is necessary for proper skeletal muscle homeostasis

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Epigenetic regulation of gene expression plays a central role in physiological or pathological processes. Although the precise mechanisms underlying epigenetic gene regulation in the pathogenesis of several diseases remain unknown, the finding that the progression of these diseases can be altered by modulating epigenetic programs, e.g. by Histone Deacetylase inhibitors (HDACi), is highly promising for medical purposes. HDACi have been used in the treatment of various cancers; in neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), and are currently in clinical trial to promote muscle repair in muscular dystrophies. However, there are many side effects associated with HDACi long-term treatments that negatively affect the patients' quality of life. The assignment of the biological functions to each individual HDAC is a prerequisite to improve pharmacological treatments of muscular diseases based on HDACi. HDAC4, a member of class II HDACs, mediates many cellular responses. In skeletal muscle, HDAC4 plays a crucial role in the regulation of skeletal muscle regeneration [1], muscle fiber identity [2], muscle mass and reinnervations following denervation [3]. Clinical reports suggest that inhibition of HDAC4 can be beneficial to cancer cachexia, dystrophic or ALS patients. However, the molecular pathways controlled by HDAC4 in these diseases are not delineated yet. With this rational, we started investigating the role of HDAC4 in skeletal muscle by implanting Lewis lung carcinoma (LLC) in mice lacking HDAC4 in skeletal muscle (HDAC4mKO), and by generating both dystrophic (mdx) and ALS mice (SOD1G93A) lacking HDAC4 in skeletal muscle. Strikingly, lack of HDAC4 in skeletal muscle resulted in a worsening of skeletal muscle atrophy induced by both LLC and neurodegeneration of motoneurons, as well as in increasing myofiber fragility and muscle wasting in dystrophic mice. The aggravation of the dystrophic phenotype induced by the absence of HDAC4 in skeletal muscle may be partially due to the impairment in skeletal muscle regeneration observed in HDAC4mKO mice. Our results indicate that HDAC4 is important and protective for maintaining skeletal muscle homeostasis and function, revealing new potential role for HDAC4 in skeletal muscle.

References:

1. Choi et al. (2014) *EMBO Rep Nov* **15**: 1175–1183.
2. Cohen et al. (2015) *Mol Cells* **47**: 122–132.
3. Moresi V et al. (2010) *Cell* **143**: 35–45.

Mitochondrial-induced oxidative stress triggers Ca^{2+}-dependent proteolysis and underlies Diaphragm Dysfunction induced by Mechanical Ventilation

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Rationale. In rodents, prolonged controlled mechanical ventilation (CMV) and associated diaphragm inactivity elicits complex pathologic changes referred to as ventilator-induced diaphragm dysfunction (VIDD). Previous workers have demonstrated that VIDD is characterized by increased mitochondrial generation of reactive oxygen species (ROS) and redox-induced dysfunction in Ca^{2+} release. Powers et al. (2011) have demonstrated that SS-31, a mitochondrial-targeted antioxidant, markedly attenuates mitochondrial ROS generation. Since, the Ca^{2+}-release pathology associated with VIDD is thought to be secondary to myofiber redox changes, we hypothesized that SS-31 should markedly attenuate these abnormalities in myofibrillar Ca^{2+} regulation.
Objective. To test the hypothesis that SS-31, a mitochondrial-targeted antioxidant, attenuates the detrimental? Effects of prolonged CMV on diaphragm myofiber Ca^{2+} homeostasis.
Method. The cross-sectional area, force, ryanodine receptor function and Ca^{2+} dependent proteolysis in muscle fibers isolated from the diaphragm of mice mechanically ventilated for 12 h untreated and treated with SS-31, a novel mitochondria-targeted antioxidant (SS-31) were compared.
Measurements and Main Results: Specific force of diaphragm fibers decreased significantly 28 ± 5 %, ($n = 10$, $p < 0.01$) following 12 h of mechanical ventilation compared to fibers from unventilated control mice. These changes were associated with a significant decrease in cross-sectional area (574 ± 19 *vs.* 673 ± 21 μm^2, $p < 0.1$), an increase in calpain activity, the oxidation of the RyR1 macromolecular complexed, RyR1 phosphorylation at Ser-2844 and the depletion of the stabilizing subunit calstabin1 resulting in an increase in sarcoplasmic reticulum (SR) Ca^{2+} leak. Fibers isolated from the diaphragm of mice treated with SS-31 were protected against all of these changes.
On the basis of these findings, we propose that SR Ca^{2+} leak via RyR1 due to mitochondrial induced oxidative stress triggers Ca^{2+}-dependent proteolysis and underlie VIDD. These results emphasize the need to develop therapeutic interventions that mitigate mitochondrial dysfunctional and thereby reduce mechanical ventilation-induced diaphragmatic weakness.

References:

Powers et al. (2011) *Crit Care Med* **39**: 7.

P2Y receptors and abnormal calcium homeostasis in dystrophic mouse myoblasts (mdx)

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Duchene muscular dystrophy (DMD) is the most common genetic muscle disease, leading to progressive muscle degeneration and premature death. It is caused by mutations in the DMD gene leading to a lack of dystrophin protein. In muscle cell development dystrophin becomes expressed after differentiation while protein is not detectable in myoblasts, irrespective of mutations in the *DMD* gene. Thus, it has not been considered important there and most of the data come from experiments on myotubes/myofibres in which dystrophin

is easily detected. In contrast to this accepted view, we have previously found substantial alterations in cellular calcium signalling, metabolism and autophagy in myoblasts derived from the *mdx* mouse (a commonly used animal model of DMD). Particularly, an increased level and activity of P2X7 receptor and activity of store-operated calcium entry have been the focus of our attention. Here we investigated whether metabotropic nucleotide receptors (P2Y) are also involved in dystrophy-related changes in calcium homeostasis in mouse myoblasts. We have found that stimulation of *mdx* myoblasts in vitro with ADP (P2Y1 agonist) under calcium-free conditions results in a rise of cytosolic Ca^{2+} concentration, which is more profound than that observed in wild-type cells. Substantially higher expression of P2Y1 protein estimated by the Western blot may, at least partially, explain higher susceptibility of *mdx* myoblasts to this nucleotide. Moreover, more intense calcium response to ATP in dystrophic myoblasts incubated in the calcium-free solution may indicate increased activity of P2Y2 and/or P2Y4 receptors, which are thought to be the most sensitive to this agonist. These observations not only confirm the presence and functionality of the various P2Y receptors in the mouse myoblasts, but also confirm the link between altered calcium signalling due to mutation in the dystrophin-encoding gene and the abnormal activity of specific P2Y receptors. Thus metabotropic G-protein-linked nucleotide receptors seem to emerge as an additional player in DMD pathophysiology. Finally, these results are in line with our previously formulated hypothesis that some cellular effects of mutations in the dystrophin-encoding gene are detectable at very early stages in muscle cell differentiation process.

CAPN3/p94/calpain-3 functions as a protease through inter-molecular complementation

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A skeletal muscle-specific protease, CAPN3 (also known as p94 or calpain-3), belongs to the calpain super family defined by the consensus protease core sequence referred to as "CysPc". An apparently Ca^{2+}-independent and exhaustive autolysis of CAPN3 has been reproducibly observed in several ex vivo protein-expressing systems. Therefore, CAPN3 is recognized as an intriguing yet intractable subject in terms of its enzymatic properties.

On the other hand, CAPN3 was identified as the responsible gene product for limb-girdle muscular dystrophy type 2A (LGMD2A), and various aspects of CAPN3′s biological functions were intensively studied. Along with the demonstration that defective CAPN3 protease activity is the primary cause for the disease, it has been shown that CAPN3 also has additional non-protease functions. Relationships between these two aspects of CAPN3 functions are yet to be defined.

With respect to the pathological mechanisms of LGMD2A, previously, we found a rare case that heterozygous mutations (two different missense mutations in *CAPN3*) results in more benign LGMD2A symptoms than those associated with homozygous single mutations. Positions of these two mutations in CAPN3 protein prompted us to hypothesize that the coexistance of two distinct CAPN3 mutants may lead to functional complementation between autolytic fragments generated from each mutant.

In fact, we found that the protease activity of CAPN3 can be reconstituted by *de novo* inter-molecular complementation (iMOC) between two autolytic fragments. Moreover, the autolytic activity of inactive full-length CAPN3 mutants was rescued by autolytic fragment-mediated iMOC. To our knowledge, such a protease activation mechanism has never been reported except for a few viral proteases.

So far, the investigation on regulatory mechanism of CAPN3 has focused on how CAPN3 circumvents autolytic degradation. With the present results, we hypothesize that the autolytic activity of CAPN3 is a positive means of self-adjustment to unique environment in skeletal muscle cells. Future studies aim to understand more comprehensively how CAPN3 exerts its function(s) and the molecular mechanism underlying LGMD2A.

Determining how mutations in the tail of β-cardiac myosin heavy chain cause skeletal muscle disease

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Mutations in the beta-cardiac myosin heavy chain are a common cause of both skeletal and cardiac muscle myopathies. Over 100 disease-causing mutations (~20 % of the total) have been identified in the filament-forming region of the myosin tail. 20 of these cause Laing's early onset distal myopathy (MPD1) and a further 65 cause hypertrophic cardiomyopathy (HCM). Intriguingly, mutations that cause MPD1 do not always appear to have a cardiac phenotype and the molecular pathogenic mechanisms are unclear.

Mutations in the coiled-coil tail could either affect the secondary structure, the ability of the myosin to form filaments, or both. First, we determined if any of five MPD1-causing mutations (E1508del, A1603P, K1617del, A1663P and L1706P) do affect the secondary structure of the coiled-coil tail. Expression constructs for the light meromyosin (LMM) region (661 residues), were generated, with and without each of these mutations. In addition, wild type (WT) and mutant 15 heptad peptides (105 residues) were also tested to investigate any localized structural changes that may be masked in the larger LMM. Four of the five mutations tested significantly decreased the helical content of the 15 heptad peptides compared to their WT counterparts, suggesting the mutations generate a local instability in the coiled-coil domain. The exception was E1508del, which did not affect the secondary structure. In the LMM constructs, only K1617del decreased the helical content. However, in thermal melting experiments, K1617del, A1663P and L1706P all increased the Tm (melting temperature) while A1603P decreased it, suggesting that the mutations do affect alpha-helical stability. We further tested the ability of GST-LMM to form filaments, which we imaged by negative stain electron microscopy. GST-LMM tends to assemble into discrete filaments that resemble native thick filaments rather than forming paracrystals. Mutant LMM isoforms tended to form filaments with much more variable lengths and widths, which is consistent with the hypervariable nature of myofibrils in MPD1 described in the literature. We have now generated adenovirus constructs of GFP-βMHC to test the ability of the mutants to incorporate into muscle sarcomeres in cultured skeletal and cardiac cells. Taken together, our results suggest that the majority of the MPD1 mutations we have tested both affect secondary structure and stability, and filament formation, which may underlie the pathogenesis of this disease.

Dystrophic cardiomyopathy is improved by deflazacort and doxycycline combined therapy

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In Duchenne muscular dystrophy (DMD) and in the *mdx* mice model of DMD, lack of dystrophin leads to progressive muscle degeneration and cardiomyopathy. Corticoids, such as deflazacort (DFZ), can delay the progression of the disease but show severe side effects. Doxycycline (DOX) has emerged as a potential therapy to dystrophy, as we previously demonstrated in skeletal muscles of the *mdx*. We evaluated whether a combined therapy of DFZ + DOX could result in greater improvement in cardiac *mdx* dystrophy than DFZ monotherapy, at later stages (17 months) of the disease. *Mdx* mice (8 months old) received the combined or monotherapy for 9 months. Control C57BL/10 and *mdx* mice (untreated) received water for the same period. Histopathological (fibrosis area), biochemical (cardiac CK), functional (electrocardiography) parameters and molecular markers (TGF-β, MMP-9, TNF-α, NF-kB, 4-HNE) of dystrophy were evaluated by Western blot. Cardiac fibrosis was greater in the right ventricle (16 ± 2) compared to the left ventricle (10 ± 2; $p < 0.05$, Anova). The combined therapy was superior in improving the dystrophic phenotype compared to monotherapy mostly for the fibrosis area (right ventricle: 60 % decrease with combined *x* 40 % decrease with DFZ alone) and TGF-β levels (6.8 ± 3.2 in *mdx*-untreated *x* 2.8 ± 1.4 in combined and 4.6 ± 1.7 in monotherapy; $p < 0.05$, Anova). 4-HNE, a marker of oxidative stress, was educed by 46 % by the combined therapy and 27 % by DFZ alone. Combined therapy better improved the electrocardiography profile compared to monotherapy. The main corrections were seen in the cardiomyopathy index (0.8 ± 0.1 for combined *x* 1.0 ± 0.2 for DFZ), heart rate (418 ± 46 bpm in monotherapy *x* 457 ± 29 bpm in DFZ), QRS interval (11.3 ± 2 in combined *x* 13.6 ± 1 in DFZ) and Q wave amplitude (-40.7 ± 21 in combined *x* -90.9 ± 36 in monotherapy). Both therapies were similar in reducing cardiac CK (about 53 %). TNF-α, NF-κB and MMP-9, markers of inflammation, were similarly reduced (about 52 % with both therapies). The combination of DFZ with DOX ameliorated the *mdx* cardiomyopathy at later stages of the disease. The present study offers data to support preclinical studies to treat cardiac failure, which is currently a significant cause of death in DMD.

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SNAIL transcription factor is a novel regulator of rhabdomyosarcoma differentiation

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Rhabdomyosarcoma (RMS) is a soft tissue non-epithelial tumor, which may originate from an impaired differentiation of skeletal muscle progenitors. Despite a lot of effort, precise mechanism of RMS development is not known. In our studies we hypothesized that RMS development, growth and differentiation is regulated by SNAIL. It is a zinc finger transcription factor, which regulates the epithelial to mesenchymal transition (EMT)—the main mechanism responsible for embryogenesis and epithelial tumor progression. Recently published data demonstrated also an important and non-canonical SNAIL role in differentiation of murine myoblasts (Soleimani VD et al., 2012, *Mol Cell* **47**: 457–468). The aim of our studies was to investigate the role of SNAIL in myogenic differentiation of RMS, which may be of significance for tumor development and growth.

Firstly, we demonstrated that SNAIL expression is elevated in alveolar subtype of RMS (ARMS), which is associated with worse prognosis, compared to embryonal RMS (ERMS). Moreover, the forced differentiation of ARMS and ERMS cells in vitro diminished SNAIL level. Subsequently, in one of ARMS cell lines (RH30), SNAIL expression was silenced by transduction with shRNA lentiviral vectors. Control cells were modified with scrambled shRNA vectors. RH30 cells with the diminished SNAIL level acquired an elongated phenotype, displayed reorganization of actin cytoskeleton and started to form structures resembling muscle fibers when they were cultured in differentiating medium with 2 % horse serum. Accordingly, silencing of SNAIL resulted in upregulation of several myogenic factors, such as for example myosin heavy chain. SNAIL silencing diminished also MyoD binding to E-box sequences, what may lead to enhanced differentiation of the cells. Similar effects of SNAIL on the level of molecular myogenic factors were also observed in human myoblasts after SNAIL silencing with siRNA. Currently, chromatin immunoprecipitation assay enable us to reveal novel targets of SNAIL, which have not been previously shown in RMS and we investigate significance of this effect in vivo.

Taken together, our results for the first time demonstrate interplay between SNAIL and myogenic factors in differentiation of RMS, what is of significance for tumor growth in vivo. We believe that in future they may open new avenues for differentiation therapies of RMS.

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Transplantation of wild type bone marrow cells repairs *mdx* mice dystrophin synthesis and neuromuscular junctions structure after non-lethal irradiation

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Mdx mice are a model of Duchenne muscular dystrophy caused by deficiency of dystrophin. Muscles of *mdx* mice are characterized by a high level of striated muscle fibers (SMFs) death and accordingly by a high level of regeneration. Neuromuscular junctions (NMJs) in *mdx* mice are altered. The cell based *mdx* mice dystrophy therapy by different stem cells is widely investigated now. A few studies are devoted to the transplantation of C57BL/6 mice bone marrow (BM) stem cells to lethal irradiated *mdx* mice. However it was shown that number of dystrophin-positive fibers in BM transplanted lethal irradiated *mdx* mice wasn't increased. Previously, we have shown that BM cells transplantation after non-lethal 3 Gy X-ray irradiation leads to growth of the number of dystrophin-positive SMFs and restoration of NMJs structures. Recovery of structure of NMJs was accompanied by recovery of the resting potential of the diaphragm end-plate membrane. In this study we investigated NMJs structure and dystrophin synthesis at 9, 12 month after BM transplantation. We observed a peak value of the part of dystrophin-positive SMFs in m. quadr. femoris *mdx* mice at 6 month after transplantation, which was accompanied by accumulation of SMFs without central nuclei. Then the part of dystrophin-positive SMFs decreased to 7.4 ± 1.5 % at 9 month and up to 5.1 ± 1.1 % at 12 month after transplantation. Similar changes of part of dystrophin-positive SMFs after BM cells transplantation were observed in diaphragm of *mdx* mice. The part of diaphragm NMJs with clusters of acetylcholine receptors (AChRs) distributed as continuous

branches was increased from 8.4 ± 2.7 % (control *mdx* mice) up to 49.1 ± 2.1 % (*mdx* mice, 6 month after transplantation) and part of NMJs with AChRs clusters distributed as islands was decreased from 90.9 ± 2.2 % (control *mdx* mice) up to 48.2 ± 2.3 % (*mdx* mice, 6 month after transplantation). Then the part of diaphragm NMJs with clusters of AChRs distributed as continuous branches was decreased to 37.4 ± 2.7 % and the part of diaphragm NMJs with clusters of AChRs distributed as islands was increased to 61.2 ± 2.7 % by 12 month after transplantation. Transplantation of wild type BM stem cells after non-lethal X-ray irradiation results in the growth of part of dystrophin-positive SMFs up to 6 month after transplantation and restoration of NMJs structure at all investigated time points.

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DYSF- and *ANO5*-related limb girdle dystrophies: genetic, clinical, and biochemical diversity in Polish patients

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The limb girdle-muscular dystrophies (LGMDs) are highly heterogeneous group of myopathies with autosomal inheritance. These disorders show progressive changes characterized by weakness and wasting of the pelvic and shoulder girdle muscles. LGMD patients exhibit phenotypic and genetic diversity, and mutations within the same gene often cause different phenotypes. Almost 30 types of LGMD are distinguished based on the causative mutation. We analyzed Polish LGMD patients and so far we found mutations in *DYSF* and *ANO5* genes.*DYSF* encodes dysferlin, a transmembrane protein that has important role in skeletal muscle repair, and *ANO5* encodes anoctamin 5 that may act as a calcium-activated chloride channel. Mutations in these genes cause of LGMD 2B and LGMD 2L types, respectively. The WES analysis showed the presence of several novel probably causative point mutations in *DYSF* in 5 patients, and two mutations in *ANO5* in one patient. Interestingly, more than one mutation in *DYSF* was found in one patient. The muscle biopsies of those patients were subjected to western blot and immunohistochemistry analyses. Western blot analysis revealed that mutations within *DYSF* though all affecting the middle and C-terminal part of the protein caused either increase or substantial decrease of dysferlin. Mutations in *ANO5* resulted in expression of the truncated protein.

Thus the WES analysis indicates that LGMDs could be in fact oligogenic and not monogenic diseases.

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Intracellular translocation of chaperones and cochaperones in human skeletal muscle myopathies: a correlative immuno-electron microscopical study

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Skeletal muscle diseases encompass a wide variety of acquired and hereditary disorders. The pathomechanisms include structural and functional changes affecting, e.g. the cellular metabolism of structural and contractile properties of sarcomeres. Heat shock proteins (HSPs) are important components of the cellular protein quality control machinery as they bind to partially unfolded client proteins, hold them in a folding-prone state, and protect them from aggregation. Several HSPs directly link to the chaperone-assist selective autophagy (CASA) and proteasomal degradation pathways. Many HSP are upregulated in skeletal myopathies.

Using pre-embedding immuno-electron microscopy we investigated at the ultrastructural level the localisation of various chaperones and co-chaperones in normal human skeletal myocytes and in disease myocytes from filaminopathy and calpainopathy (LGMD2a) patients. Correlative high resolution microscopy revealed that in the myopathy samples several small HSP translocate from the cytosol to the myofibrils, where they bound to the z-disc and/or I-band. The two small HSPs HSP27 and alpha B-crystallin, translocated in disease myocytes preferentially to the titin springs, as seen in stretched sarcomeres. In the LGMD2a and filaminopathy myocytes we observed a subcellular translocation of ATP-dependent HSP such as HSP90 from the cytosol to the elastic regions of titin.

This findings suggest that the HSPs may have a protective effect on the structural and mechanical sarcomeric proteins to prevent their aggregation and loss of function. The protective effect of alpha B-crystallin and HSP27 on the titin springs includes the prevention of titin aggregation-induced increases in titin-based passive tension. Other heat shock proteins presumably help maintain the structural and functional integrity of the z-disc.

Marker proteins of muscular dystrophy in mildly and severely affected *mdx* mouse muscles characterized by proteomic and immunofluorescence analyses

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Duchenne muscular dystrophy (DMD) and the homologous mouse model *mdx* are recessive X-linked hereditary diseases characterized by the loss of the cytoskeletal protein dystrophin. Despite of the same primary abnormality *mdx* muscles can be differently affected by dystrophic processes, such as fiber degeneration and fibrosis (severe to mild phenotype: *diaphragm* (DIA), *soleus* (SOL), *extensor digitorum longus* (EDL), *flexor digitorum brevis* (FDB), *interosseous* (INT)). In order to compare and quantify secondary alterations in differently affected skeletal muscles we performed proteomic and immunofluorescence analyses of *mdx* and wildtype (WT) muscles. Total muscle protein extracts from 100 d old male C57BL/10ScSn Dmd^{mdx}—and WT mice were used for label-free mass spectrometry analysis. The proteomic analysis revealed an altered abundance in 35, 16, 18, 23, and 14 proteins in *mdx* DIA, SOL, EDL, FDB and INT muscles, respectively. Changed proteins are associated with distinct functional families, such as the cytoskeleton, the extracellular matrix, the contractile apparatus, the cellular stress response, Ca^{2+} homeostasis and metabolism. Proteomic analysis of *mdx* muscles revealed amongst other changes increased levels of lamin A/C in all dystrophic tissues investigated,

being > 3-fold higher in DIA and EDL, a marked increase in collagen isoform VI (preferentially in DIA) as well as substantially lower levels of parvalbumin (SOL, DIA and FDB). Immunofluorescence of the nuclear envelope protein lamin (isoform A/C) showed higher numbers of nuclei in cross sections of *mdx* EDL muscles in agreement with increased numbers of nuclei in regenerating muscle tissue. We did not find evidence for morphological abnormalities of myonuclei in dystrophic muscles except for the fact that most *mdx* fibers contained central nuclei. First data on muscle fiber type composition showed altered fiber type distribution in dystrophic muscles. *Mdx* SOL fibers stained for MHC I appeared to be more clustered in the center of the muscle. In *mdx* DIA type IIA and IIX fibers seemed to be more clustered than in WT muscles. Quantification of fiber types should reveal if the reduction of parvalbumin, can be explained by fiber type shifts from type IIB/X to oxidative type IIA and type I fibers. As expected collagen immunostaining showed an increased area in DIA cross sections of *mdx* mice. We conclude that the combination of proteomic analysis and immunofluorescence offers the opportunity to identify new muscle proteins involved in the pathomechanism of *mdx* muscular dystrophy and new biological disease markers. Since dystrophic muscle remodeling is complex due to ongoing degeneration, regeneration and fibrosis, a set of proteins rather than single protein markers will be needed to distinguish moderately affected from severely affected dystrophic muscles and moderate phases from severe phases in the course of the disease.

Antioxidant treatment prevents skeletal muscle dysfunction in adjuvant-induced arthritis rats

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Patients with rheumatoid arthritis (RA) show significant muscle weakness and this has a major impact on the disability in these patients. Here we examined mechanisms underlying arthritis-induced muscle weakness, focusing on the role of redox stress on contractile properties in fast-twitch extensor digitorum longus (EDL) muscles of rat with adjuvant-induced arthritis (AIA). AIA was induced in the knees of rats by injection of complete Freund's adjuvant and was allowed to develop for 21 days. EDL muscles from AIA rats showed decreased tetanic force per cross-sectional area and slowed twitch contraction and relaxation. Actin aggregates were observed in AIA muscles and these contained high levels of 3-nitrotyrosine protein adducts. Daily administration of EUK-134 (3 mg/kg/day), a superoxide dismutase/catalase mimetic, prevented both the decrease in tetanic force and the formation of actin aggregates in AIA muscles without having any beneficial effect on the arthritis development. These data imply that antioxidant treatment can be used to effectively counteract muscle weakness in inflammatory conditions.

Conditional Abrogation of p38α in myofibers attenuates denervation-induced muscle atrophy

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Loss of muscle mass increases the risk of falls and fractures, and could severely compromise the activities of daily living in the elderly. It is, therefore, important to learn more about the prevention and treatment for muscle atrophy (sarcopenia). However, the mechanisms underlying muscle atrophy still remain poorly understood. The purpose of our study is to analyze the potential role of p38α, one of the mitogen activated protein kinases that are responsive to stress stimuli, in muscle atrophy.

We first asked if p38α was activated in the muscle tissue following denervation. We performed western blot analysis using the tibialis anterior muscle collected from mice with sciatic nerve transection and found that phosphorylation of p38α was highly enhanced in the injured limbs compared to the untreated control limbs, indicating that p38α may play a role in muscle atrophy. To investigate the function of p38α in the process of muscle atrophy in vivo, we next generated mutant mice in which the floxed p38α alleles were excised under the control of a human muscle actin promoter ($Mapk14^{HMA}$). The $Mapk14^{HMA}$ mice showed no obvious abnormalities and grew normally under unchallenged conditions. However, we found that these mice were more resistant to denervation-induced muscle loss than wild type control mice. Of note, we found that the expression levels of two E3 ubiquitin ligases, Trim63/MuRF1 and MAFbx/Atrogin-1, early markers for muscle atrophy, were significantly decreased in the $Mapk14^{HMA}$ tibialis anterior muscle compared with that in control animals following sciatic nerve transection. Moreover, phosphorylation of FoxO3a, a transcription factor that regulates the transcription of these two E3 ligases, was highly increased (and hence the activity of FoxO3a was decreased) in the $Mapk14^{HMA}$ tibialis anterior muscle compared to that in the control mice following sciatic nerve transection.

Taken together, the present study showed that p38α was activated in the early stage of muscle atrophy and that $Mapk14^{HMA}$ mice were less sensitive to denervation-induced muscle atrophy than wild type animals. There was a decrease in the expression levels of E3 ubiquitin ligases and in the activity of FoxO3a in the $Mapk14^{HMA}$ tibialis anterior muscle following sciatic nerve transection, indicating that p38α functions as a positive regulator for FoxO3a. These data suggest that p38α may be a potential therapeutic target for muscle atrophy.

MicroRNA-99b reduces muscle protein synthesis in human primary myotubes

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Introduction: Sarcopenia is the natural and progressive loss of skeletal muscle mass that occurs with age and is linked to loss of

independence and quality of life. The onset and progression of sarcopenia is associated with an attenuation of muscle protein synthesis (MPS) and Akt/mTOR signalling in response to anabolic stimuli. MicroRNAs (miRNAs) play an essential role in muscle health and disease, including sarcopenia, but cause-and-effect relationships have not been investigated. Our group identified a number of miRNAs that were dysregulated in the muscle of an elderly population following an anabolic stimulus (PMID 25460913). One such miRNA, miR-99b, is known to target members of the Akt/mTOR signalling pathway. The aim of this study was to validate the role of miR-99b as a negative regulator of MPS and Akt/mTOR signalling in human primary myocytes.

Methods: Primary myocytes were isolated from the biopsies of 3 young (23.5 ± 5.0 y.o.) and 3 old (64.6 ± 4.0 y.o.) subjects. Myotubes were transfected for 8 h with either a scramble sequence or a miR-99b mimic. *In vitro* protein synthesis and protein degradation was assessed by the direct incorporation of 3H-tyrosine into the myotubes and the release of 3H-tyrosine from the myotubes respectively. The gene and protein levels of the members of the Akt/mTOR pathway and miR-99b predicted targets, mTOR, RPTOR and IGF-1R, were determined by qPCR and western blot. Differences were determined by 2-way ANOVA.

Results: Protein synthesis was measured over 24 h. While there is no change during the transfection period, protein synthesis is significantly reduced with miR-99b mimic for 16 h after transfection in young and old myotubes ($p < 0.001$) with no change to protein degradation. During this period there is also a significant decrease in mTOR mRNA expression ($p < 0.001$). No difference were observed in mTOR protein expression, RPTOR and IGF-1R mRNA ad protein expression. No changes were observed in the phosphorylation levels of Akt/mTOR signalling proteins.

Conclusion: The miR-99b mimic negatively regulates in vitro protein synthesis in young and old myotubes. Although one predicted target for miR-99b, mTOR, was downregulated at the mRNA level, no differences were observed in predicted target protein expression and Akt/mTOR signalling proteins. Further investigation is required to elucidate the mechanisms of MPS regulation by miR-99b. This study provides the first evidence for the role of a miRNA in the regulation of MPS in vitro.

Session 4: Smooth Muscle in Health and Disease

Oral presentations

Synaptopodin 2, a dual compartment protein, shuttles between nucleus and cytoplasm upon phenotype switch of vascular smooth muscle cells

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Objective: The actin binding and nucleating protein Synaptopodin 2 (Synpo2) is a dual compartment protein. Although Synpo2 is abundant in smooth muscle cells, its role in smooth muscle cells is not fully understood. We now investigated (i) shuttling of Synpo2 during phenotype switching in primary vascular smooth muscle cells (VSMCs) and (ii) the interaction of Synpo2 with importin alpha and 14-3-3 proteins in HEK293 cells.

Methods: Primary VSMCs from 8–10 week old rats were isolated and cultivated on acrylamide matrices, coated with different extracellular matrix proteins. Subcellular localization of Synpo2 was determined by specific antibodies, nuclear stains, and Alexa555-conjugated phalloidin. Expression of Synpo2 in differentiated and dedifferentiated VSMCs was determined by qRT-PCR. For the investigation of 14-3-3 and importin alpha mediated trafficking in HEK293 cells, a bimolecular fluorescence complementation assay (BIFC) was employed. Fluorescence signals were visualized by confocal laser scanning microscopy.

Results: In differentiated VSMCs Synpo2 localized mainly to the cytoplasm, while it shuttled to the nucleus upon dedifferentiation. Cytoplasmic localization in differentiated VSMCs went along with high expression levels of Synpo2. Cultivation of dedifferentiated VSMCs on laminin coated acrylamide gels restored the differentiated phenotype, while cells cultivated on collagen I matrices maintained the dedifferentiated phenotype. Similarly Synpo2 was detected in the nucleus in wire injured carotid arteries. In uninjured carotid arteries it was in cytoplasm. Using the BIFC assay we observed in HEK293 cells that trafficking of Synpo2 is accomplished together with importin alpha and the 14-3-3beta protein. Synpo2—14-3-3beta and Synpo2—importin alpha complexes were observed in the nucleus, indicating that Synpo2 forms a ternary complex with 14-3-3beta and importin alpha within the nucleus.

Conclusions: The close correlation of Synpo2 expression and localization during phenotype switching of VSMCs suggests that Synpo2 is involved in the differentiation process of VSMCs both in vitro and in vivo. Whether this is accomplished by stabilization of the actin cytoskeleton in the cytosol or whether Synpo2 has a more immediate function in regulating gene expression is presently not known.

Knock-down of caldesmon is not compatible with life and leads to fetal bladder hypocontractility

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Background: The putative smooth muscle regulatory protein caldesmon (CaD) binds actin, myosin, calmodulin and tropomyosin and is expressed in two isoforms generated by alternative splicing from the same gene: *h*-CaD present in differentiated smooth muscle (SM) and *l*-CaD present in dedifferentiated SM and a wide variety of non-muscle cells. Despite a large body of biochemical investigations CaD's role for contractile function of smooth muscle remained elusive. Suggested functions were inhibition of contraction and/or organization of the contractile apparatus of smooth muscle as it is localized to the actomyosin domain in differentiated smooth muscle.

Methods: To elucidate the in vivo function we generated a mouse model with targeted deletion of all CaD splice variants. Loss of CaD was verified by PCR-genotyping and southern and western blotting. E17-E18.5 longitudinal bladder strips were triton-skinned and mounted isometrically in a myograph, cross-sectional area (CSA) was determined from the length and weight of the strips.

Results: KO animals died perinatally whereas the life span of heterozygotes (Het) was normal. The most prominent morphological anomaly of E17-18.5 animals was an abdominal wall defect resulting in herniation of the gut and liver in 22/27 KO compared to 2/44 WT, but no other gross macroscopic or microscopic pathologies were observed. Mendelian distribution was maintained at E14 ($n = 112$ total, 29 WT, 51Het, 27 KO, 5 resorbed) but not at E18 ($n = 162$

total, 44 WT, 80Het, 27 KO, 11 resorbed). The body weight and the bladder to body weight ratio of KO at E18.5 were reduced by respectively ~14 and 25 % ($p < 0.05$) compared to WT. Maximal force/CSA (pCa 4.3) was significantly lower in KO compared to WT and was intermediate for Het (F_{max}/mm^2 WT 3.6 ± 1.6, n = 16, Het: 2.7 ± 0.33, n = 5, KO: 1.6 ± 0.2, n = 14, $r^2 = 0.9943$, $p < 0.05$). Ca^{2+}-independent contractions elicited by microcystin in respectively KO and WT bladder strips had similar amplitudes as those elicited by pCa 4.3. There was no major effect on relaxation kinetics nor on Ca^{2+}-sensitivity but the slope of the force-pCa relation was steeper (n_H WT 2.7 ± 0.2 *vs.* KO 3.4 ± 0.3, $p < 0.02$).

Conclusions: Ablation of caldesmon was perinatally lethal likely due to the abdominal wall defect. Force bearing capacity of skinned fetal bladder strips was significantly reduced consistent with the notion that caldesmon is important for the structural and functional integrity of the contractile machinery.

Molecular mechanisms of the muscarinic cholinergic excitation of intestinal smooth muscles

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Acetylcholine, the main excitatory neurotransmitter released by enteric motor neurons, plays a key role in the regulation of intestinal smooth muscle motility. It causes membrane depolarization and contraction of gastrointestinal myocytes by activating the M_2 and M_3 subtypes of muscarinic receptors [1]. While the M_2 receptor is preferentially coupled to pertussis toxin (PTX) sensitive $G_{i/o}$ proteins causing adenylyl cyclase inhibition and reduction in intracellular cAMP concentration, the M_3 receptor is predominantly coupled to PTX-insensitive $G_{q/11}$ proteins causing stimulation of phospholipase C (PLC), that results in the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) and formation of inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). The primary mechanism of the muscarinic cholinergic smooth muscle excitation is the opening of cation-selective channels, which produces inward cation current termed mI_{CAT}. This causes membrane depolarisation and secondary Ca^{2+} influx via L-type voltage-gated Ca^{2+} channels for smooth muscle contraction.

mI_{CAT} has a characteristic doubly-rectifying U-shaped I-V relationship, which is similar to the voltage-dependence of receptor-operated TRPC4/5 channels. Testing the hypothesis that these channels may mediate mI_{CAT} we found, using mouse gene knockout models, that indeed TRPC4 is the main molecular counterpart of the current [2]. Importantly, activation of both M_2 and M_3 receptor subtypes was indispensible for mI_{CAT} activation [3]. The molecular basis of such strong synergy between different muscarinic receptor subtypes is complex. First, there is a strong cross-talk between voltage and agonist sensitivity in this system [4]. Second, mI_{CAT} generation requires simultaneous activation of $G_{i/o}$ proteins (M_2-effect) and PLC (M_3-effect) [5, 6]. Signal transduction downstream of PLC activation is also complex and remains incompletely understood. It involves potentiation of mI_{CAT} via activation of IP_3 receptors and intracellular Ca^{2+} release [7] and PIP_2 inhibition of TRPC4-alfa isoform [8]. Intriguingly, PIP_2 depletion *per se* is not sufficient for TRPC4 activation, while the shorter TRPC4-beta isoform is insensitive to PIP_2.

Thus, in intestinal smooth muscles TRPC4 is activated in synergy by M_2 and M_3 receptors via several complexly intervening pathways. TRPC4 channel can therefore effectively integrate voltage-, receptor- and calcium signaling being an important focal point of cholinergic excitation–contraction coupling in gastrointestinal smooth muscles.

References:

1. Bolton TB et al. (1999) *Annu Rev Physiol* **61**: 85–115.
2. Tsvilovskyy VV et al. (2009) *Gastroenterol* **137**: 1415–1424.
3. Zholos AV, Bolton TB (1997) *Br J Pharmacol* **122**: 885–893.
4. Bolton TB, Zholos AV (2003) *Science STKE* pe52.
5. Yan HD et al. (2003) Br J Pharmacol **139**: 605–615.
6. Zholos AV et al. (2004) *Br J Pharmacol* **141**: 23–36.
7. Gordienko DV, Zholos AV (2004) *Cell Calcium* **36**: 367–386.
8. Otsuguro K et al. (2008) *J Biol Chem* **283**: 10026–10036.

Posters

Getting closer to the elucidation of the mechanism of curcumin-induced vascular smooth muscle cells senescence

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Curcumin is a natural polyphenol for centuries widely used not only in Indian cuisine but also in natural medicine. Basic research as well as clinical studies revealed that it can be considered antinflammatory, antioxidant, anticancer and antiaging agent. However, it is also able to induce cellular senescence in cancer cells as it has been documented in our laboratory. Our recent findings indicate that in some range of concentrations curcumin can also induce senescence of primary cells—namely vascular smooth muscle cells (VSMCs).

The aim of this study is to elucidate the mechanism of curcumin-induced senescence of VSMCs. DNA damage is a common cause of cellular senescence. We observed that after curcumin treatment the levels and activation of some components of the DNA damage response (DDR) pathway are elevated. At the same time, analysis of 53BP1 foci revealed decrease in the number of double strand breaks in VSMCse cells, suggesting that senescence is DNA damage independent.

Despite well proven antioxidant properties, curcumin, in the first mode of action, can act as a prooxidant. It was suggested that reactive oxygen species (ROS) can directly induce ATM autophosphorylation what is followed by activation of the DDR pathway. Therefore, we treated VSMCs with ROS scavengers (NAC or trolox) prior curcumin treatment and assessed the number of senescent cells and the level/activation of selected DDR pathway components. No differences were observed suggesting that ROS do not play a central role in the curcumin-induced senescence of VSMCs. Subsequently, we pursued to verify the involvement of ATM in the process. ATM silencing did not prevent senescence of VSMCs after curcumin treatment which let us conclude that ATM is not the main player in the curcumin-induced senescence.

We also observed transient increase in the level and activation of p38—mitogen activated protein kinase involved in another pathway leading to cellular senescence. Using p38 inhibitor (SB203580) we decreased the number of senescent cells, however, only slight. Therefore we suppose that senescence of curcumin-treated VSMCs is driven not by one but several complementary pathways.

Summarizing, despite its well documented beneficial effects, curcumin, in relatively low concentrations, can induce senescence of VSMCs. Our results indicate that attempts to increase bioavailability of curcumin should be carried out with great caution especially if the aim is the use in a diet supplementation.

Vascular Smooth Muscle (VSM): Modulation of the BK channel current by phosphorylation/dephosphorylation-linked cellular events

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Background: VSM excitability is regulated by large conductance, calcium-activated K + (BK) channels. Our research group has previously reported that type I cGMP-dependent protein kinase (cGKI) phosphorylates the BK channel at key serine residues, leading to augmented BK channel current magnitude [1]. Here, we investigate the cellular mechanisms regulating the temporal pattern (i.e. onset and decay) of stimulus-evoked, phosphorylation-dependent enhancement of BK channel current in VSM.
Procedures. **1.** BK currents were recorded from primary and cultured rat VSM myocytes by whole-cell patch clamp using a 500 ms step-protocol (−20 to 100 mV) delivered at five second intervals. **2.** Proximity ligation assay (PLA) was used to examine BKα subunit co-localization with other proteins, including cGKI (spatial restriction < 40 nm). **3.** For pressure myography measurements, isolated rat arterial segments were mounted in a myography chamber and examined under constant intraluminal pressure (i.e. 70 mmHg).
Results: Voltage clamp recordings demonstrated that VSM BK channel current was reversibly enhanced by the nitrovasodilator sodium nitroprusside (SNP, 100 mM) and sensitive to the highly-selective BK channel inhibitor, penitrem A (100 nM). The broad-spectrum phosphatase inhibitor, calyculin A (30 nM), augmented the SNP-mediated current enhancement, and further slowed the decline of this elevated current. Fostriecin (30 nM), a selective inhibitor of the protein phosphatase PP2A isoform, similarly enhanced the BK channel current magnitudes and slowed the decay. PLA fluorescence staining demonstrated the basal co-distribution of BK channels with cGKI, which was observed to increase following SNP treatment. PLA staining also confirmed co-distribution of BK channels with PP2A protein. In myogenically-active cremaster arteries, SNP evoked a reversible inhibition of myogenic tone that was significantly inhibited by penitrem A.
Conclusions: These findings suggest that cGKI and PP2A form a complex with BK channels to regulate the magnitude and time course of the NO-mediated BK current enhancement in VSM.

References:

1. Kyle et al. (2013) F*ASEB J* **5**: 2027–2038. doi: 10.1096/fj.12-223669.

Knock down of Caldesmon leads to hypocontractility in fetal (E17.5) murine aorta and basilar artery

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Background: Caldesmon is an actin and myosin binding protein which is localized in smooth muscle to the actomyosin domain. Hence, it may serve an important function for the structural organization of the contractile apparatus and contractility. To explore the function of caldesmon a mouse model with targeted deletion of the caldesmon gene (*Cald1*) was generated. This mutation was perinatally lethal in homozygous animals (KO), likely because of an abdominal wall defect, but heterozygous mice had a normal life span (Hets).
Methods: Ring segments of the abdominal aorta and basilar arteries from E17 to E18 fetuses were dissected and isometrically mounted in a wire myograph in bicarbonate buffered physiological saline solution.
Results: The passive length tension relations of the abdominal aorta were shifted to the right in KO compared to WT. Basal tone developed after pre-stretching to ~900 μM was 2.4 ± 0.4 mN in KO compared to 3.8 ± 0.5 mN in WT ($n = 3$). Inhibition of eNOS with 100 μM L-NAME increased basal tone to 2.8 mN in KO *vs.* 4.8 mN in WT mice ($n = 3$). Maximal U46619 elicited force was less in KO compared to WT aortas (3.4 ± 0.5 mN in KO *vs.* 5.2 ± 0.2 mN in WT; $p < 0.05$), but the potency in presence or absence of L-NAME was similar in both groups (pEC_{50} ~7.5). The rate of force development was slower in KO. Similar changes in passive length tension relations, basal or U46619 induced tone were observed in ring segments from fetal KO basilar artery (BA). In BA from aged, 24 month old, Hets the passive length tension relation was also shifted rightward compared to WT mice.
Conclusion: Caldesmon deficient smooth muscle is hypocontractile which may be explained by an impaired structural organization of the contractile apparatus, a possibility that we are currently exploring.

New myometrium plasma membrane calcium pump suppressor – calix[4]arene C-90 and its structural peculiarities which determine efficiency of its action

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Background: Current problem in nowadays research is the design of affine selective inhibitor for the plasma membrane Mg^{2+}, ATP-dependent Ca^{2+}-pump (PMCA). According to our results it could be calix[4]arene C-90 (5,11,17,23-tetra(trifluoro) methyl(phenyl-sulfonylimino)-methylamino-25,26,27,28-tetrapropoxycalix[4]arene). The influence of C-90 structure analogs was used to elucidate structural peculiarities which are important in its suppressor action.
Observations: All calixarenes (obtained in Institute of organic chemistry, prof. Kalchenko) structure used in this research was characterized by infrared spectroscopy and NMR methods. C-90 inhibition coefficient $I_{0.5}$ for PMCA ATPase activity was 20.2 ± 0.5 μM and PMCA Ca^{2+}-transport activity was also inhibited. The inhibition efficiency grew with increased number of phenylsulfonylimino groups in upper macrocycle rim and its distal orientation is more effective ($I_{0.5} = 53.4 \pm 3.6$ μM) than proximal ($I_{0.5} = 53.4 \pm 3.6$ μM). Absence of phenylsulfonylimino groups caused a loss of inhibitory power, nevertheless, substitution of phenyl in phenylsulfonylimino groups by methyl did not significantly influence inhibitory efficiency ($I_{0.5} = 29.2 \pm 0.9$ μM). Modification of macrocycle lower rim by octanol instead of propanol groups increased affinity of inhibitor ($I_{0.5} = 15 \pm 0.5$ μM) that points on the dependence of inhibition on calixarene hydrophobicity. Additionally, changes in ANS probe fluorescence denoted that C-90 is inbuilt into plasma membrane and could interact with PMCA transmembrane domains. Inhibitory action of C-90 on PMCA did not depend on the plasma membrane polarization.

Conclusions: PMCA activity inhibition by C-90 was associated with the cooperative action of four sulfonylimino groups, spatially oriented on the macrocycle upper rim and its hydrophobicity. Investigated structural peculiarities can be used for further construction of pharmacological agent on the base of C-90.

Are NOX4-derived ROS detrimental for induction of senescence?

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Cellular senescence is an irreversible proliferation arrest of the cells and is suggested to drive organismal aging. The most common trigger of senescence are double-strand DNA breaks (DSBs). One of the intrinsic factors that leads to DSBs are ROS produced mainly by mitochondria. Indeed increased level of oxidative stress has frequently been linked to aging. Although mitochondria are considered as the main source of ROS in the cells, the involvement of other sources like NADPH oxidases (NOXs) has also been linked to cellular senescence as well as age-related diseases such as atherosclerosis and cancer. The aim of our study was to determine the role of NOXs in the regulation of the cell cycle and cell arrest in different cellular contexts. The experiments were performed using normal cells derived from the vasculature—smooth muscle cells (VSMCs) and endothelial cells (ECs) as well as using human colon cancer cells (HCT116). To this end we treated cells with the NOX inhibitor—DPI or downregulated one of its isoforms—NOX4. Inhibition of NOX activity led to a diminished level of ROS as well as a significant growth arrest of both normal and cancer cells. Interestingly, temporal DPI treatment caused irreversible growth arrest—senescence, even when DPI was removed from the medium. To address the NOX4 exclusive role in these processes we compared the effect of NOX4 silencing in VSMCs, ECs and HCT116 cells. Decreased expression of NOX4 led to a rapid growth arrest of VSMCs, which underwent senescence within a few days. In contrary proliferation of both ECs and HCT116 cells was only temporarily inhibited and no significant induction of senescence was observed. The induction of senescence of VSMCs was correlated with decreased production of ROS, upregulation of the cell cycle inhibitor $p27^{Kip1}$ and dephosphorylation of pRb. This finally led to an escape from the cell cycle, as revealed by the lack of the expression of Ki-67. Our results show, for the first time, that apart from the detrimental role of ROS in the induction of senescence, diminished ROS signaling derived by NADPH oxidases led to permanent growth arrest. The role of a particular oxidase could differ depending on the cell type. Defining the signaling pathways affected by ROS produced by NOX in the context of senescence induction still remains under investigation.

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Calix[4]arene C-90 selectively inhibits Ca^{2+}, Mg^{2+}-ATPase of myometrium cell plasma membrane

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Calixarenes are macrocyclic oligophenolic compounds which are perspective for design of biologically-active substances. Previously it was found that calixarene C-90 efficiently inhibited ATPase activity of PMCA in myometrium cell plasma membrane fraction selectively compared with other ATPases located in plasma membrane. The aims of our investigation were to research molecular mechanisms of C-90 inhibition effect and elucidate the influence of noted compound on physiological function of PMCA.

We have studied kinetic characteristics of the Ca^{2+}, Mg^{2+}-ATPase enzymatic activity inhibition by calixarene C-90 and have shown the luck of competition of calixarene C-90 with ATP, Ca^{2+} and Mg^{2+} during the interaction with enzyme. Computer simulation of the calixarene C-90 interaction with Ca^{2+}, Mg^{2+}-ATPase allowed us to show that the most probable site of calixarene association with enzyme locates on transmembrane domain of mentioned enzyme. According to the results of confocal microscopy study calixarene C-90 increased Ca concentration in cytoplasm of unexcited myometrium smooth muscle cells by 45 ± 9 %. However, during the next 1.5–2 min the calcium concentration was returned to initial level. Additionally, with method of laser correlation spectroscopy an effective hydrodynamic diameter of smooth muscle cells was determined. Calixarene C-90 (50 μM) reduced noted parameter by 26 %, which is similar to uterotonic oxytocin impact (100 nM). Also calixarene C-90 (10 μM) decreased maximum relaxation velocity of uterine smooth muscle normalized on contraction amplitude on 20 % measured in spontaneous muscle activity with tensometric method.

Obtained data can be useful for subsequent prediction and construction of new effective and selective inhibitors of PMCA on the base of calixarene C-90. Calixarene C-90 is perspective for creation of new pharmaceuticals in order to regulate muscle contractility and tone.

Session 5: Excitation–Contraction Coupling

Oral presentations

Alterations in Store Operated Calcium Entry mechanism modifies SR calcium release

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The intracellular Ca^{2+} store depletion-induced calcium entry mechanism (SOCE) of flexor digitorum brevis (*FDB)* muscles of 4–6 months old mice of two different mouse models was examined. The Compact (*Cmpt*) mice with a 12 bp deletion in the myostatin gene exhibit a remarkable hypermuscular phenotype due to both myofiber hypertrophy and hyperplasia. However, they have greater propensity for metabolic acidosis, impaired respiratory and cardiovascular systems, and fatigue more readily. The genetic mouse model in which the embryonic type calcium channel of $Ca_V1.1$ ($Ca_V1.1$DE29) is permanently expressed in adult displays a significant calcium influx component during EC coupling. Although overall motor activity of $Ca_V1.1$DE29 mice was normal, isolated muscles exhibited reduced muscle strength and increased fatigue resistance. Consistent with these physiological changes the fiber type composition of slow and fast muscle types was shifted towards slower fiber types.

Fibers were loaded with the fluorescence calcium indicator Fluo-8. Sarcoplasmic reticulum (SR) calcium stores were first depleted by applying a cocktail of calcium release activators and re-uptake

blockers in a zero-calcium solution. Then extracellular calcium was re-applied to allow calcium influx and refilling of the stores. When re-applying calcium in the external solution, a slowly activated secondary increase of fluorescence was detected in the control fiber, indicating, presumably, activation of SOCE and Ca^{2+}-influx via Orai1 channels. This, however, was much less pronounced in case of fibers of *Cmpt* mice. Preincubation with 10 μM BTP2, a potent SOCE inhibitor, significantly blocked SOCE activation in *wt* but not in the *Cmpt* mutant. While in *wt* muscle fibers store depletion-induced calcium influx was not affected by the application of the L-type calcium channel blocker nisoldipine, in $Ca_V1.1$DE29 muscle fibers it was dramatically reduced, indicating that in $Ca_V1.1$DE29 expressing muscle SR refilling is predominantly carried by calcium influx through L-type channels. A simple three compartment model predicted an exponential decay of SR calcium content during depleting stimulation and enabled the estimation of the difference in SOCE rate between the different mouse models.

Maturation and contractile properties of induced pluripotent stem cell derived cardiomyocytes from a Duchenne Muscular Dystrophy patient

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Duchenne Muscular Dystrophy (DMD) is a wasting disease of striated muscle resulting from membrane fragility. Cardiac complications in DMD patients became prominent only recently and the molecular sequelae due to the lack of dystrophin are still poorly understood. To this aim, we utilized human induced pluripotent stem cells derived cardiomyocytes (hiPSCs-CMs) from DMD patients as a platform for modeling DMD cardiomyopathy. Here we used an approach to develop hiPSC-CMs in culture that more closely resemble adult cardiomyocyte dimensions and morphology. We compared the contractile properties of hiPSC-CMs (generated from urine-derived cells) from a healthy individual (WT-CMs) with those from a DMD patient (DMD-CMs) with a mutation that results in total lack of dystrophin (deletion of Exon 50). To dissect the role of the sarcomere, we isolated myofibrils from hiPSC-CMs with a newly designed procedure to assess both force and kinetics of activation and relaxation with a custom-built apparatus.

Day 20 post-differentiated hiPSC-CMs were replated onto fibronectin-coated nanogrooved surfaces and cultured for 3 months. Both DMD- and WT-CMs were usually $> 100 +$ μm in length with clearly defined myofibrils and aligned Z-bands. Interestingly, 3D confocal images for caveolin 3 revealed finger-like invaginations tightly associated to Z-bands, likely representing T-tubules, in WT-CMs but absent in age-matched DMD-CMs. Intact DMD-hiPSC-CMs paced at 1 Hz showed no difference in the rate of cell shortening but depressed response under inotropic intervention (post rest potentiation) and prolonged relaxation (t_{50}). Ca^{2+} transients showed higher amplitude, prolonged decay and increased diastolic Ca^{2+} level at any pacing frequency (0.5–12 Hz). Preliminary data for myofibril mechanics of DMD-CMs showed lower force development and prolonged relaxation phase (increased duration of slow phase t_{REL}, slow, slower rate of fast phase k_{REL}, fast) but no differences in the other kinetic properties. Moreover, DMD-CM myofibrils exhibited increased calcium sensitivity of force development (decrease pCa50). We are investigating the structural maturation and protein composition of myofibrils to elucidate mechanisms that lead to slower relaxation in DMD-CMs.

We conclude that hiPSC-derived DMD-CMs (1) can be a viable model for mechanical studies of the development of DMD cardiomyopathy in vitro (2) showed diastolic dysfunction due to both myofibril properties and Ca^{2+} handling alterations.

Posters

Calcium regulatory proteins and the relation to muscle myalgia before and after a physical exercise intervention

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Aim of Investigation: Trapezius myalgia is the most common type of chronic neck pain. While physical exercise reduces pain and improves muscle function, the underlying mechanisms remain unclear. The symptoms of work related chronic pain is muscle stiffness, weakness and tension. The aim of the present study is to elucidate the underlying mechanisms behind chronic work related muscle pain utilizing muscle biopsies investigating the difference in abundance of calcium regulatory proteins determining the contractile properties of the muscle.

Methods: Forty-one women clinically diagnosed with trapezius myalgia (MYA) and 18 healthy controls (CON) were included in the case–control study. MYA were randomly assigned to either 10 weeks of specific strength training (SST, $n = 18$), general fitness training (GFT, $n = 15$), or health information (REF, $n = 8$). Abundance of calcium regulatory calsequestrin proteins connected to the excitation contraction coupling mechanisms in the muscle were investigated for their abundance using immunohistochemistry and western blot analysis.

Results: Distribution of fiber type, cross-sectional area did not differ between MYA and CON. The abundance of calsequestrin was lower in MYA $p = 0.010$; and the distribution of calsequestrin-1 in different fiber types differed in accordance to fiber type.

Conclusions: The lower abundance of calsequestrin protein in myalgic muscle presented with this analysis could be an indication of an adaptation due to peripheral sensitization or increased neuronal signaling shown in previous studies on myalgic muscle, altering the contrability of the muscle presumably producing the symptoms of myalgia.

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Altered sarcoplasmic reticulum Ca^{2+} release in muscle fibers from MTM1-deficient mice: spatially discrete defects and partial rescue by phosphatidylinositol 3-kinase inhibition

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Myotubular myopathy is due to mutations in the gene encoding the phosphoinositide (PtdIns*P*) phosphatase MTM1 that dephosphorylates PtdIns3*P* and PtdIns(3,5)P_2. Excitation–contraction (EC) coupling is disrupted in muscle fibers from MTM1-KO mouse and this likely makes a major contribution to the disease-associated fatal muscle weakness. The reason why MTM1 is necessary for proper EC coupling function remains though unclear. Using confocal line-scan detection of rhod-2 Ca^{2+} transients in combination with voltage-clamp, we found that the spatially averaged sarcoplasmic reticulum (SR) Ca^{2+} release flux in muscle fibers from MTM1-KO mice exhibits a decreased maximum amplitude and an increased time to peak over the full range of voltage activation. For instance, in response to a pulse from −80 to +10 mV, mean values for maximum amplitude and time to peak of Ca^{2+} release were 46.5 ± 5 and 17.8 ± 3 μM ms^{-1} and 10.2 ± 4 and 26.4 ± 3 ms in wild-type (WT, n = 20) and MTM1-KO fibers (n = 15), respectively. Exposure of muscle fibers to the two pan-PtdIns 3-kinase inhibitors Wortmannin and LY294002 had no effect in WT fibers but enhanced peak Ca^{2+} release in MTM1-KO fibers by 60–80 %, suggesting that PtdIns 3-kinase activity acutely contributes to the depressed Ca^{2+} release. The effect occurred though with no significant concurrent reduction of the time to peak. The promoting effect of Wortmannin and LY294002 on Ca^{2+} release was not reproduced by 3-methyladenine, a specific inhibitor of class III PtdIns 3-kinase. In MTM1-KO fibers, examination of the rhod-2 confocal images revealed strong heterogeneity of the Ca^{2+} transient along the scanned line, due to spatially discrete failing regions yielding either reduced peak amplitude and/or delayed activation. Local defects re-occurred at the same positions upon repeated activation of Ca^{2+} release, showing these alterations to be stably associated with specific groups of triads. The spatial heterogeneity in MTM1-KO fibers appeared to be unchanged by treatment with the pan-PtdIns 3-kinase inhibitors. Expression of a mCherry-tagged PtdIns(3,5)P_2 binding probe gave no indication that PtdIns(3,5)P_2 distribution and density are altered in MTM1-KO fibers. Overall results demonstrate that MTM1 deficiency generates complex and spatially inhomogeneous Ca^{2+} release alterations. Although triadic structure defects may be involved, increased level of PtdIns 3-kinase products also seems to play an immediate role in the decreased Ca^{2+} release.

The preload-dependent regulation of Ca^{2+} transient decay is species-specific

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The right ventricular (RV) myocardium of healthy rat has unique preload-dependence of Ca^{2+} transient (CaT) decay: while monotonous in non-stretched isolated muscle, this decay exhibits the phase of brief deceleration ("bump") in stretched muscle. Moreover, the magnitude and duration of the "bump" are progressively elevated with preload. The putative mechanism of the "bump" is the length-dependent change in the interaction between regulatory protein troponin C and Ca^{2+}. An intervention affecting free cytosolic calcium may therefore intensify or alleviate the expression of the "bump". The investigation of this effect is important to clarify its role in the preload-dependent regulation of myocardial contractility in rats and other mammals.

We examined the influence of preload (which affects Ca^{2+} utilization by contractile proteins) and temperature (which affects Ca^{2+} sequestration into sarcoplasmic reticulum via SERCA2a) to the expression of "bump" in rat and guinea pig RV trabecula. Additionally, the effect of SERCA2a inhibition by thapsigargin was tested. CaTs were measured in trabecula at variable preload (from 0.8 to 1.0 Lmax) and temperature of saline solution (25, 30, 35 °C). The pacing rate was 1 Hz and physiological Ca^{2+} concentration in the solution was used.

We following results were obtained: i) temperature does greatly affect the shape of CaT decay in guinea pig RV myocardium (monotonous decay at 25 °C, the "bump" phase at 30 °C, the "plateau" phase at 35 °C) but not in rat RV myocardium (the basic characteristics remain unchanged); preload does affect the shape of CaT decay in rat RV myocardium (the "bump" magnitude and duration elevate in parallel with progressive stretch of a muscle) but not in guinea pig RV myocardium (if existing, the "bump" did not change magnitude or kinetics); iii) an increase in temperature does accelerate CaT decay in rat and guinea pig RV myocardium; iv) an inhibition of SERCA2a substantially decelerates CaT decay in rat and guinea pig RV myocardium.

We concluded that both indirect (temperature) and direct (thapsigargin) effects on CaT decay are expressed in healthy RV myocardium of rats and guinea pigs. In contrast, strong preload-dependent modulation of the shape of CaT decay is observed in rat RV myocardium but not in the guinea pigs. This species-specific difference in the preload-dependent regulation of CaT decay may arise from the totally faster $[Ca^{2+}]_i$ in rat myocardium compared to the guinea pig myocardium. Such fast Ca^{2+} removal from cytosol in rat myocardium may facilitate to observe the length-dependent changes in the shape of CaT decay during cardiac relaxation. On the other hand, relatively slow Ca^{2+} removal from cytosol in guinea pig myocardium masks changes in CaT decay which are related to the length-dependent regulation of Ca^{2+} dissociation from troponin C.

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The calcium homeostasis may change in some myotonic dystrophies

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In skeletal muscle excitation contraction coupling the voltage-gated L-type calcium channel plays a crucial role as voltage sensor. The embryonic type of this calcium channel (CaV1.1e) displays a modified voltage-dependence and gating kinetics as compared to that expressed in adult mammalian skeletal muscle. The latter opens slowly at strong depolarizations, and it has negligible role as a source of calcium influx during a short action potentials. In contrast, calcium influx through the embryonic CaV1.1e substantially contributes to depolarization-induced calcium transients in cultured myotubes and in fetal skeletal muscles.

We generated a genetically modified mouse (CaV1.1DE29), which exclusively expresses the embryonic CaV1.1e variant also in adult muscle. In this animal the external calcium influx component through CaV1.1e is

maintained lifelong. This is similar in some human myotonic dystrophy, which is a clinically and genetically heterogeneous muscle disorder.

Using this mouse, elementary calcium release events (ECRE) were recorded in enzymatically isolated, intact adult skeletal muscle fibers from the *m. flexor digitorum longus* using the fluorescent calcium probe fluo-8 and the fast confocal microscope.

While control animals did not display such events, CaV1.1DE29 mice spontaneously generated ECRE with a frequency of $9.2 \times 10^{-4} \pm 6 \times 10^{-4}$ Hz/μm^2 (4, mice, 19 fibers; mean ± SEM) in elevated (5 mM) external calcium concentration. The role of external calcium as the trigger was proved by either decreasing the extracellular calcium concentration to normal (1. 8 mM) or by applying 5 μM nizoldipine to block the calcium current through CaV1.1e. Both interventions resulted in a complete loss of the calcium events. Identified ECREs were classified as short and long events. The short, spark like events (n = 125) were characterized by an average amplitude (DF/F_0) of 0.41 ± 0.01, a full-width at half-maximum of 1.65 ± 0.05 μm, and duration of 46.0 ± 1.5 ms. The long, ember like events (n = 218) were characterized by an average amplitude of 0.32 ± 0.01, and duration of 473 ± 14 ms. These parameters are clearly different from the properties of calcium sparks and embers on saponin-permeabilized adult mammalian skeletal muscle fibers.

These changes originated the sustained expression of the CaV1.1e splice variant and may be present in myotonic dystrophy and could contribute in increased muscle tone and fatigability.

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Decline of S-glutathionylation of troponin I fast isoform induces prolonged low-frequency force depression in the late stage of recovery

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Vigorous muscle contractions lead to a greater loss of force at low compared to high stimulation frequency and prolonged recovery is required for restoring force, which is called "prolonged low-frequency force depression (PLFFD)". Our previous study suggested that *S*-glutathionylation (*S*-glut) of troponin I fast isoform (TnIf) offsets a decrease in myofibrillar (myo-) Ca^{2+} sensitivity in the early stage of recovery from PLFFD. The purpose of this study was to examine the contribution of *S*-glut of TnIf to myo-Ca^{2+} sensitivity during recovery from PLFFD. Intact rat gastrocnemius (GS) muscles were electrically stimulated via the sciatic nerve until force was reduced to ~50 % of the initial. The superficial regions of GS muscles were excised 0, 0.5, 2, 6 and 12 h after the end of fatiguing stimulation and used for skinned fiber and biochemical analyses. In whole GS muscles, the ratio of force at 20 Hz to that at 100 Hz was decreased at 0.5, 2 and 6 h of recovery. Skinned fibers were activated in heavily buffered Ca^{2+} solutions to assess myo-Ca^{2+} sensitivity. A Ca^{2+} concentration at half-maximum force ($[Ca^{2+}]_{50}$) was unaltered at 0.5 and 2 h of recovery, whereas it was increased at 0 and 6 h of recovery. Then skinned fibers were subjected to *S*-glut by successive treatments with 2,2′-dithiodipyridine and glutathione (*S*-glut treatment). $[Ca^{2+}]_{50}$ is more increased by *S*-glut treatment in fibers rested for 6 h than those for 0.5 and 2 h. After *S*-glut treatment, fibers were applied for dithiothreitol (DTT treatment). Immediately after fatiguing stimulation, $[Ca^{2+}]_{50}$ with DTT treatment was higher than that of control. Western blotting demonstrated that the level of *S*-glut of TnIf decreased at 0 and 6 h of recovery while it increased at 0.5 h of recovery. These results indicate that PLFFD is attributable to decreased myo-Ca^{2+} sensitivity in the late stage of recovery and that the decrease is due to the decline of *S*-glutathionylated TnIf.

Session 6: Molecular Motors

Oral presentations

Stretch-induced conformational changes in the myosin heads in relaxed skeletal muscle

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In relaxed skeletal muscle at near-physiological temperature and myofilament lattice spacing, the majority of the myosin heads have the long axis of their regulatory light chain (RLC) domains roughly parallel to the thick filament, consistent with the electron microscopy model of the OFF state of myosin, with a minority in a distinct conformation with their RLC regions roughly perpendicular to the filament axis (Fusi et al., 2015, *Biophys J*, under revision). However it is still unclear how the myosin molecule is switched on during contraction. In this study we tested the hypothesis that a stress sensor in the thick filament regulates its activation by measuring the changes in myosin conformation produced by stretching relaxed muscle fibres. We used fluorescence polarisation from bifunctional rhodamine probes at four sites on the RLC to measure changes in its orientation produced by step increases in length or load in relaxed fibres from rabbit psoas muscle (T = 25 °C, 5 % dextran). At sarcomere lengths above 2.7 μm, where passive force becomes substantial, a length stretch produced a change in RLC orientation towards the more perpendicular conformation seen during calcium activation that was partially reversed during stress relaxation after the stretch. The kinetics of the change in RLC orientation produced by a stepwise force increase to ~20 % of the isometric force applied to the relaxed muscle fibre has fast (~300 s^{-1}) and slower (~80 s^{-1}) components, with rate constants similar to those of the viscoelastic elongation of the fibre. These results indicate that the orientation of myosin heads in relaxing conditions is sensitive to thick filament stress, suggesting the existence of a mechano-sensing mechanism in the thick filament that might regulate the release of the myosin motors from their OFF state.

EB1-dependent microtubule plus-end tracking by kinesin-14 Ncd—formation of EB1:Ncd:microtubule complex

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Ncd is a kinesin from *D. melanogaster*. Ncd belongs to the family of kinesins-14 which move towards the minus end of microtubules (MTs). Ncd dimer contains 2 sets of MT-binding sites: one in the C-terminal motor domain, the other in the N-terminal tail. Ncd crosslinks and slides MTs during mitotic spindle formation and is present in the central part of the spindle, but also at the plus ends of growing MTs, despite the minus-end directed movement of the motor. Ncd achieves this localization by the interaction with EB1, a protein which specifically binds and follows the ends of polymerizing MTs. This activity is called "plus-end tracking" and for Ncd the occurrence of this activity depends on the presence of the N-terminal SRLP motif on the kinesin. However, the detailed mechanism of MT plus-end tracking by kinesins-14 is unknown.

We measured Ncd-EB1 affinity in the solution using FRET between chromophores attached to the proteins and found that the Ncd-EB1 K_d was ~9 μM, indicating a rather weak interaction. To analyze the behavior of Ncd and EB1 on dynamic MTs, we prepared 2 Ncd mutants with lowered affinity to MTs and one which didn't bind to MT. We reconstituted MT dynamics using purified and fluorescently labeled proteins and observed the localization of those proteins under the TIRF microscope. In the presence of unlabeled EB1 all the mutants were able to track the plus ends of MTs. However, in the case of the Ncd mutant which didn't bind to MTs, the tracking was less efficient, required higher EB1 concentration and was not always visible. Next, we measured the efficiency of the tracking for all the mutants as a function of increasing EB1 concentration. We observed an inverse correlation between the affinity of the Ncd mutant to MT and the concentration of EB1 required to obtain high tracking efficiency.

We also studied single molecules of Ncd on the dynamic MTs under the TIRF microscope. In the presence of unlabeled EB1, some Ncd molecules switched between two states: diffusion along the MT lattice and MT plus end tracking. The dwell time of Ncd molecules at the MT end (2.6 s) was longer than that for EB1 (0.4 s). The dwell time of labeled EB1 molecules in the presence of unlabeled Ncd tail was 10–30 % longer than without Ncd. These results suggest that Ncd, EB1 and MT form a ternary complex crucial for the plus-end tracking.

Design and testing of a simple, single alpha helical domain: factors that affect solubility and stability

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Single alpha helical (SAH) domains are abundant protein motifs that, surprisingly, are still relatively mysterious in terms of function and structural properties. SAH domains are specialised alpha helices that do not require interactions with neighbouring domains for stability, are monomeric in solution and are extraordinarily stable over a range of NaCl concentrations and pH. Their sequence contains a repeating pattern of oppositely charged amino acid residues (K, R, E) in the general form of $((R/K)XXXEXX)_n$ (where X is any amino acid). Intrahelical K-E/R-E interactions and formation of salt bridges likely stabilise the helix. The best studied SAH domains are found in the motor proteins Myo6 and Myo10 in which they elongate the lever arm and may act as constant force springs. We have analysed the amino-acid content of a range of natural SAH domains, and used this information to test the properties of several *de novo* designed SAH domains. Each one is 96 aa residues long, and composed of a repeating 7mer pattern that contains E, K or R and A only. Our results show distinct differences between K and R in the stability and solubility of these *de novo* helices. These data have allowed us to successfully design and test a simple *de novo* SAH domain that behaves in the same way as the well-characterised SAH domain from Myo6, and which should be useful in synthetic biology applications in the future.

Structural dynamics of myosin 5a

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Myosin 5a is a molecular motor that moves along actin filaments by taking 74 nm steps. Although its enzymatic cycle is well understood, there is inconclusive and conflicting information on the motion that describes individual steps. Studies aimed at revealing the stepping mechanism have either reported periods of increased flexibility or proposed partitioning of the step into one or multiple sub-events. All resulting models involve a forward aiming power stroke followed by a Brownian search of the unbound head. We used interferometric scattering microscopy (iSCAT) to track the head of myosin 5a with simultaneous nanometer spatial and millisecond temporal precision. We observed a single, spatially constrained transient state of the detached head. Within individual traces, the position of the transient always occurred on the same side of actin filament but the sidedness could change when myosin molecule switched tracks. Simultaneous tracking of both heads revealed that myosin moves in a "spinning" hand-over-hand fashion, where both heads follow the same path. We also show that, in contradiction to the Brownian search hypothesis, the detached head reaches the desired binding site in a highly controlled manner. Improving the spatial precision to the sub-nm regime revealed a structural transition in the motor domain associated with the power stroke in the leading head, which may correspond to strain release. Taken together, our results demonstrate how the motor structure tightly controls its movement (Andrecka et al., *Elife*. 2015 Mar 6; 4).

Posters

Inhibition of myosin II in cytokinesis reveals force generation thresholds

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Cytokinesis is the final event of cell division, which can be divided into two distinct processes. The first is the furrow ingression; this processleads to the maturation of the intercellular bridge. After this event there is a secondary ingression which defines the site of the cellular abscission. The molecular mechanisms of these processes remain still elusive. Furthermore the recent theories regarding the said processes do not include myosin-II, although this protein is thought to have a pivotal role at leastin the furrow ingression. To investigate the role of the myosin-II in cytokinesis, we treated HeLa cells with different concentrations of the myosin specific inhibitor para-nitroblebbistatin. At low inhibitor concentration (EC_{50} ~5 μM) the furrowing slowed down, but it was not completely hindered. However, in this concentration range the abscission failed, leading to the formation of multinucleated cells. At higher concentrations (EC_{50} ~20 μM) even the cleavage furrow failed to form. These two phenotypes in the function of blebbistatin concentration are well separable, which indicates that a different threshold in force generation is required for the furrowing and for the abscission. The initial furrow ingression requires lower force, while the formation of the functional intercellular bridge requires higher force.

Nucleotide-induced interaction between the motor and regulatory domains of myosin subfragment 1

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Myosin head (myosin subfragment 1, S1) consists of two major structural domains, the motor (or catalytic) domain and the regulatory domain. The motor domain is responsible for ATP hydrolysis and actin binding, and the regulatory domain is a long α-helix stabilized by non-covalent interactions with the essential (ELC) and the regulatory (RLC) light chains. The functioning of the myosin head as a molecular motor is believed to involve a rotation of the regulatory domain (lever arm) relative to the motor domain during the ATPase cycle. According to predictions, this rotation can be accompanied by an interaction between the motor domain and the C-terminus of ELC associated with the regulatory domain. To check this assumption, we applied differential scanning calorimetry (DSC) combined with temperature dependences of fluorescence to study the changes in the thermal unfolding and the domain structure of S1, which occur upon formation of the ternary complexes S1-ADP-AlF_4^- and S1-ADP-BeF_x that mimic the S1 ATPase intermediate states $S1^{**}$-ADP-P_i and $S1^{*}$-ATP, respectively. To identify the thermal transitions on the DSC profiles (i.e. to assign them to the structural domains of S1), we compared the DSC data with temperature-induced changes in fluorescence of either tryptophan residues, located only in the motor domain, or recombinant ELC mutants (light chain 1 isoform), each containing a single cysteine residue at different positions in the C-terminal half of the ELC molecule (Cys-99, Cys-127, Cys-142, Cys-160, or Cys-180), which were first fluorescently labeled at these cysteines and then introduced into the S1 regulatory domain. Using this approach, we have shown that formation of the ternary complexes S1-ADP-AlF_4- and S1-ADP-BeF_x significantly stabilize not only the motor domain, but also the regulatory domain of the S1 molecule. Moreover, we showed that the thermal unfolding of S1 is accompanied by dissociation of ELC from the S1 heavy chain, which occurs at higher temperature in the complexes S1-ADP-BeF_x and S1-ADP-AlF_4 than in the nucleotide-free S1. All these results testify in favor of the proposed nucleotide-induced interaction between the motor domain and ELC associated with the regulatory domain, which leads to significant increase in the thermal stability of the S1 regulatory domain and may play an important role in functioning of the myosin head as a molecular motor.

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Myosin VI localization in the nuclei: a search for its role in the nucleolus

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Myosin VI (MVI), the only known myosin walking towards the minus end of actin filaments, is involved in numerous cellular processes associated with the actin cytoskeleton. Remarkably, MVI was also found within the nuclei of several cell types, including neuronal PC12 cells, and the C2C12 myotubes and rat skeletal muscle as well as in neonatal rat cardiomyocytes. Its nuclear role still remains poorly understood. Since MVI acts through interactions of N-terminal motor domain with actin filaments and of C-terminal globular tail (GT) domain with cell/tissue specific binding proteins, we performed a search for MVI partners by means of affinity chromatography using GST-tagged MVI-GT as a bait. It was followed by mass spectrometry that allowed for identification of numerous proteins involved in interaction with nucleic acid and the related processes that are potential novel MVI binding partners. In PC12 cells, among the identified putative partners was nucleolin, a major nucleolar protein which is implicated in the ribosomal DNA (rDNA) transcription, rRNA maturation and ribosome assembly. MVI-nucleolin interaction was further confirmed by immunocytochemistry and proximity ligation assay. Additionally, we showed that other nucleolar proteins involved in ribosome biogenesis, namely fibrillarin and RNA polymerase I engaged in ribosome biogenesis also interact with MVI. As revealed by the immunogold technique, MVI is present within the all nucleolar compartments, also at the nucleoplasm–nucleoplasm border, indicating it involvement in the shuttling between these nuclear compartments and in nucleolar processes. Indeed, immunocytochemistry and electron microscopy revealed profound changes in organization of the endoplasmic reticulum in MVI-depleted PC12 cells. Moreover, similar observations we made for MVI-depleted C2C12 myoblasts. In light of these data, we speculate that MVI could be engaged in regulation of ribosome biogenesis, and thus in the ER network organization.

Muscle plasticity in response to resistance training is mainly controlled by a myosin ATPase adaptation

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Introduction: Skeletal muscle shows a remarkable plasticity in structural and functional characteristics in response to different stimulations. The classical theory predicts that contractile and energetic properties depend on the myosin heavy chain isoforms content that is primarily responsible for the speed of contraction and the myosin ATPase activity (m-ATPase) [1]. Nevertheless, experimental evidences suggest the existence of adaptive mechanisms that occur directly in the contractile unit [2]. Thus, the aim of the present study was to detail the adaptations in m-ATPase, for a given fiber type, in response to resistance exercise in rats.

Methods: After a 4-week resistance training program based on a climbing model, animals performed a climbing boot at maximal load. Then, flexor digitorum profundus, deltoid and biceps muscles were removed and the myofibrils were purified. Myofibril ATPase activity in Ca^{++}-fully activated condition was followed over 5 s by Rapid Flow Quench method and the ADP production over time was measured by High Performance Liquid Chromatography. Changes in myosin heavy chain expression were evaluated by RT-qPCR. The cross-bridge cycle was modeled to identify the steps that hold adaptations to training.

Results and Discussion: In the trained group, beyond the phenotypic shift to IIA fibers and hypertrophy, a mean increase of 123 % m-ATPase activity was observed compared to the control group in the three muscles studied. Kinetics analysis with a simple scheme of the cross-bridge cycle revealed that adaptations responsible of the enhancement of the m-ATPase activity come mainly from an acceleration of the step including the main myosin head isomerization (*i.e.* powerstroke) and the liberation of the hydrolysis product (i.e. ADP and Pi) and to a lesser extent, during ATP hydrolysis step. These results indicate that the mechanical power gain developed during climbing in loading situations (+135.7 %) is essentially dependent on the myosin ATPase adaptation process (+123 %) that occurs principally during the liberation of the products of ATP hydrolysis.

References:

1. Canepari M et al. (2010) Acta Physiol **199**: 465–476.
2. Roels B et al. *(2008)* Am J Physiol Regul Integr Comp Physiol **294**: R1911–R1918.

A kinase anchoring protein 9 (AKAP9): a novel myosin VI binding partner linking myosin VI with the PKA pathway in myogenic cells and skeletal muscle

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We showed recently that myosin VI (MVI), a unique actin-based motor protein walking towards the minus end of actin filaments, could play important roles in striated muscle and myogenic cells. MVI acts through interactions of its C-terminal globular tail (MVI-GT) domain with its tissue specific partners so we performed a search for its partners in myoblasts and myotubes. By means of a pull down assay with GST-tagged MVI-GT domain as a bait, followed by mass spectrometry analysis, we identified a number of proteins that could interact with MVI. Among them were talin, involved in cell adhesion, and A kinase anchoring protein 9 (AKAP9), a regulator of PKA activity, and these interactions were found both in the myoblasts and myotubes.

We confirmed MVI-AKAP9 interaction in myoblasts by the co-immunoprecipitation and proximity ligation assay as well as by immunocytochemistry. MVI and AKAP9 colocalized at Rab5-containing early endosomes and the amount of AKAP9 decreased, similarly to MVI, during myoblast differentiation. However, we found a substantial increase of the AKAP9 level in skeletal muscle of *Snell's waltzer* (*SV*) mice lacking MVI and in MVI-depleted (MVI-KD) myoblasts. Interestingly, in MVI-KD cells both cAMP and PKA levels were increased, and a change in the MVI motor-dependent AKAP9 distribution was observed. We confirmed functional relevance of the MVI-AKAP9 interaction by showing that in vitro PKA phosphorylated the MVI-GT domain. This novel interaction linking MVI with the PKA pathway could be important for targeting the AKAP9-PKA complex within the cell and/or providing PKA to phosphorylate the MVI tail domain.

Expression of myosin VI in mouse testes

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Myosin VI (MVI) is a unique unconventional motor protein which moves toward the slow growing, minus (pointed) end of actin filaments unlike all other known myosins, which are the plus (barbed) end oriented molecular motors. MVI has three major domains: an N-terminal motor domain (head) with conserved ATP- and actin-binding motifs; a neck region (lever arm), which has two light chain binding sites and is important for reverse direction movement; and a multi-domain tail, which plays a role in stepping and also binds cargoes and/or adaptor proteins. Due to the presence of two different inserts in the C-terminal part of the tail, small (SI) and large (LI) ones, four alternatively spliced variants of MVI could be formed in mammalian cells: isoforms with SI or LI and protein variants with both or without inserts. It has been postulated that presence or lack of these specific inserts could determine cellular localization and biological role of MVI [1].

MVI has been implicated in a large number of different cellular processes, including endocytosis, stabilization of Golgi apparatus and exocytosis, morphology of hair cells in Corti organ, cell adhesion and migration, cytokinesis, nuclear processes, autophagy, myogenesis, and iris development [2]. It was also showed that lack of MVI in *Drosophila* testes leads to male infertility, what proves that MVI's function is crucial in the process of spermatogenesis in invertebrates [3]. However, it is still unknown whether this protein plays an important role in the sperms formation or maturation in mammals. Using protein immunoblot (western blot) and molecular approach (RT-PCR) we have showed that in mouse testes MVI is expressed at relatively high level compared to other organs, such as heart or brain. Moreover, we have revealed that in mouse testes the MVI variant with LI is present. These studies have been supplemented by structural and cytochemical/immunocytochemical analysis. All obtained results reported here suggest that MVI may play also an important role in spermatogenesis of mammals.

References:

1. Tomatis VM et al. (2013) *J Cell Biol* **3**: 301–320.
2. Zakrzewski P, Lenartowska M (2014) *Postępy Biochemii* **60**: 323–332.
3. Noguchi T et al. (2006) *Moll Biol Cell* **17**: 2559–2571.

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Session 7: Muscle Cytoskeleton

Oral presentations

Breaking sarcomeres in vitro using electrical pace stimulation

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Eccentric exercise leads to myofibrillar disruptions, described as Z-disc streaming or sarcomeric "lesions". These lesions are thought to be responsible for the post-exercise weakness, and are supposed to represent areas where new sarcomeres are assembled during the remodelling response. However, these structures have only been investigated using tissue sections of exercised skeletal muscles, which precludes examination of their generation, development, or behaviour during muscle activity. To overcome this, we use electrical pace stimulation (EPS) to mimic exercise in C2C12 myotubes. In stimulated myotubes EPS induces sarcomeric lesions similar to those observed in animal models, with increasing prevalence with time of application. We also follow lesion formation and maturation using live cell imaging, and show that it is a progressive process. Finally, our study of the behaviour of these lesions live during EPS-induced tetanic contractions indicates that during stimulation, in contrast to the flanking sarcomeres which contract, lesions lengthen. Therefore, these structures provide slack to the myofibrils, explaining how such small ultrastructural changes can lead to post-exercise weakness. Furthermore, the slack provided by the lesions may protect the muscles from further damage, possibly contributing to the repeated bout effect. Our results highlight the use of EPS of cultured myotubes for the study of the dynamic processes that occur in skeletal muscles during exercise and sarcomere damage.

Molecular network in sarcomeric M band: implication in skeletal and cardiomyopathy

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Titin (or connectin) is the largest single polypeptide protein, tethering myofilaments from Z-disc to M-band. It is composed of many modular domains known as Ig (Immunoglobulin like), Fn3 (Fibronectin type 3) domains and also signalling domains, e.g. a MLCK-like kinase domain. The gigantic size and modular structure of the titin molecule confer multiple functions and enable it to perform multiple roles in the sarcomere, from development (sarcomerogenesis), to maintenance and adaptation.

At the M-band titin forms a ternary complex with obscurin (and its small cousin obscurin-like-1 (obsl1)) and myomesin that is indispensable for the function of M-band protein network as mechanical link.

Intriguingly, the titin M-band region, especially the last Ig domain (M10), is one of the hotspots of disease-related TTN mutations. Tibial muscular dystrophy (TMD) and limb girdle muscular dystrophy 2 J (LGMD2 J) causative mutations all reside in M10, while several truncation mutations in M5 and Is6 (insertion between M7 and M8) are reported in Salih myopathy.

Here, we will show our findings concerning the structural M-band protein network and the consequences of TMD related mutations on this network, by using biochemical and microscopic analyses and X-ray crystallography of titin/obscurin (or obsl1) complexes.

In most cases, the interaction between titin M10 and obscurin/obsl1 were weakened or abolished by introducing TMD mutations. And as a result, these would trigger the mis-location of obscurin/obsl1 in sarcomere, which was confirmed in the affected muscles. The complex structures solved by x-ray crystallography provide detailed insight into the way of the two giant proteins interaction and showed how mutations in titin lead to a failure of their association.

References:

Fukuzawa A et al. (2008) *J Cell Sci* **121**: 1841–1851.
Pernigo S et al. (2010) *Proc Natl Acad Sci U S A* **107**: 2908–2913.
Sauer F et al. (2010) *EMBO Rep* **11**: 534–540.
Pernigo S et al. (2015) *J Mol Biol* **427**: 718–736.

Mechanically driven unfolding and refolding transitions in full-length titin

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Titin, a giant filamentous intrasarcomeric protein, is a serial chain of more than 300 globular domains and numerous unique sequences. Although force-dependent unfolding has been extensively investigated in recombinant homopolymeric constructs of titin domains, neither the global kinetics, nor the spatial pattern of mechanically-driven domain unfolding is known within the complexity of the full-length molecule. To follow the global kinetics of domain unfolding, we stretched individual titin molecules isolated from rabbit *M. longissimus dorsi* using high time- and force-resolution optical tweezers in force- and velocity-clamp modes. When clamped at high forces, the molecule extended in discrete steps via unfolding of its constituent globular domains. In an apparent violation of mechanically-driven activation kinetics, however, neither the global domain unfolding rate, nor the folded-state lifetime distributions of titin were sensitive to force. The contradiction can be reconciled by assuming a gradient of mechanical stability so that domains are gradually selected for unfolding as the magnitude of force increases. To explore whether there is a spatial pattern in this gradient of domain unfolding, we carried out a topographical screening of individual titin molecules stretched to varying degrees with receding meniscus. We found that unfolded domains were distributed homogenously along the entire length of the overstretched titin molecule.

In order to investigate the mechanically-driven refolding process of titin, we manipulated previously unfolded molecules in force-feedback optical tweezers experiments. After quenching the force to low levels the extension fluctuated without resolvable discrete events. The amount of refolding scaled both with the extension and time of the relaxation. In experiments where one terminus of the molecule was held in a constant position, force fluctuations were observed, suggesting that the molecular chain contacts against force due to domains hopping between an extensible unfolded and a compact molten-globule state. Under mild denaturing conditions (0.5 M urea) that favor the molten-globule state, the length and force fluctuations appeared even in constant-velocity experiments, indicating that this intermediate is part of the folding trajectory. Because the transition from the unfolded to the molten-globule state shortens the chain more extensively than a purely entropic collapse, an additional sarcomeric contractility may arise under stressed conditions leading to domain unfolding.

Posters

Reorganization of the contractile system in rat neonatal cardiomyocytes is regulated by extracellular matrix

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Freshly isolated neonatal cardiomyocytes preserve highly organized myofibrillar system. However, during long-term cultivation, their contractile system undergoes reorganization, with the reversible conversion of typical myofibrils into structures of non-muscle type, and the loss of contractility. We have previously shown that during this conversion cardiomyocytes produce extracellular matrix (ECM), which production is not typical for these cells. The present study aims to correlate the reorganization of actin structures with the production by cardiomyocytes of ECM proteins.

PCR analysis revealed that the reorganization of the contractile system is accompanied by switching of actin isoforms. At the early stage of cultivation, when the myofibrillar organization is preserved, only cardiac isoform is revealed. Disassembly of myofibrils correlates with the expression of α-smooth muscle actin. The restoration of myofibrils is accompanied by the predominant expression of cardiac isoform, with the low level of smooth muscle isoform. Western-blot analysis and immunofluorescent staining confirmed that the appearance of α-smooth muscle actin precedes the conversion of myofibrils into structures of non-muscle type. The restoration of myofibrillar system is accompanied by the reduction of α-smooth muscle actin, where α -smooth muscle actin is not detected in the newly formed myofibrils. Western-blot analysis also revealed the gradual accumulation of ECM proteins collagen and laminin during the reorganization process, wherein the rise of these proteins corresponds to the reduction of α-smooth muscle actin. These results indicate that the enhanced expression of α-smooth muscle actin may be induced by the lack of extracellular matrix, whereas the accumulation of the appropriate matrix may be a signal for cardiac isoform expression.

Taken together, our results suggest an inverse correlation between α-smooth muscle actin and ECM production by neonatal rat cardiomyocytes in culture. We believe that the rearrangement of cardiomyocyte contractile system in long-term culture is due to the ECM synthesis by cardiomyocytes themselves to maintain specific organization of their contractile system, and ECM is a key regulator of the reorganization process.

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Impaired passive force development in skeletal muscles lacking arginyl-tRNA-protein transferase (Ate1)

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Introduction: Arginylation is a post-translational process mediated by the enzyme arginyltransferase (ATE1) that transfers arginine from tRNA onto proteins. Recently, we showed that striated muscles lacking ATE1 experience a drastic reduction in the isometric force, due mostly to modifications in the kinetics of myosin molecules. In this study, we investigated the role of arginylation on passive force development, and its connection with titin molecules.

Methods: We used atomic force microscopy to evaluate the passive force development of myofibrils isolated from ATE1 KO (Ckmm-Ate1) and wild type (WT) skeletal muscles from mice. Myofibrils were stretched at 3 μm/sec by steps of 25 % of the initial myofibril length (WT 2.39 ± 0.001 μm, $n = 13$ and Ckmm-Ate1 2.40 ± 0.003 μm, $n = 12$), and held for 25 s between stretches in order to reach a steady state. We used mass spectrometry and electrophoresis to investigate possible sites or modifications in titin in both sets of muscles.

Results: Both viscoelastic and steady-state forces after stretches developed by Ckmm-Ate1 myofibrils were lower than those developed by WT myofibrils. Ckmm-ATE1 myofibrils also presented a faster rate of force decay after the peak force reached during the stretches. The average sarcomere length during the steady state was significantly higher in Ckmm-Ate1 myofibrils than WT, suggesting a higher compliance in Ckmm-Ate1 myofibrils. Mass spectrometry revealed five sites for arginylation within titin, located in the A-band portion of the molecule. Electrophoresis showed no increase in mobility of titin or isoform changes due to lack of arginylation. Finally, electron microscopy showed that the ultrastructure, cross-sectional area and number of filaments in Ckmm-Ate1 were not altered.

Conclusion: Arginylation is an important process of passive force regulation in skeletal muscles. Since arginylation does not affect directly the spring-like segments of titin, we suggest that the anchorage of proteins associated with the A-band of titin may be impaired in muscles lacking arginylation. Arginylation may therefore be required for proper lodging of titin within the A-band, with consequences for passive force development.

The effects of cofilin and non-muscle tropomyosin isoforms Tpm3.2 and Tpm3.4 on the regulation of actin filaments dynamics

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Two protein families, tropomyosin and ADF/cofilin, are central regulators of actin filaments dynamics. Tropomyosins stabilize the filament and control access of other actin-binding proteins to the filament. Cofilins sever the filaments and accelerate depolymerization through actin monomers sequestering. In the nervous system, dynamics of the actin cytoskeleton is fundamental to cell morphology and formation of a branched network of neurons. The short non-muscle isoforms of tropomyosin, Tpm3.2 and Tpm3.4, derived from human TPM3 gene, are abundant in neurons, but during cell development they sort to different compartments (Vrhovski B et al., 2003). Non-muscle cofilin 1 (Cfl1) was reported as a regulatory factor of actin dynamics in neurite formation (Flynn KC et al., 2012). The goal of this project was to define effects of Tpm3.2 and Tpm.3.4 on the regulation of Cfl1 activity.

Human Tpm3.2 and Tpm3.4 and mouse Cfl1 were expressed in *E. coli* BL21 strain and purified using standard methods. Actin was isolated from chicken skeletal muscle. The regulation of actin filaments dynamics was analyzed with the use of co-sedimentation and turbidymetric assays. Direct contacts between Cfl1 and tropomyosins were tested with the use of zero-length cross-linker $CuSO_4$, and 10.5 Å cross-linker o-PDM.

Tpm3.2 differs in sequence from Tpm3.4 only in the 27-C-terminal amino acid residues encoded respectively by exons 9d and 9c. This

difference affected the apparent actin binding constant (K_{app}). For Tpm3.2 $K_{app} = 4.5 \times 10^6$ M^{-1} and for Tpm3.4 $K_{app} = 1.5 \times 10^6$ M^{-1}. Cfl1 bound to actin with $K_{app} = 4.0 \times 10^6$ M^{-1} and it competed with both tropomyosins in binding to actin filament by reducing actin affinity and partial removal of bound tropomyosins. The presence of Tpm3.2 reduced the initial rate of actin polymerization 3-fold. In contrast, Tpm3.4 had little effect on the polymerization rate. Cfl1 reversed the inhibiting effect of Tpm3.2 and accelerated polymerization in the presence of Tpm3.4. These effects were achieved by allosteric conformational changes, because neither $CuSO_4$ nor o-PDM cross-links revealed direct interactions between both proteins. In conclusion, non-muscle products of the TPM3 gene diversify the actin filaments by differential regulation of Cfl1.

References:

Vrhovski B et al. (2003) *J Neurosci Res* **72**: 373–383.
Flynn KC et al. (2012) *Neuron* **76**: 1091–1107.

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Session 8: Heart and Heart Failure

Oral presentations

The working stroke of cardiac myosin studied with fast half-sarcomere mechanics in intact trabeculae from rat ventricle

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Heart performance depends on power developed by the muscle and mutations of sarcomeric proteins which affect power output have been proposed to be responsible for dilated (decrease in power) or hypertrophic (increase in power) cardiomyopathy (Spudich JA, 2014, *Biophys J* **106**: 1236–1249). Understanding the molecular basis of the alterations in power related to mutations in cardiac myosin II requires the definition of its mechano-kinetic properties as a single motor and of the emergent properties of its array arrangement in each half-sarcomere. Here we apply fast sarcomere-level mechanics to intact trabeculae from the right ventricle of the rat to record for the first time the isotonic velocity transient elicited by a stepwise drop in force. The trabecula is connected to the levers of the force and motor-length transducers (Lombardi & Piazzesi, 1990, *J Physiol* **431**: 141–171) via double-hooked 50 μm wires and electrically stimulated at 0.5 Hz (27 °C; 1 mM external [Ca^{2+}]). Sarcomere shortening during force development (60–100 nm per half-sarcomere, hs) can be prevented with a feedback based on the changes in sarcomere length (SL) recorded by a striation follower. Force steps (rise time, 200 μs) are superimposed on the peak force of the twitch (T_p) at different SL (range 1.9–2.3 μm). We find that (*i*) the attachments contribute by ~40 % to the total compliance; (*ii*) T_p almost doubles with SL (Length Dependent Activation, LDA, e.g. ter Keurs et al., 1980, *Circ Res* **46**: 703–714); (*iii*) the amplitude and the rate of the early phase of the velocity transient, the mechanical manifestation of the working stroke in the myosin motors, increase from 3 to 8 nm and from 1000/s to 6000/s respectively with the reduction of the load from 0.8 to 0.2 T_p and are independent of SL; (*iv*) the force–velocity relation determined during steady shortening scales in proportion to T_p, while the unloaded shortening velocity (V_0) remains constant at ~8 μm/s/hs independent of SL (see Daniels et al., 1984, *J Physiol* **355**: 367–381); (*v*) the maximum power output increases with SL. We conclude that, in the SL range studied, the working stroke is independent of SL suggesting that LDA is an emergent property of the ensemble of myosin motors in the half-sarcomere. These experiments demonstrate the unequalled power of our approach to define the mechano-kinetics of cardiac myosin opening the possibility for in situ studies of the molecular basis of cardiomyopathies.

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Defining the role of the stress sensors MLP and CARP for DCM development

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Muscle LIM protein (MLP, Csrp3) and muscle ankyrin-repeat proteins (MARPs) are known to be involved in mechanosignaling in cardiomyocytes. MLP knockout mice were the first genetically engineered animal model for dilated cardiomyopathy (DCM) and have been intensely used to investigate disease mechanisms. However, it remains elusive how absence of MLP is causative of the disease. Another protein family that gained prominence as molecular marker for cardiomyopathy and in heart failure are the MARPs, consisting of CARP1/Ankrd1, CARP2/Ankrd2 and CARP3/Ankrd23.

We set out to investigate whether ablation of MARP expression would have any beneficial effect on the MLP knockout phenotype and generated double knockout animals for MLP and either CARP1, CARP2 or CARP3. We analyzed cardiac function of these mice at 2 and 6 months of age using trans-thoracic echocardiography. Changes to well-characterized molecular markers for cardiomyopathy were evaluated by qPCR, immunoblot assays, and immunofluorescence.

CARP1xMLP and CARP2xMLP double knockout mice show a complete or partial reversal of the DCM phenotype with normal heart morphology and rescue of their physiological function. In contrast, CARP3xMLP mice display no improvement of cardiac function. Paradoxically, classical markers for cardiomyopathy, like ANF and BNP are still upregulated in the double-knockout mice. In addition, healthy double knockout hearts still display increased levels of fibrosis compared to controls.

Because MARPs are predominantly expressed in striated muscle, interference with their expression may be of huge therapeutic promise to attenuate the development of heart failure.

Is the catalytic base of titin kinase required for muscle function?

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The correct functioning of muscle relies on the precise arrangement of proteins within the sarcomere. The giant sarcomeric muscle protein titin has an essential role as a molecular ruler in determining sarcomere length. Furthermore, titin has multiple signalling domains, including a C-terminal kinase domain (TK) located in the M-band. Force spectroscopy experiments and molecular dynamics simulations have demonstrated that TK could be activated by physiological forces pulling the autoinhibitory domain out of the active site, suggesting a role for the kinase in mechanosensing. A signalling complex has previously been reported where the open form of titin kinase interacts with the autophagy adaptor protein Nbr1 in a multi protein complex implicated in the regulation of load-dependent muscle protein turnover. However, a functional role for titin kinase activity has yet to be demonstrated in vivo, amid controversy whether TK is an active kinase or an inactive scaffold.

We therefore generated a knock-in mouse where the catalytic aspartate in TK, crucial for phosphotransfer in all active protein kinases and completely evolutionary conserved in all titin kinases, was mutated to alanine with the intention of rendering the kinase inactive (TK-DA). The crystal structure of the mutant kinase was solved and shows no changes to the conformation of the active site. Genetic interaction assays and cellular FRET were used to verify the unchanged interaction with the autoinhibitory tail and Nbr1. These results suggest that any purely scaffolding functions of TK are unaffected by the D > A mutation. TK-DA mutant mice develop normally and seem to have normal life expectancy. However, detailed physiological investigations showed that the knock-in animals had significantly enlarged baseline left atria, thinner interventricular septa and left ventricular posterior walls along with elevated heart rates. Furthermore, upon subjecting the mice to adrenergic stress, extensive cardiac remodelling and increased mortality was observed in TK knock-in mice but not wild-type controls or unstressed TK-DA animals. These results indicate that the titin kinase catalytic aspartate is required for the maintenance of cardiac muscle and cardiac stress response. Analysis of the proteome and phosphoproteome identified novel candidate targets of TK involved in muscle maintenance and turnover.

New approach to assess contractile function of sarcomeres within human embryonic stem cell-derived cardiomyocytes

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The aim of this study is to evaluate the myosin-driven contractile function of human embryonic stem cell-derived cardiomyocytes (hESC-CMs) with different myosin isoform composition. Using typical differentiation protocols, hESC-CMs express significant levels of the fast cardiac myosin heavy chain (αMyHC), while in human ventricular cardiomyocytes the slow βMyHC predominates. To determine the effect of the two myosin isoforms on hESC-CM function, cells were differentiated and propagated as "cardiac bodies" in suspension culture and plated on laminin-coated coverslips for subsequent functional analysis. To switch the cells towards αMyHC-expression (αMyHC-CMs), some hESC-CMs were treated with triiodothyronine. We used chemically demembranated hESC-CMs (d-hESC-CMs) to dissect sarcomeric contractile function from intracellular Ca^{2+} handling mechanisms that modulate contractility, as chemical skinning exposes the entire sarcomeric structure to experimental solutions with defined Ca^{2+} concentrations. To understand how βMyHC and αMyHC expression affects sarcomere contractility of d-hESC-CMs, we measured in an nN-sensitive micromechanical setup isometric force (F_{ACT}) generated by cross-bridges and two key parameters to characterize cross-bridge cycling: the rate constant of force re-development (k_{TR}) and the rate constant of the isometric phase of force relaxation (k_{LIN}). While F_{ACT} was similar (42—43 kPa) for both, βMyHC-CMs and αMyHC-CMs, k_{TR} was $0.67 \pm 0.10\ s^{-1}$ for βMyHC-CMs and $2.44 \pm 0.30\ s^{-1}$ for αMyHC-CMs, and k_{LIN} was $0.30 \pm 0.09\ s^{-1}$ for βMyHC-CMs and $1.47 \pm 0.38\ s^{-1}$ for αMyHC-CMs (mean ± S.D.). In the absence of added inorganic phosphate, these data yield about $0.4\ s^{-1}$ and $1.0\ s^{-1}$ for f_{app} and $0.3\ s^{-1}$ and $1.5\ s^{-1}$ for g_{app} of the βMyHC-CMs and αMyHC-CMs, respectively. These force kinetic parameters are compared with the corresponding ones of myofibrils isolated from native human ventricular tissue. The faster k_{LIN} (g_{app}) of αMyHC-CMs indicates higher tension cost compared to ventricular-like βMHC-CMs. This should be considered when using cardiomyocytes expressing mainly αMyHC for cellular disease models, tissue engineering or for testing effects of new drugs. Our approach of using d-hESC-CMs to directly assess contractile function at the sarcomeric level, correlated with the type of myosin isoform, could be further used with human induced pluripotent stem cell-derived CMs as a cellular model for hypertrophic cardiomyopathy caused by missense mutations in the cardiac MyHC.

Increasing cardiac 2 deoxy-ATP improves performance in small and large animal models of systolic heart failure

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Heart Failure results in more deaths and hospitalizations than almost any other single cause. We are developing a gene therapy treatment strategy that increases 2 deoxy-adenosine triphosphate (dATP) in heart muscle via the cardiac specific expression of ribonucleotide reductase (RNR). dATP is produced by RNR. In vitro studies show dATP increases cross bridge binding and cycling, and that elevated dATP increases the magnitude & rate of contraction in rodent, and failing human heart muscle without altering calcium handling or slowing relaxation. We have over-expressed RNR selectively in heart muscle with a viral vector that contains a cardiac specific enhancer/promoter of human cardiac troponin T (AAV6-cRNR) and demonstrated it increases cardiomyocyte contraction and cardiac performance in normal and infarcted rodents. Here we report that AAV6-cRNR rescues left ventricular function in a large animal (pig) model of myocardial infarct and provide proof of concept for effectiveness in both a mouse and dog model of dilated cardiomyopathy (DCM). In the pig model, infarcts were generated by balloon occlusion of the left anterior descending artery and AAV6-cRNR was delivered via coronary catheterization. At two months post-treatment, left ventricular (LV) ejection fraction and systolic LV dimension improved in the high-dose groups, despite further deterioration in the saline controls. Hemodynamic parameters including LV end-diastolic pressure, +dP/dt, and −dP/dt improved in the high-dose group, with

low and medium doses showing intermediate responses that improved over time. In adult mice carrying the D230 N alpha Tm DCM mutation, pCa50 was decreased (5.47 ± 0.01) compared to WT (5.59 ± 0.03), but dATP rescued this defect (5.55 ± 0.03). In isolated myofibrils, force at pCa = 5.6 decreased from 85 ± 14 mN/mm^2 in WT myofibrils to 52 ± 7 mN/mm^2 ($p < 0.05$) in D230 N Tm myofibrils, and this deficit was improved to 72 ± 14 mN mm^2 ($p = 0.47$) with dATP. The activation rate (kACT) was slower in D230 N Tm (1.6 ± 0.2 s^{-1}) *vs.* WT (3.5 ± 0.3 s^{-1}) myofibrils, and was partially corrected by dATP (2.5 ± 0.4 s^{-1}). dATP also corrected the early, slow relaxation phase of relaxation in myofibrils from D230 N Tm mice. Similar results were found for myofibrils from dog hearts that had idiopathic DCM. These studies demonstrate that elevated cardiac dATP can treat diseased and damaged hearts and that AAV6-cRNR may be an effective therapeutic strategy as a preventative agent in progressive heart failure.

Posters

Heme induced contractile dysfunction in human cardiomyocytes by myofilament protein oxidation

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Introduction: Under pathological conditions free intracellular heme predisposes for oxidant-mediated tissue damage. Previous results in our laboratory showed alteration in contractility machinery and structure of the sarcomere function. The aim of this project was to elucidate the mechanism beside the dysfunction of the contractile protein machinery of human cardiomyocytes.
Methods: Force measurements were performed in skinned human cardiomyocytes isolated from left ventricle. Ca^{2+}-activated active force (F_{active}), Ca^{2+}-independent passive force ($F_{passive}$) were monitored before and after 20-min-long incubations in the presence of increasing heme concentrations (1–100 μM) with or without added H_2O_2 (30 μM) and/or the antioxidant DTT (10 mM). A protein biotinylation assay was performed to determine the relative levels of sulfhydryl (SH) group oxidation for individual proteins in the presence of increasing heme concentrations (1–300 μM).
Results: Following 100 μM heme exposures (without H_2O_2), F_{active} decreased to 11 ± 2 % and $F_{passive}$ increased to 568 ± 61 % (mean ± SEM, $P = 0.05$, n = 8) with structural disturb. Nevertheless with H_2O_2 F_{active} was only 0.5 ± 0.5 % and $F_{passive}$ was 456 ± 71 % ($P = 0.05$, n = 8). The half maximal inhibitory concentration of heme dependency (IC_{50}) on F_{active} was shifted to the left (from 19 μM to 4 μM) by H_2O_2. SH group decreased after 30 μM of heme concentration to almost complete oxidation of all myofilament proteins at 300 μM. Partial restoration of SH content was observed in cardiac myosin binding protein C after DTT (from 48.56 ± 11.63 to 71.07 ± 16.39 %, $P = 0.05$). However other proteins (titin, myosin heavy chain and filamin C and alpha actinin) did not show reversibility to DDT.
Discussion: Our observations suggest that free heme modify the SH groups in cardiac contractile machinery proteins, specifically on thick filaments; leading to an important mechanical limitations in their physiological functions. The heme induced antiparallel changes in F_{active} and $F_{passive}$ that may potentially explain part of the systolic and diastolic cardiac dysfunctions not only in haemolytic diseases, but also during heart failure and acute myocardial infarction.

Metformin stimulation affects titin isoform switching and phosphorylation status in embryonic rat cardiomyocytes

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Question: In embryonic cardiomyocytes passive sarcomere stiffness is mainly defined by the expression levels of large N2BA titin isoforms. After birth these isoforms are subsequently replaced by the shorter N2B isoform. To study titin isoform switching the cell culture model of embryonic rat cardiomyocytes (ERC) is a well-established tool. Stimulation of ERC with insulin or the thyroid hormone T3 accelerates titin isoform switching towards the adult N2BA/N2B composition in a PI-3-Kinase (PI3 K) dependent manner. The PI3 K/AKT pathway is also responsible for the activity of several protein kinases that can phosphorylate titin and thereby modify titin-based passive stiffness. Here we analyzed the impact of insulin and the well-established anti-diabetic drug metformin on titin isoform composition and phosphorylation.
Methods: Titin isoform composition was analyzed by 2.1 % SDS-PAGE in ERC cultured under hormone depleted conditions with or without insulin or metformin for up to 8 days. Titin phosphorylation was tested by Western blot analysis using phospho-specific antibodies targeting titins N2-B or PEVK domain in ERC treated with insulin or metformin for 15–45 min. Activation of the PI3 K/AKT-pathway was estimated using phospho-specific antibodies targeting pathway members. Titin splicing activity was rated by the expression levels of the splicing factor RBM20.
Results: Insulin and metformin treatment of ERC increased titin phosphorylation in the N2-B and in the PEVK domain, probably involving activation of ERK1/2, PKG and PKCα. Both effects can be blocked by simultaneous application of the PI3 K-inhibitor LY294022. As shown for insulin stimulation ERC treated with metformin showed a significantly higher content of N2B titin within the first days of cultivation compared to control cells. Titin isoform switching correlated with significant higher levels of AKT activation and RBM20 expression. Metformin treatment starting at day 7 of ERC culture resulted in lower levels of N2B-titin compared to control cells, and also significantly decreased the expression levels of the splicing factor RBM20.
Conclusions: Our results indicate that the oral anti-diabetic drug metformin activates pathways in ERC similar to insulin and induces modification of titin composition and posttranslational modification. We further show that insulin and metformin-induced phosphorylation of the elastic titin domains seem to be at least partly regulated by the PI-3-Kinase signaling pathway.

Development of hypertension-induced left ventricular hypertrophy is associated with impairment of lipolysis in cardiomyocytes

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Many recent studies underline the important role of lipid metabolism genes in the regulation of heart function. The aim of the present study

was, therefore, to determine the role of lipolysis in the pathogenesis of hypertension-induced cardiac hypertrophy. We investigated the expression of lipolytic factors involved in triglyceride (TG) utilization in the heart of 6 and 18 weeks of age spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY). Echocardiography analysis revealed proper cardiac structure and function in SHR rats at 6 weeks compared with age matched WKY rats. Plasma and cardiac TG levels were significantly higher in SHR than in WKY rats at 6 weeks of age. Interestingly, accumulation of TG in the heart of SHR rats progressed with age, possibly accounting to increase of the left ventricle wall thickness in 18 weeks of age SHR rats compared with WKY controls. The TG accumulation was accompanied by decreased protein levels of adipose triglyceride lipase (ATGL), the rate-limiting enzyme of TG hydrolysis in the heart and its activator α/β-hydrolase domain containing 5 (CGI-58). Diacylglycerol (DAG) generated by ATGL is subsequently hydrolyzed by hormone-sensitive lipase (HSL). Phosphorylation of HSL at Ser^{563} stimulates its activity, whereas phosphorylation at Ser^{565} inhibits HSL activity. The level of phosphorylation of HSL at Ser^{563} was decreased and at Ser^{565} was increased resulting in reduced rate of DAG lipolysis. DAG is next hydrolyzed to monoacylglycerol by DAG lipase (DAGL). The protein level of DAGL was also decreased in the left ventricle of 18 weeks SHR rats when compared with WKY rats and consistent with data regarding ATGL and HSL. Combined, obtained results suggest that decreased rate lipolysis leads to the TG accumulation in the myocardium of SHR rats and contributes to development of hypertension-induced left ventricular hypertrophy.

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Characterization of cTnI-R170G/W, new mutations in TNNI3 associated with restrictive cardiomyopathy

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Introduction: Several mutations leading to restrictive cardiomyopathy (RCM) have been identified in the gene encoding cTnI, the inhibitory subunit of the troponin complex. It is suggested, that the phenotype of mutations with restrictive physiology is defined by changes in Ca^{2+}-sensitivity and alterations of protein–protein interactions within the sarcomere. Mutations leading to cTnI-R170G/W amino acid exchanges were recently found in young RCM patients, who died within 6 months. The exchanges are located in the C-terminus of cTnI, which is thought to modulate the function of its inhibitory region and interacts calcium-dependently with the thin filament.

We investigated the effects of R170G/W on interactions of MyBP-C domains C0-C2 with actin, of cTnI with actin and tropomyosin (Tpm), as well as on Ca^{2+}-sensitivity.

Methods: cTnI-R170G/W have been introduced into the cDNA of cTnI via site-directed mutagenesis. Proteins were expressed in *E. coli* and chromatographically purified. cTnI-variants and wt-cTnI (control) were reconstituted with the two other troponin subunits (cTnC, cTnT) to form a functional troponin complex. Actomyosin-S1 ATPase activity dependent on the free Ca^{2+}-concentration was measured in presence and absence of C0-C2 using a coupled enzymatic assay by detection of P_i released from ATP. Formation of the thin filament was analyzed via cosedimentation. Interaction with Tpm as well as actin was monitored by surface plasmon resonance.

Results and Discussion: While Ca^{2+}-sensitivity of the actin-myosin interaction was preserved for R170 W, it was strongly reduced for R170G. Consistently, the inhibitory capacity of the latter was increased. C0-C2 increased the maximal ATPase activity for wt and R170 W filaments, but not for R170G. Ca^{2+}-sensitivity was increased by C0-C2 for wt and R170G, while R170 W lacked this shift. Additionally, formation of thin filaments was severely impaired for R170 W only. Accordingly, the association rate of R170G to Tpm was similar or even enhanced compared to cTnI-wt. However, dissociation rates were significantly reduced, indicating a stabilization of the interaction with Tpm. In contrast, R170 W strongly reduced both association and dissociation rates, underlining impairment of proper thin filament formation.

Thus, we could show for the first time that mutants, though located at the same position, have different effects on Ca^{2+}-sensitivity influenced by MyBP-C or lead to a severe impairment of thin filament formation.

Impact and relative contribution of cardiac Troponin-I and Myosin Binding Protein C phosphorylation in modulating myofilament length dependent activation

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β-Adrenergic stimulation results in activation of protein kinase A (PKA) and the subsequent phosphorylation of cardiac contractile proteins, including Myosin Binding Protein C (MyoBPC) and Troponin I (TnI). PKA mediated phosphorylation has been shown (Konhilas et al., 2003; Sequeira et al., 2013) to modulate myofilament length dependent activation (MLDA). MLDA is thought to be the cellular basis of the Frank-Starling regulatory property of the heart (de Tombe et al., 2010). The molecular mechanisms underlying MLDA are the subject of intense investigation and not completely resolved. Since both TnI and MyoBPC are targeted by PKA, it is not known which phosphorylation target causes altered MLDA and/or to what extent. Evidence has accumulated that TnI PKA mediated phosphorylation, by itself, is sufficient to enhance MLDA in the cardiac sarcomere (Biesiadecki et al., 2007). Accordingly, the aim of the current study was to determine the impact of MyoBPC PKA mediated phosphorylation on MLDA, and to assess whether MyoBPC and TnI PKA mediated phosphorylation affect MLDA independently or synergistically.

For these studies, two separate mouse models were employed where endogenous MyoBPC was replaced by either phospho-mimic charge mutated MyoBPC (S273D,S282D,S302D; DDD model) or by phospho-null MyoBPC (S273A,S282A,S302A; AAA model), as compared to the wild-type (WT) litter mate controls. Complete replacement was ensured by cross-breeding with a MyoBPC genetic knockout mouse model. Hearts were removed from anesthetized mice (~8 weeks of age) and flash frozen. Skinned myocyte sized fragments were prepared from frozen heart samples by mechanical isolation using previously published methods (Konhilas et al., 2003). Force-$[Ca^{2+}]$ relationships were determined at two sarcomere lengths (SL = 1.9 μm and SL = 2.3 μm), measured by video image FFT analysis. Data were fit to a modified Hill equation for each individual cell preparation at each SL. MLDA was indexed as DEC_{50}, the

difference in [Ca^{2+}] required to achieve 50 % force activation at the two SL. Recombinant PKA was applied for 30 min at room temperature to assess the impact of PKA mediated phosphorylation on MLDA.

In a first series of experiments we studied the impact of MyoBPC phospho-null or phospho-mimic and subsequent PKA treatment (n = 9 for all groups). In WT, baseline MLDA was 0.63 + 0.05 μM, which increased to 0.90 + 0.10 μM upon PKA treatment, confirming the enhanced MLDA upon PKA treatment as reported previously. In AAA myocytes, MLDA was significantly blunted to 0.39 + 0.06 and 0.51 + 0.08 μM pre- and post PKA treatment, respectively. In contrast, in DDD myocytes, MLDA was already enhanced at baseline (1.12 + 0.15 μM) and not further affected by subsequent PKA treatment (1.01 + 0.07). Hence, these data suggest that MyoBPC PKA mediated phosphorylation plays a dominant role in modulating MLDA of the cardiac sarcomere. Analysis of MyoBPC and TnI phosphorylation (using site specific phospho antibodies Western blot of SDS/PAGE) revealed the expected increase in MyoBPC and TnI phosphorylation upon PKA treatment in WT, and only TnI in both AAA and DDD myocytes. However, a significant and variable level of TnI phosphorylation on the PKA sites was also detected at baseline and in all groups (WT, AAA, DDD), a finding that, therefore, precludes the assessment of the relative impact of either TnI or MyoBPC PKA mediated phosphorylation on MLDA. Accordingly, in a follow-up second series of experiments, AAA or DDD myocytes were exchanged with recombinant TnI in which the two PKA sites were mutated to either phospho-null (S22A,S23A; AA) or phospho-mimic (S22S,S23D; DD). SDS/PAGE analysis (TnT-*myc* method) revealed 93 + 0.5 % (range = 84–97 %) exchange efficiency. MLDA was 0.33 + 0.05 μM in AAA + AA (n-17) and 0.57 + 0.05 in AAA-DD (n = 12), while in DDD-AA we found 0.81 + 0.05 μM (n = 16) compared to 1.05 + 0.04 μM in DDD-DD (n = 15). Thus, on average, PKA mediated phosphorylation of MyoBPC increased MLDA by ~0.48 μM, while phosphorylation of TnI contributed ~0.24 μM. From these results we conclude that PKA mediated phosphorylation of MyoBPC and TnI independently contribute to enhanced myofilament length-dependent activation properties of the cardiac sarcomere with relative contributions of ~65 % for MyoBPC and ~35 % for TnI. Our results indicate that β-Adrenergic stimulation affects the Frank-Starling regulatory mechanism of the heart predominantly via MyoBPC PKA mediated phosphorylation at the molecular/cellular level.

References:

Biesiadecki et al. (2007) Circ Res **100**:1486–1493.
De Tombe et al. (2010) *JMCC* **48**: 851–858.
Konhilas et al. (2003) *J Physiol* **547**: 951–961.
Sequeira et al. (2013) *Circ Res* **112**:1491–1505.

Interaction of estrogen receptor alpha with cardiac myosin essential light chain isoform 4 as a new regulatory mechanism in the heart

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Estrogen (17β-estradiol, E2) is a central player in regulation of growth, differentiation, and physiological function in different tissues, including the heart. The effects of E2 are mainly mediated by estrogen receptor (ER) alpha and beta, which act in concert with many cofactors to mediate estrogenic effects. So far, only a few cofactors of ER have been described in the human heart. To gain a better understanding of E2-mediated ERα action in the human heart, we identified and characterized the novel interaction partner of ERα.

Yeast two hybrid screening of a human heart cDNA library revealed that ERα interacts with the cardiac myosin essential light chain isoform 4 (ALC1) in presence of E2. ALC1, as a member of contractile proteins, is expressed in the fetal heart and becomes restricted to the atria of the adult heart under physiological conditions, and is re-expressed in ventricle of adult hypertrophic hearts. This switch is accompanied by alteration of contractile performance, thus improving the heart function. Retransformation experiments showed that ALC1 interacts with full-length ERα and ERα-EF domain in presence of E2. The interaction of ERα with ALC1 was also confirmed by Co-IP in human atrium. Double immunofluorescence (IF) analysis of paraffin-embedded sections from human atrium tissues showed co-localization of ERα and ALC1 proteins in a striated sarcomeric pattern. Co-localization corresponds most likely to the A-zone of sarcomere. IF analysis of AC16 cells (a human cardiomyocyte cell line) showed a translocation of cytoplasmic ERα and ALC1 into the nuclei of AC16 cells in presence of E2. Luciferase reporter assays in AC16 cells with two different lengths of the ALC1 promoter sequence (−834 bp to +1 bp and −1405 bp to +1 bp) showed an upregulated transcriptional activity by 94 % due to co-transfection of ERα vector. However, treatment of transiently transfected AC16 cells with E2 led to a significant reduction of ALC1 promoter activity by 60 %. This regulatory effect of E2 was similar for both ALC1 promoter constructs.

In this project, we characterized for the first time an E2-regulated interaction of ALC1 with ERα in cardiomyocytes that may be crucial in physiological and/or pathological processes by regulating transcriptional activity of ERα in the heart and/or by modulating contractile properties of cardiomyocytes.

Myocardial microvascular inflammatory endothelial activation in heart failure with preserved ejection fraction

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Background: Metabolic risk is associated with diastolic LV dysfunction and heart failure with preserved ejection fraction (HFpEF). The present study investigates if systemic, low-grade inflammation of metabolic risk contributes to diastolic LV dysfunction and HFpEF through coronary microvascular endothelial activation, which alters paracrine signalling to cardiomyocytes and predisposes them to hypertrophy and high diastolic stiffness.

Methods and results: We explored inflammatory endothelial activation and its effects on oxidative stress, NO bioavailability and cGMP-PKG signalling in myocardial biopsies of HFpEF patients and validated our findings by comparing obese ZSF1-HFpEF rats to ZSF1-Control (Ctrl) rats. In myocardium of HFpEF patients and ZSF1-HFpEF rats we observed: (1) E-selectin and ICAM-1 to be upregulated, (2) NOX2 expression to be raised in macrophages and endothelial cells but not in cardiomyocytes and (3) uncoupling of eNOS which was associated with reduced myocardial nitrite/nitrate concentration, cGMP-content and PKG-activity.

Conclusions: HFpEF is associated with coronary microvascular endothelial activation and oxidative stress. This leads to a reduction of NO-dependent signalling from endothelial cells to cardiomyocytes, which can contribute to the high cardiomyocyte stiffness and hypertrophy observed in HFPEF.

Soluble adenylyl cyclase, a new player in cardiac hypertrophy

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Background and Purpose. In contrast to membrane bound adenylyl cyclases, soluble adenylyl cyclase (sAC) is localized in the cytosol and in organelles of cells from various tissues. sAC can be activated by bicarbonate and calcium and plays an important role in cell death and growth. However, its role in heart muscle is unknown. We investigated whether sAC is involved in isoprenaline induced hypertrophy applying in vitro and in vivo models.
Methods: In isolated ventricular adult rat myocytes hypertrophy was induced by treatment for 24 h with isoprenaline (ISO, 5 and 10 μM) and the β_2-adrenoceptor antagonist (ICI 118,551; 0.05 μM). To evaluate the contribution of sAC in cardiomyocyte hypertrophy, either the activity of sAC was suppressed using a specific inhibitor, KH7 (12.5 μM), or expression of sAC was attenuated (adenoviral shRNA transfection). Cell size, RNA/DNA and protein/DNA ratios, expression of alpha-skeletal actin were used as hypertrophic markers. Phosphorylation of CREB, Erk1/2 and BRaf was analyzed by Western blot. In wild type (WT) and sAC knock out (KO) mice pressure overload was induced by transverse aortic constriction (TAC) for 2 weeks. Hemodynamic parameters, heart, body- and lung weight as well as atrial natriuretic peptide (ANP) expression were analysed.
Results and Conclusion: Long-term treatment of cardiomyocytes with ISO/ICI resulted in elevated cytosolic Ca^{2+} concentration and led to significant hypertrophy, characterized by increased cross sectional area, RNA/DNA and protein/DNA ratios and amount of alpha-skeletal actin. Pharmacological inhibition or knockdown of sAC blunted hypertrophy. Mechanistic analyses revealed that CREB is activated upon induction of hypertrophy, but is only marginally affected by sAC inhibition. In contrast, activity of BRaf/Erk1/2 was suppressed upon inhibition of sAC. Comparable results have been obtained using TAC treated mice. In WT mice, TAC led to rise of heart weight and ANP expression. In contrast, no hypertrophic response could be found in KO mice. Our data suggest sAC as a novel pivotal contributor to hypertrophy in the heart acting via BRaf/Erk pathways in ISO/ICI induced hypertrophy.

Compensatory mechanism of MuRF E3 ubiquitin ligases in heart muscle

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The adult mammalian muscle cell is terminally differentiated, and especially cardiac cells lack efficient mechanisms to self-renew. Therefore, efficient maintenance and quality control of muscle proteins are essential to allow healthy muscle growth and consistent force production. To control protein quality as well as quantity within a muscle cell, continuous protein turnover takes place through precise balancing of protein synthesis and protein degradation. The muscle-specific RING finger protein family (MuRF) act as E3 ubiquitin ligases and thereby label proteins for degradation, an important role not only in normal muscle development, but during various disease conditions of heart and skeletal muscle. However, the target specificity, complementarity and in vivo functions of MuRF ligases are not fully understood. MuRFs comprise three isogenes: MuRF1/TRIM63, MuRF2/TRIM55 and MuRF3/TRIM54. These are evolutionary highly conserved, similar in structure and expression pattern and therefore expected to function similarly. Mice lacking one of the isogenes are phenotypically normal, suggesting compensation by other family members. However, the absence of two isogenes causes diverse phenotypes. In order to clarify the degree of redundancy within the MuRF family, we generated homozygous triple knockout mice that form the null background to understand the role of MuRFs in the heart. Comparisons to all possible MuRF double knockout combinations reveal isogene-specific targets of MuRFs and shows that MuRF proteins regulate cardiac hypertrophy not only through pathological target protein accumulation.

Structural and biochemical characterisation of human cardiac troponin C mutations associated with genetic cardiomyopathies

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Troponin C mutations have been linked to genetic hypertrophic and dilated cardiomyopathies. We aimed to understand, at the molecular level, how several cardiomyopathies associated mutations in troponin C (Y5H, A8 V, L29Q, A31S, E59D, D75Y, E134D and D145E) affect troponin structure and its biochemical properties. Circular dichroism, co-sedimentation with actin and ATPase assays demonstrated that these mutations had little or no effect on the folding or the thermal stability of the troponin complex. ATPase assays, fluorescence spectroscopy and transient kinetics were used to assess the effect of these mutations on the Ca^{2+} dependent inhibition and activation of the acto-myosin ATPase, the size of the cooperative unit, the transition between the blocked and closed state and the affinity and kinetics of Ca^{2+} interaction with troponin and thin filaments. We found that all troponin C mutations did not change the affinity of the troponin complex for actin. Troponin C mutations also did not affect the proportion of thin filaments in the blocked state in the absence of Ca^{2+}. In contrast several troponin C mutations dramatically reduced the amount of thin filaments switched to the closed state in the presence of Ca^{2+}. This effect is correlated with a noticeable effect on the Ca^{2+} dissociation rate constant. Overall these results provide insight into the mechanism by which troponin C mutations affect contractility in hypertrophic and dilated cardiomyopathy.

Ahnak deletion leads to mitochondrial remodeling in murine hearts

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Ahnak is a ubiquitous protein, which is implicated in cell type specific functions. In cardiomyocytes, it plays a role in the modulating of the cardiac calcium channel activity and therefore, it modulates the contraction of the heart muscle. In recent studies, we observed mitochondria abnormalities in skeletal muscles of Ahnak knock-out (KO) mice. Our preliminary data showed that the hearts of 10–12 months old female and male Ahnak KO mice developed concentric cardiac hypertrophy in comparison to age-matched wild type (WT) mice. We hypothesize that the Ahnak deletion influences the mitochondrial homeostasis in an age-dependent manner resulting in cardiac hypertrophy. Therefore, in the present study we aimed to analyze the effect of Ahnak deletion on the mitochondrial remodeling and function in mouse heart. For this purpose, we analyzed the number and size (surface) of the mitochondria in the left ventricular sections of female and male WT and Ahnak KO mice at different ages (3 and 12 months) using transmission electron microscopy. The electron microscopy study showed that female and male Ahnak KO hearts from young mice (3 months) revealed significantly higher mitochondrial number per unit area (♂134.4 and ♀132.5, respectively) in comparison to those of WT mice (♂114.6 and ♀102.4, respectively). Moreover, the mitochondrial remodeling in the heart of the KO and WT mice is different. Male and female young (3 months) KO mice hearts showed moremitochondria with the size $> 1\ \mu m^2$ (♂ 25.7 and ♀21.8 %) compared to age-matched WT mice hearts (♂16.2 and ♀13.9 %). The percent of small mitochondria ($< 0.5\ \mu m^2$) was less in male and female KO mice hearts (♂36.2 % ♀35.8 % *vs.* ♂46.0 and ♀44.1 % in WT). Interestingly, the mitochondrial remodeling in old KO hearts differs from young KO hearts. Our data showed significantly lower mitochondrial number per unit area (♂79.2 and ♀90.9, respectively) in old (12 months) KO mice hearts compared to WT hearts (♂116.5 and ♀145.3, respectively). However, the old KO mice hearts have significantly more large ($> 1\ \mu m^2$) mitochondria (♂37.2 and ♀35.8 %) compared to WT hearts (♂9.1 and ♀10.4 %). In old KO mice hearts, less small mitochondria (♂28.3 and f 28.2) were detected than in age-matched WT mice hearts (♂64.2 and ♀56.8 %). Our results suggest that Ahnak plays an important role in the mitochondrial remodeling in the heart.

Altered titin properties in ischemic and remote myocardium after acute myocardial infarction

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Question: Titin is a major determinant of myocardial passive stiffness. Titin-based passive stiffness is modulated by the expression levels of the cardiac isoforms N2BA (3.2–3.7 MDa) and N2B (3.0 MDa) and is further modified by several modifications including phosphorylation of the elastic titin domains PEVK and N2B. Phosphorylation in the PEVK domain by e.g. PKCα or CaM-Kinase δII leads to an increase, phosphorylation in the N2B domain by PKA or PKG cause a decrease in titin-based myofilament stiffness. Myocardial ischemia (MI) is known to activate several protein kinases, including PKCα, thereby regulating the onset and extent of cell injury and death. Here, we tested whether myocardial ischemia induces changes of titin phosphorylation in ischemic and remote myocardium and affects its elastic properties.

Methods: We analyzed titin parameters in models of MI: (I) *Langendorff*-perfused mice hearts, 20′ global Ischemia (I)/30′ Reperfusion (R); (II) adult mice hearts with either 3 days permanent LAD ligature or (III) 10 days permanent LAD ligature. Titin isofom composition was tested by 2.1 % SDS-PAGE. Titin phosphorylation was analyzed by Western blot using phospho-specific antibodies targeting the titin N2B or PEVK domain. Kinase activities were estimated by using phospho-specific antibodies detecting activated kinases or kinase substrates. Passive stiffness was measured using isolated skinned cardiac myocytes. Results: In model I and II PEVK phosphorylation was significantly increased. Changes occurred within 20′ after beginning of ischemia (I) and persisted even after 3 days (II). After 10 days LAD ligature (III) PEVK phosphorylation was unchanged. PKG-dependent N2B phosphorylation was significantly decreased in model I and unchanged in model II. In model III PKG-dependent N2B phosphorylation was unchanged but PKA dependent phosphorylation was significantly reduced. Consistent with increased titin PEVK and reduced N2B phosphorylation passive stiffness was elevated in model I and model II. Despite of the phosphorylation status in model III passive stiffness is still significantly increased in almost the same range. Conclusion: Titin-based passive stiffness is rapidly increased early after myocardial ischemia and is adversely modulated after the acute phase. Our data suggest that modification of titin stiffness may play an important role in both the early adaptational and the remodeling phase of the ischemic and remote myocardium after myocardial ischemia.

The characterization of action potential and Ca^{2+} transient in right ventricular myocardium of young male and female rats under monocrotaline-induced heart failure

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It is known that heart failure is accompanied by the alteration of excitation–contraction coupling both in human myocardium and in different animal models. We examined action potentials (AP) and Ca^{2+} transients (CaT) measured under variable preload in the experimental model of monocrotaline-induced right ventricular (RV) failure.

The study conforms to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1985). 2-month Wistar rats were arranged to males/females injected by a physiological solution (CONT, $n = 6$ in both groups) and males/females injected by monocrotaline (50 mg/kg) one month before the killing (MCT, $n = 10$ in both groups). The development of heart failure in the MCT-treated rats was confirmed by the significant increase in RV weight together with the loss of LV weight. AP and CaT were measured in thin RV trabecula and papillary muscles held under low (85 % Lmax) and high preload (95 % Lmax), at 25 °C and 0.33 Hz pacing rate.

At low preload (85 % Lmax), AP duration at 90 % repolarization (APD_{90}) significantly increased from $147.8 \pm 16.9/154.4 \pm 16.4$ ms in CONT males/females to $273.1 \pm 12.2/317.9 \pm 12.4$ ms in MCT males/females. The CaT amplitude (expressed as F/F_0) was attenuated from $0.79 \pm 0.13/0.58 \pm 0.11$ in CONT males/females to $0.25 \pm 0.03/0.29 \pm 0.07$ in MCT males/females; this relative attenuation was larger in MCT males. CaT was substantially prolonged in

both MCT males and females. The CaT time-to-peak increased from 62.0 ± 5.6/60.3 ± 2.9 ms in CONT males/females to 104.8 ± 8.5/95.0 ± 5.9 ms in MCT males/females. The duration of CaT decay (estimated from peak time to the drop to 10 % CaT amplitude) rose from 462 ± 26/52 ± 42 ms in CONT males/females to 597 ± 29/522 ± 31 ms in MCT males/females.

At high preload (95 % Lmax), the similar differences in APD_{90} and CaT characteristics were observed between CONT and MCT groups. The increase in preload resulted in ~10 % shortening of APD90 in each CONT and MCT groups. CaT amplitude was preload-independent in each group while CaT time-to-peak was preload-independent in CONT groups only, 15 % decrease *vs.* ~7 % increase in CaT time-to-peak was shown in MCT males *vs.* females, respectively. The CaT decay was prolonged in each CONT and MCT group, on average by > 10 % in CONT groups and by ~5 % in MCT groups.

In conclusion, the relative prolongation of AP is similar in the monocrotaline-treated young male and female rats, regardless the preload. In contrast, the relative attenuation and prolongation of CaT are more prominent in MCT males, both at low and high preload. Therefore, the mechanisms of adaptation of myocardial contractility to the heart failure involve both electrophysiological changes (in sex-independent manner) and alterations in calcium handling (with some extent of sex-dependence). The latter may originate from the upcoming effect of sex hormones in the early stage of puberty in the young rats.

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Unravelling the 3D structure of myosin binding protein C by cryo-electron microscopy of mammalian cardiac muscle

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We have previously shown that the effect of chemical fixation on striated muscle fibres is induction of small random changes in the fine structure and that the averaging of several electron micrographs of these samples can considerably recover the native state (Luther et al., 2008, *J Mol Biol* **384**: 60–72). We have also shown that cryosections of such fibres upon thawing have improved mechanical character and are ideal for cryo-electron microscopy capable of revealing high resolution detail (Luther & Morris, 2003, *J Struct Biol* **142**: 233–240). Here we use these techniques to understand the 3D structural organisation of cardiac myosin binding protein C in relaxed cardiac muscle. We have used cryosections of cardiac muscle, which were thawed and refrozen and then examined by cryo-electron microscopy. We show 2D projection views which illustrate the potential of the technique. We have also carried out cryo-electron tomography of these samples followed by sub-tomogram averaging. We compare our results with the tomography of plastic sections of rapidly-frozen/freeze-substituted skeletal fibres (Luther et al., 2011, *PNAS* **108**: 11423–11428).

Nuclear translocation of the C-terminal of the L-type voltage-gated calcium channel Cav1.2 is regulated by sex and sex hormone estrogen

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The cardiac L-type voltage gated Ca^{2+} channel (Cav1.2) constitutes the main entrance gate for Ca^{2+} that triggers cardiac contraction. Several studies provided evidence that the cleaved C-terminal fragment (CCt) of Cav1.2 translocates into the nucleus and regulates transcription. It has also shown that the nuclear translocation of CCt is regulated both developmentally and by changes in intracellular calcium. However, the effects of sex and sex hormone estrogen on CCt nuclear translocation are still unexplored. To investigate the sexual disparity in the CCt nuclear translocation, we first generated an antibody (α_{1C}-dCT)directed against the sequence around serine 1928 (MCA-GLY-R–R-A-S-F–H-L-E-Amid) located down-stream of the predicted truncation site and used it to probe ventricular myocytes isolated from female and male mice. Western blot analysis showed that α_{1C}-dCT antibody recognizes CCt fragment. Antibody specificity has been evaluated by using blocking peptide. Immunocytochemistry on isolated mouse ventricular cardiomyocytes revealed decoration of two compartments, namely nuclear staining and structured punctuate staining outside the nucleus. Line scan analysis showed cardiac nuclear localization close to that of RyR2. Staining intensities for both proteins showed regular spacing of ~2 μm consistent with the expected localization of α_{1C}–dCT in the T-tubular sarcolemma facing the RyR2 embedded in junctional sarcoplasmic reticulum. Surprisingly, we observed a remarkable sex-disparity in nuclear labeling intensity. Indeed, ratio of staining (I_{nuc}/I_{cyt}) was significantly higher in isolated female cardiomyocytes (1.42 ± 0.05) compared to male cardiomyocytes (1.05 ± 0.02). As shown by others, we also found a significant decrease in nuclear staining intensity of CCt, when the cardiomyocytes were subjected to serum withdrawal for 18 h (I_{nuc}/I_{cyt} 0.89 ± 0.02). Interestingly, subsequent application of estrogen for 8 h normalized the staining intensity ratio to basal values in male cardiomyocytes (I_{nuc}/I_{cyt} 1.04 ± 0.02), while it remained decreased in the vehicle treated male cardiomyocytes (I_{nuc}/I_{cyt} 0.88 ± 0.02). By contrast, an estrogen treatment has no effect on CCt nuclear translocation levels in female cardiomyocytes. Taken together, our results indicate that the levels of CCt in the nucleus and the cleavage of Cav1.2 are regulated in a sex-dependent manner and estrogen may play an important role in the nuclear shuttling of CCt in the heart.

Effects of structural changes in the region of residues Glu180–Cys190 of tropomyosin on the properties of its molecule

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Tropomyosin (Tm) is a coiled-coil actin-binding protein that plays a key role in the regulation of muscle contraction. Two highly conserved residues, Glu180 and Cys190, are of particular interest as they are located at a troponin T–binding region of Tm. Disulphide cross-linking of Cys190 residues between the two chains of cardiac Tm was shown to occur upon human end-stage heart failure (Canton M et al., 2011, *J Am Coll. Cardiol* **57**: 300–309), whereas mutation E180G is associated with Hypertrophic Cardiomyopathy (HCM) which leads to severe cardiac hypertrophy (Thierfelder L et al., 1994, *Cell* **77**: 701–712). More recently, a novel mutation, E180 V, associated with HCM was described in the cardiac α-Tm gene (Regitz-Zagrosek V et al., 2000, *Circulation* **102**: e112–e116). In the previous works the effects of mutation E180G on structural and functional properties of

Tm, as well as the structural effects of the disulphide cross-linking of Cys190 residues, were studied in detail. In particular, it was shown using differential scanning calorimetry (DSC) that the cross-linking between the two Cys190 residues strongly increases the thermal stability of C-terminal part of Tm molecule, whereas mutation E180G decreases the stability of this part of the molecule (Kremneva E et al., 2004, *Biophys J* **87**: 3922–3933). However, until now it was absolutely unclear how the disulphide cross-linking affects the functional properties of Tm; as to the properties of Tm with mutation E180 V, they were not studied at all. We have shown using DSC that mutation E180 V, unlike mutation E180G, substantially increases the thermal stability of the C-terminal part of Tm molecule. As to functional effects of the disulphide bonds, it has been shown using cosedimentation assays that their formation strongly decreases the affinity of Tm for F-actin. On the other hand, this cross-linking enhanced maximal sliding velocity of regulated actin filaments containing Tm and troponin in the in vitro motility assay at high Ca^{2+} concentrations, with no appreciable effect on Ca^{2+}-sensitivity of the actin-myosin interaction underlying this sliding. These results indicate that the changes in the region of residues Glu180–Cys190 of the Tm molecule may have a significant effect on structural and functional properties of Tm. This can explain, at least partly, why these changes in Tm are associated with human heart diseases.

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Some biochemical parameters in serum and tissues of lamb with white muscle disease

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White Muscle Disease (WMD) is muscular dystrophy resulted from deficiency of selenium (Se) causes nutritional myodegeneration in newborn animals like lambs, goats, calf and affecting skeletal and cardiac muscle. In the presented study 16 lambs 3–50 days aged were used as research materials. Eighth lambs were clinically diagnosed as WMD because all had typical symptoms of WMD like motor disturbance, recumbency, in breathing during movement. The lambs with WMD as kept for all analysis in a separate boxes. Control group, 8 lambs, were selected from the same area at the same ages. The blood samples were taken all animals. Then we gave the vitamin E + Se injection the lambs with WMD (Yeldif, CEVA-DIF) at the dose of 1 ml per lamb im. Blood samples and postmortem tissue samples were taken. The collected sera were analyzed for Ca, P, T3, T4, albumine, globuline, total protein, GSH in serum by ARCHITECT plus 160000 autoanalyzer. MDA, GSH concentrations of liver and thyroid tissues were determined before and after treatment. Liver and thyroid tissues were homogenized by addition of BHT, then centrifuged at 10000 rpm for 15 min. Supernatant analyzed for the GSH determination by spectrophotometer at 412 nm using Ellman's reagent (GSH μmol/g protein). The MDA levels of liver and thyroid tissues were determined by Thiobarbituric acid (TBA) methods. Tissue MDA amounts were measured spectrophotometrically at 532 nm and presented as nmol/g protein. In the presented study Ca ions was decreased in WMD than increased after vitamin E + Se injections. This could be because of the intracellular Ca accumulation and formation of Zenker necrosis. Similarly the levels of P followed the same pattern, decreased in lambs with WMD then rose to normal levels. As WMD didn't affect the blood protein parameters, significantly. But we showed a positive increased after treatment. In the selenium deficiency Type 1 deiodinase, a Se containing enzyme, activity would be inhibited. The circulating T4 levels will be increased and the concentration of T3 will be decreased. Se deficiency in also effective on the iodine depletion. We found that formation of WMD decreased T3 but increased T4 levels in the lamb serum. After the vitamin E + Se administration serum T4 turn to normal levels. GSH is an important antioxidant compound prevents cells from oxidative damages. In our study the tissue levels of GSH in liver and thyroid were decreased but only significance was found in liver GSH content after treatment ($p < 0.05$). The MDA levels in liver and thyroid tissue were slightly increased in sick group and turn to normal level after treatment with vitamin E + Se preparations. As conclusion; serum Ca, P, albumin, globulin, total protein, T3, T4, GSH, tissue GSH and MDA differences were statistically important between groups. As results serum Ca/P balances, protein amounts, thyroid hormone levels and GSH affected by WMD.

Key words: Blood parameters, GSH, MDA, Liver, Thyroid, WMD

Collagenase, elastase, leptin, nitrite and nitrate levels and histopathological examinations in lambs with white muscle disease

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White muscle disease (WMD), has received considerable attention in the lamb and calf. Lambs mostly affected with the congenital from either born death or die a few day after birth following physical exertion. Myocardial and liver lesions are seen. Myofiber are particularly sensitive to nutritional deficiencies that result in the loss of antioxidant defense mechanism. It is generally known as Se-Vitamin E deficiency. The delayed form occurs between 3–8 weeks of age. This study has been done in the Eastern part of Turkey, in the city of Van and surroundings. WMD is common problem in this area. For this purpose 9 healthy controls and 9 WMD diagnosed 3–45 days old lambs were used as research materials. The blood and postmortem heart tissue samples were taken. Collected sera were analyzed for nitrite, nitrate and leptin concentrations and heart tissue were used for collagenase, elastase activity determinations. The heart tissues were taken after postmortem examination and homogenized with PBS at pH 7.4 than centrifuged at 10000 rpm for 10 min. Supernatants were used for the collagenase and elastase activity using commercial kits. In those analysis, collagenase I (ELISA Sunmed Bio) neutrophil elastase (NE Kit ELISA Sunmed Bio) and for leptine (Cusabio Sheep Leptin ELISA) commercial kits were used. Serum nitrite and nitrate levels were determined spectrophotometrically using a coupling reagent The changes of collagenase activity was significantly important in white muscle diseased lamb ($p \leq 0.001$). Elastase activity was slightly increased but there was no statistical importance. Serum nitrite and nitrate levels were slightly increased in sick animals but no statistical importance were found ($p > 0.05$). Leptin induces oxidative stress in endothelial cells but here we didn't find any importance between the groups. Histopathologically oedematous muscle, homogeneous pink colour, necrotic nuclei, hyperemic and locally haemorrhagic blood vessels were found. Also muscular dystrophic calcification in necrotic areas as well as hyaline degeneration and Zenker necrosis was determined, in the calcified region showed a mononuclear cell infiltration mainly macrophages, were observed. As conclusion in white muscle disease lamb heart tissue collagenase

activity was an important parameters because of degenerative processes in heart.

Key words: Collagenase, elastase, nitrite, nitrate, leptin, white muscle disease

Fat soluble vitamins in white muscle disease

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Vitamin are essential for the health of all kind living organisms. Fat soluble vitamin are vitamin E, A, D and K. Deprivation of Vit E causes different disturbances such as disorder of reproduction, muscle function, cardiovascular system. Vitamin A has different biological function such as vision, growth, reproduction and differentiation of epithelial cells. In vitamin A deficiency there is keratinisation of epithelium, disturbances, metaplasies of genital and genitourinary system. Vitamin D is defined as prohormone and Vitamins are essential for the health of all kind living organisms. Fat soluble vitamins are vitamin E, A, D and K. Deprivation of vitamin E causes different disturbances such as disorder of reproduction, muscle function, cardiovascular system. Vitamin A has different biological function such as vision, growth, reproduction and differentiation of epithelial cells. In vitamin A deficiency there is keratinization of epithelium, disturbances, metaplasies of genital and genitourinary system. Vitamin D is defined as prohormone and responsible for the Ca homeostasis, bone metabolism, bone structure, maintain cellular and neural functions of Ca. Clarifying the cause of White Muscle Disease (WMD) in lambs, serum fat soluble vitamins, retinol, α-tocopherol and vitamin D3 levels were determined in 16 lambs at 3–50 days ages from different flocks. Eight of them were healthy and the rest of were diseased lambs. The gluteal and heart tissue samples from 30 lambs with WMD were also taken. Vitamins were analyzed by HPLC. Serum α-tocopherol and retinols and vitamin D3 levels were low in diseased animals but only retinol ($p < 0.001$) and α-tocopherol ($p < 0.0011$) level differences were statistically important. Macroscopically Zenker necrosis was determined in 17 lambs only in heart, 6 lambs in gluteal and chest muscle and in 7 lambs both in heart and gluteal muscle. Microscopically they were analyzed and similar findings were found both in gluteal, chest and heart muscle such as swollen homogeneous pink muscle, pycnotic nuclei, hyperemic and haemorrhagic blood vessels. Hyaline degeneration and Zenker necrosis, dystrophic region in necrotic areas, mononuclear macrophage infiltration in calcified area were also determined in thirty samples. As conclusion, in this case WMD was detected as a severe lamb disease at the early life with severe degeneration in different muscles. It was also detected fat soluble vitamin deficiency in sick animals.

Key words: Fat soluble vitamins, Zenker necrosis, WMD

Primary effects of HCM mutations in humans and cats

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Hypertrophic cardiomyopathy (HCM) is the most commonly inherited cardiomyopathy. In cats it is even more common: 15 % of a large population of unselected outbred cats has HCM. HCM-causing mutations in sarcomeric proteins have been proposed to increase myofilament Ca^{2+}-sensitivity but may have additional primary effects.

We have found that in dilated cardiomyopathy (DCM)-causing mutations in thin filament proteins the relationship between phosphorylation at Ser 22 and 23 of cardiac troponin I (TnI) and Ca^{2+}-sensitivity was abolished. This phenomenon has been termed 'uncoupling' and has been found for all thin-filament protein DCM-mutations investigated in vitro and in skinned myofibrils. It is associated with a blunted response to β1-adrenergic stimulation and reduced cardiac reserve that is potentially disease-causing.

Interestingly, uncoupling might also be a characteristic of HCM. Uncoupling has been demonstrated in a number of HCM mutants in TnI and we observed uncoupling in the cardiac actin mutation E99 K in vitro. To determine whether either of these phenomena is common to HCM mutations we have studied troponin and tropomyosin (Tm) from HCM mutant tissue in humans, mice and cats using single reconstituted cardiac muscle thin filaments moving on skeletal myosin in the in vitro motility assay (IVMA).

The HCM ACTC E99 K mutation has been extensively studied in both transgenic mice and in a human sample, the mutation caused an increase in Ca^{2+}-sensitivity (2.3 fold in mice and 1.3 fold in humans) and also abolished the change in Ca^{2+}-sensitivity due to TnI phosphorylation (uncoupling) using IVMA. We also studied force production in single myofibrils and observed a partial uncoupling: Ca^{2+}-sensitivity was increased 1.2 ± 0.1 fold by dephosphorylation compared with 1.8 ± 0.2 fold in wild-type.

A homozygous HCM mutation TnT K280 N was also studied. The mutation itself caused a Ca^{2+}-sensitivity increase (using recombinant proteins) but in the patient sample, the mutation had no affect on Ca^{2+}-sensitivity. However, the K280 N mutation also showed uncoupling with both recombinant and native K280 N TnT. Two further HCM mutations were studied, Tm E180G and TnT R92Q. Both of these mutations increased Ca^{2+}-sensitivity and also showed uncoupling. Five further TnT HCM mutations are under investigation.

Preliminary experiments were carried out with troponin from a Ragdoll cat heart with HCM due to the R820 W mutation in MYBPC3 and a non-affected cat as control. The thin filaments showed an increase in Ca^{2+}-sensitivity and it was also uncoupled. Thus, troponin from HCM cats was abnormal even though the mutation was in MyBP-C that was not present in the assay.

Overall these results suggest the Ca^{2+}-sensitising and uncoupling properties of HCM mutations may be more widely distributed than previously thought.

Assessment of 'force–velocity' relationship in cardiac muscle preparations and in the in vitro motility assay

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Two types of myosin heavy chains (MHC)—α and β determine cross-bridges (Xb) kinetics underlying mechanical characteristics of mammalian ventricular myocardium. The amino acid sequence identity between α and β MHC is 93 %, 7 % nonidentical amino acid residues located in the actin-binding domain, the ATPase pocket and converter domain determine differences in the functional properties of these isoforms. Myosin isoforms V1 and V3 are homodimers of α- and β-heavy chains respectively. Expression of isomyosins V1 and

V3 is different in cardiomyocytes from various layers of the ventricle wall, depends upon species, age, and hormonal state of the animal. Changes in the expression of these isoforms are also associated with cardiac pathologies.

The aim of this work is to compare 'force–velocity' relationships registered in rabbit cardiac muscle preparations with predominating either V1 or V3 isomyosins and with respective relationships obtained in the in vitro motility assay. Shift to predominate V1 or V3 cardiac myosin isoform expression was produced in hyper- and hypothyroid rabbits. Papillary muscles or trabeculae isolated from the rabbit right ventricles were exposed to series of after loaded contractions, and peak shortening velocity in each one was plotted *vs*. the afterload. In the in vitro motility assay using the actin-binding protein alpha-actinin as a load, we recorded the 'force–velocity' relationship (plotting alpha-actinin concentrations *vs.* respective reconstructed thin filaments movement velocities) for both cardiac myosin isoforms at two calcium concentrations in solution, pCa 6.5 and 7.0.

We found 'force–velocity' relationships for both myosin isoforms V1 and V3 in the in vitro motility assay and for both types of muscle preparations corresponded to hyperbolic Hill's equation under low loads and deviated from it under high loads demonstrating opposed concavity. Observed deviation presumably may result from effects of the Xb-CaTnC-cooperativity for both V1 and V3 isomyosins. Registered unloaded velocities in preparations with predominating V1 isoforms were $\sim$2-fold higher than in V3 muscles. Similar distinction was found in the in vitro motility assay. We conclude MHC composition described features of the 'force–velocity' curves in myocardium.

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Functional impact of stearoyl-CoA desaturase 1 deficiency on thyroid hormone signaling

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Stearoyl-CoA desaturase 1 (SCD1) is the rate limiting enzyme in monounsaturated fatty acid synthesis. Studies on mouse strains that have a mutation in the SCD1 gene provided evidence that SCD1 is an important control point in lipid metabolism and body weight regulation. Mice with a targeted disruption in the SCD1 gene have increased energy expenditure, reduced body adiposity and increased insulin sensitivity. Thyroid hormone (TH) plays an important role in the regulation of lipid metabolism. TH negatively regulates expression of genes involved in lipid synthesis, i.e. fatty acid synthase and sterol regulatory element-binding protein 1-c (SREBP-1c). Moreover, TH downregulates SCD1 gene expression, without direct binding of the thyroid receptor (TR) to the SCD1 gene promoter. The aim of the presented study was to determine the functional impact of SCD1 gene deletion on thyroid hormone effect on lipid metabolism. Our study show that SCD1 deficient mice are characterized by increased triiodothyronine (T3) plasma level, whereas thyroid stimulating hormone (TSH) and thyroxine (T4) concentrations were decreased, when compared to SCD1 +/+ mice. TH plasma levels are regulated by enzymes deiodinases. We observed decreased type 3 deiodinase protein and mRNA levels in cardiomyocytes of SCD1 −/− mice (what is consistent with increased T3 concentration), when compared to SCD1 +/+ controls. Furthermore, TH action in tissues is mediated by TR which function as transcription factors binding to TH response elements and change gene expression. TRβ protein and mRNA levels were increased in the heart of SCD1 −/− mice compared to SCD1 +/+ mice. Mice with a targeted disruption in the SCD1 gene are resistant to diet-induced obesity. On the other hand, hypothyroidism is frequently associated with elevated levels of cholesterol, and in particular, triglyceride levels in serum. We induced hypothyroidism in SCD1 −/− and SCD1 +/+ mice and analyzed lipid content by thin-layer chromatography. Interestingly, SCD1 ablation leads to steatosis of liver, adipose tissue and heart of hypothyroid mice. Obtained results show that SCD1 deficiency affects TH signaling pathway. Moreover, TH seems to be important for triggering of anti-steatotic effect of SCD1 ablation.

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Epigallocatechin-3-gallate reverses the defects in modulation of Ca^{2+}-sensitivity by Troponin I phosphorylation caused by hypertrophic and dilated cardiomyopathy mutations in cardiac muscle

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Heart muscle contraction is switched by Ca^{2+} and modulated via the β-adrenergic response that leads to phosphorylation of Troponin I (TnI) at Ser 22/23, which changes the Ca^{2+}-sensitivity of the cardiac myofilament. Previously it has been shown that mutations found in Dilated Cardiomyopathy (DCM) and Hypertrophic Cardiomyopathy (HCM) patients abolish the relationship between TnI phosphorylation and Ca^{2+}-sensitivity (uncoupling).

Ca^{2+}-sensitisers and Ca^{2+}-desensitisers that act upon troponin alter the Ca^{2+}-sensitivity of the myofilament but their relationship with TnI phosphorylation has never been studied before.

Epigallocatechin-3-gallate (EGCG) is a major extract of green tea and it also acts as a Ca^{2+}-desensitiser by binding to Troponin C of the myofilament. 100 μM EGCG decreased Ca^{2+}-sensitivity of phosphorylated and unphosphorylated wild-type thin filaments equally (by 2.15 ± 0.45 and 2.80 ± 0.48-fold, respectively), retaining the coupling. In contrast, EGCG reduced Ca^{2+}-sensitivity of phosphorylated but not unphosphorylated thin filaments containing 8 DCM (*TPM1* E54 K and E40 K, *TNNC1* G159D, *TNNI3* K36Q, *ACTC* E361G) and HCM (*TPM1* E180G, *TNNT2* K280 N, *ACTC* E99 K)-causing mutations. As a result the dependence of Ca^{2+}-sensitivity upon TnI phosphorylation of uncoupled mutant thin filaments was restored in every case.

Furthermore, 30 EGCG analogue compounds were studied using the in vitro motility assay for their ability to recouple HCM and DCM thin filament mutations. Some of these analogues indicated potent recoupling properties, which may be independent of Ca^{2+}-desensitizing activity, and their effects were preserved across three mutations causing DCM or HCM.

The effect of EGCG and its analogues demonstrates that it is possible to reverse the pathological defects in troponin caused by DCM and HCM mutations pharmacologically.

αB-crystallin increases the passive stiffness of skinned cardiac trabeculae via interaction with titin

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Abnormally stiff or compliant cardiac muscle is commonly observed following acute damage or disease. Cardiac stiffness is primarily modulated by the extracellular matrix protein collagen and intracellularly by the giant sarcomeric protein titin. However, it is not clear, particularly in the absence of fibrosis, how stiffness is altered in disease conditions. Recently, a mutation in the small heat shock protein αB-crystallin (R157H) was shown to cause inherited dilated cardiomyopathy. This abundant protein (3–5 % of total soluble protein in the heart) is thought to bind titin and may regulate its stiffness. To test this, we measured the passive stiffness of mouse skinned cardiac trabeculae (with endogenous αB-crystallin extracted) by extending the sarcomere length from 2.0 to 2.6 μm in relaxing solution and measuring the resulting tension; addition of 1 mg/ml recombinant WT αB-crystallin significantly increased stiffness. Addition of the R157H mutant produced a significantly weaker effect. We have shown using mass spectrometry that a nine amino acid peptide, that includes Arg157, interacts with titin; in skinned cardiac trabeculae this peptide increased passive stiffness whereas the mutant R157H peptide showed a diminished effect on stiffness. These data suggest that reduced cardiac stiffness may directly underlie the cardiomyopathy caused by this mutation, and moreover this novel means of cardiac muscle regulation may have implications for the pathogenesis of other cardiac diseases.

Knock out of soluble Guanylyl Cyclase affects myocardial titin phosphorylation and myofilament stiffness

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Question. Soluble guanyl cyclase (sGC) has a crucial role in smooth muscle but also in heart muscle. Soluble GC regulates activity of PKG which has been shown to phosphorylate titin and thereby affects the elasticity of the heart. Here we analyzed how heart-specific and global depletion of the sGC alters titin phosphorylation and titin based passive myofilament stiffness. We further hypothesized that due to potential crosstalk with the PKG signalling pathways other kinases that phosphorylate titin (PKA, PKCα) could be affected by the sGC depletion.

Methods: Titin isoform composition, phosphorylation and titin-based passive stiffness were analyzed in mouse hearts from global soluble guanylyl cyclase KO (sGCKO) and cardiac specific KO (MHC-sGCKO). Titin isofom composition was tested by agarose-strengthened 2.1 % SDS-PAGE. Titin phosphorylation was analyzed by Western blot analysis using phospho-specific antibodies targeting titin's N2B or PEVK domain. Kinase activity was estimated by using phospho-specific antibodies targeting the activated kinases itself or kinase substrates. Passive stiffness was measured using isolated skinned cardiac myocytes.

Results: Titin isoform composition was unchanged in all analyzed groups. sGCKO hearts showed reduced relative N2-Bus phosphorylation at S4010 (PKA-dependent) and S4099 (PKG-dependent). MHC-sGCKO showed reduced S4010 phosphorylation, whereas S4099 phosphorylation was unchanged. PEVK phosphorylation at S11878 and S12022 (PKCα- and CaM-Kinase 2δ-dependent) was unchanged in sGCKO, but reduced in MHC-sGCKO. PKG and PKA activity, assumed from Troponin I phosphorylation, were strongly reduced in sGCKO and unaltered in MHC-sGCKO. PKCα activity was slightly reduced in sGCKO and unchanged in MHC-sGCKO. Instead of an increase in titin based passive stiffness, which could be expected from the observed changes in titin domain phosphorylation we determined a mild but significant decrease in sGCKO hearts compared to wild-type controls. In MHC-sGCKO hearts passive stiffness was unchanged.

Conclusion: Our data demonstrate that sGC depletion impairs not only PKG- but also PKA-dependent titin phosphorylation. The unchanged PKG dependent titin phosphorylation in MHC-sGCKO is unexpected and raises the question of a putative intercellular cross-talk or intracellular mechanism compensating for sGC depletion.

Lack of essential myosin light chain phosphorylation impairs adaption of cardiac function in response to physical stress

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Cardiomyopathy represents a heterogeneous group of diseases characterized by functional abnormalities of the cardiac muscle, resulting in deficient circulation and thereby insufficient oxygen and metabolite supply to the body by the heart. The understanding of underlying mutations of motor or regulatory muscle proteins leading to this cardiac insufficiency helps to design better strategies to overcome such abnormalities.

Mutations in the essential myosin light chain (ELC) have been linked to hypertrophic cardiomyopathy (HCM) with hugely varying phenotypes and high incidences of sudden death. Yet, the exact role of ELC in regulating heart function and especially in the pathogenesis of HCM is still elusive. We used heterozygous adult mutant zebrafish, *lazy susan* (*laz*^{m647}), that carry a nonsense mutation resulting in a 11-amino acid truncation of the C-terminus of ELC and thus the removal of a highly conserved phosphorylation site, S195. By echocardiography we found that heterozygous adult *laz* zebrafish display signs of systolic dysfunction. When subjected to physical stress by forced swimming, heart function severely deteriorated causing heart failure and sudden death. We used native heart tissue to show that upon beta-adrenergic stimulation ELC becomes phosphorylated. Additionally, in vitro motility analysis of zebrafish actin sliding on ventricular myosin derived from wildtype (wt) and *laz* mutant zebrafish after rest or physical stress reveals that C-terminal phosphorylation critically modulates cross-bridge activity, cycling kinetics and filament velocity specifically after stress. Our model enabled us to analyse acto-myosin interaction in native composition of wt and mutant protein. Further, calcium-dependent force measurements and calcium transient recordings demonstrates impaired calcium utilization and handling in *laz* mutant cardiomyocytes, again specifically after physical stress.

Our study thereby for the first time utilizes adult zebrafish to model human relevant cardiomyopathy and uncovers novel mechanistic insights into ELC phosphorylation of S195, which enhances interaction of myosin heads to actin through supporting the binding of the ELC N-terminus to actin filament.

Investigation of obscurin mutations and haploin sufficiency in hearts of patients with dilated cardiomyopathy

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Introduction: It is estimated that 40 % of dilated cardiomyopathy (DCM) cases occur due to inherited mutations in sarcomeric proteins. Recent studies have shown that truncating titin mutations cause 25 % of familial DCM cases. However, studies of the functional consequences of DCM-causing mutations have been limited to a few cases where patients with known mutations had heart transplants. In order to increase the number of potential tissue samples for direct investigation we performed whole exome sequencing of explanted heart muscle samples from 30 patients in the Sydney Heart Bank and screened for potentially disease-causing mutations in 58 HCM or DCM-related genes.
Results: We identified 4 novel potentially disease-causing mutations in OBSCN gene that codes for obscurin. All the mutations were missense and located in highly conserved parts of the *OBSCN* gene except for one, which was judged to be not disease-related. Also identified were 6 truncating mutations in *TTN* gene coding for titin, 3 mutations in *MYH7*, 2 in *DSP* and one each in *TNNC1*, *TNNI3*, *MYOM1*, *VCL*, *GLA*, *PLB*, *PKP2* and *LAMA4*.
We subsequently characterised obscurin expression in human heart tissue samples. By immunofluorescence we showed that obscurin is located at the level of the M-line, co-localising with the myomesin label and complementary to the α-actinin (Z-disc) label. We did not detect any differences in obscurin expression pattern between samples with obscurin mutations and donor heart samples.

We next quantified obscurin protein expression in human heart myofibrils and report that there were no significant differences between donor hearts, DCM samples without *OBSCN* mutations and myectomies obtained from hypertrophic cardiomyopathy (HCM) patients. In contrast, the 3 samples with *OBSCN* mutations had significantly lower levels of obscurin (45 ± 7 %, $p < 0.0001$, 48 ± 3 %, $p < 0.0001$, 72 ± 6 %, $p < 0.014$, respectively) compared with the other DCM samples and controls. Parallel studies with the *TTN* mutations-harbouring DCM samples showed no titin haploin sufficiency.
Conclusions: Disease-related *OBSCN* mutations may result in the development of a DCM phenotype via haploin sufficiency; therefore, mutations in the obscurin gene could be a significant causal factor of DCM, alone or in concert with other mutations.

Altered phosphorylation of cardiac troponin I through mutant R145G

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Background: The amino acid exchange R145G in human cardiac troponin I (hcTnI) causes familial hypertrophic cardiomyopathy. Overexpression of hcTnI-R145G in adult rat cardiomyocytes leads to suppression of contractile parameters. Suppression is also present upon $beta_2$- but not $beta_1$-adrenoceptor (AR) stimulation, where defects are rescued. Therefore, we investigated the influence of the mutation on phosphorylation of cTnI and other sarcomeric proteins.
Methods: Adult rat cardiomyocytes overexpressing mutant or wild-type hcTnI were either not stimulated or subjected to general beta-, or specific $beta_1$- or $beta_2$-AR stimulation and subsequently homogenized to preserve momentary phosphorylation states. Total cellular lysates were studied by western blot analysis, sequential fluorescence staining of one-dimensional (1-D) gels using Pro-Q® Diamond and SYPRO® Ruby, two-dimensional (2-D) gel electrophoresis and phosphate-affinity SDS/PAGE using Phos-tag™.
Results: Western blot analysis of protein kinase A (PKA)-sites Ser23,24 showed a reduction of phosphorylation in the mutant, whereas analysis of global phosphorylation using Pro-Q® Diamond showed no difference between mutant and wild-type cTnI. Separation and quantification of hcTnI phospho-isoforms with Phos-tag™ revealed that specific $beta_2$-stimulation leads to decreased bis- and elevated de-phosphorylation in the mutant compared to wild-type. Elevated de-phosphorylation of mutant hcTnI was also observed in unstimulated cardiomyocytes. Phosphorylation patterns after general beta- and specific $beta_1$-AR stimulation did not differ between mutant and wild-type hcTnI. Investigation of the phosphorylation background of other sarcomeric proteins as myosin light chain 2 and cTnT using Pro-Q® Diamond and 2-D gel electrophoresis did not reveal any differences due to the mutation.
Conclusions: Expression of hcTnI-R145G in adult rat cardiomyocytes leads to an alteration of the phosphorylation pattern of hcTnI at baseline as well as after $beta_2$-AR stimulation. These results are in line with the suppressing effects of the mutant on contractile parameters and may be their underlying cause. Explicit identification of all phospho-isoforms of wild-type and mutant hcTnI generated upon beta-adrenergic stimulation of cardiomyocytes is currently under investigation via mass spectrometry.

Expression of inflammation/remodeling-related genes in skeletal muscle of patients with chronic heart failure after different exercise training protocols

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Introduction: The pathophysiology of chronic heart failure (CHF) involves peripheral myopathy in which pro-inflammatory cytokines play an important role. Nevertheless, inflammation has also beneficial effects on skeletal muscle remodeling, by activating angiogenic and hypertrophic pathways in muscle tissue. Moreover, it remains a matter of discussion whether exercise training interferes with local inflammatory factors involved in the pathogenesis of the CHF myopathy. The aim of this study was to examine and compare the effects of two high-intensity interval training (HIIT) programs on the expression profile of inflammation/remodeling-related genes in skeletal muscle of CHF patients.

Methods: Thirteen male, stable CHF patients (age: 51 ± 13 years; BMI: 27 ± 4 kg/m^2) participated, after stratified randomization, in either a HIIT (n = 6) or HIIT combined with strength training (CT) program (n = 7), consisting of 36 sessions (3/week). The patients of the HIIT program exercised for 3 min at 50 % of peak oxygen uptake (VO_{2p}) followed by 4 cycles alternating 4 min of exercise at 80 % VO_{2p} with 3 min at 50 % VO_{2p}. The patients assigned to the CT program exercised for 3 min at 50 % of VO_{2p} followed by 2 cycles alternating 4 min of exercise at 80 % of VO_{2p} with 3 min at 50 % of VO_{2p}, for a total duration of 17 min followed by 14 min of strength training (60–70 % of the 1-RM). Both exercise regimens were of the same total duration (31 min). All patients performed a cardiopulmonary exercise test and underwent percutaneous needle biopsies of the vastus lateralis muscle before and after the completion of the exercise program. Changes in the mRNA expression profile of the inflammation and/or tissue remodeling-related factors IL-6, TNF-α, uPA, uPA-R, and TGF-β were evaluated in the exercised muscle after the completion of the exercise programs.
Results: An increased expression of TNF-α, uPA-R and TGF-β was found in the total number of patients following the exercise training compared with the pre-exercise expression levels ($p < 0.05$). Moreover, TNF-α, uPA-R and TGF-β exhibited a significant increase particularly after the HIIT program ($p < 0.05$).
Conclusion: The findings of this study suggest that high-intensity interval exercise alters the expression profile of inflammatory/remodeling factors in skeletal muscle of CHF patients, possibly to drive a remodeling process in the exercised muscle. Moreover, HIIT program may interfere more with inflammatory and remodeling factors compared to CT program; thus, the incorporation of strength training in a therapeutic exercise regimen for CHF patients does not appear to induce an excessive inflammatory response in skeletal myopathy of CHF patients.

Cardiac sarcomere function and Ca^{2+} handling are affected by matrix stiffening

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Background: Cardiac stiffening via altered extracellular matrix (ECM) properties is predominantly described as a passive inhibitor of cardiac function. However, as properties of the extracellular environment are sensed inside the cell through costameres that link he ECM directly with the sarcomeres, we hypothesize that cardiac stiffening not only passively but also actively impairs cardiomyocyte function. So far little is known about the effects of ECM stiffness on sarcomere proteins and function.
Methods: To explore the effects of matrix stiffness on sarcomere function, cardiomyocytes isolated from adult rats were plated on laminin-coated polyacrylamide substrates of defined stiffness (8 kPa, 15 kPa, 100 kPa). 24 h after isolation and plating of the cardiomyocytes the cells were detached form their substrates and sarcomere shortening and calcium dynamics were measured.
Results: Interestingly, relative sarcomere shortening and shortening velocity were optimal on substrates which stiffness closely resembled stiffness of the healthy heart (20 kPa), while these parameters were all reduced on a more elastic (8 kPa) and stiffer (100 kPa) substrate. Additionally, relaxation rate was impaired on the stiffer substrate. These findings were not solely explained by sarcomere changes, as Ca^{2+} release velocity and re-uptake rate were highest on the substrate that resembled the healthy heart, but altered in cells plated on the more compliant and stiffer substrates.
Conclusion: Our data show that within a relatively brief period changes in ECM stiffness alter cardiomyocyte sarcomeric and calcium handling properties. Sarcomere function as well as calcium dynamics are affected by stiffness of the extracellular environment and optimal under physiological conditions.

The role of intraluminal reactive oxygen species in endothelin-1 and angiotensin II signaling in the heart

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Hypothesis. Due to a cross-talk between cytoplasmic superoxide (O_2^-) and intraluminally expressed xanthine oxidase (XO), intraluminal O_2^- is a key player in mediating intraluminal and cytosolic vascular oxidative stress induced by endothelin-1 (ET-1) and angiotensin II (AT-II).
Methods and Results: Isolated guinea-pig hearts were subjected to 10-min agonist perfusion causing a burst of an intraluminal O_2^-. ET-1 antagonist, tezosentan, attenuated AT-II-mediated O_2^-, indicating its partial ET-1 mediation. ET-1 and Ang-T (AT-II + tezosentan) triggered intraluminal O_2^-, endothelial dysfunction, MAPKs and p47phox phosphorylation, and NADPH oxidase (Nox) and XO activation. These effects were: (i) prevented by blocking PKC (with chelerythrine), Nox (with apocynin), mitochondrial ATP-dependent K^+ channel (with 5-HD, 5-hydroxydecanoate), complex II (with TTFA, thenoyltrifluoroacetone), and XO (with allopurinol) and (ii) mimicked by the activation of Nox (with NADH); and mitochondria (with diazoxide, 3-NPA). The effects evoked by NADH were prevented by 5-HD, TTFA and chelerythrine, and those evoked by diazoxide and 3-NPA by apocynin and chelerythrine. These results suggest that the agonists coactivate Nox and mitochondria, which further amplify their activity via PKC. The effects by ET-1, AT-II + tezosentan, NADH, diazoxide and 3-NPA were opposed by blocking intraluminal O_2^- (SOD) and XO, and were mimicked by XO activation (hypoxanthine). Also, apocynin, TTFA, chelerythrine, and SOD opposed the effects by hypoxanthine.
Conclusion: Oxidative stress by agonists involves cellular inside-out and outside-in signaling, in which Nox-mitochondria-PKC system and XO mutually maintain their activities via the intraluminal O_2^-.

Rescue of a Tropomyosin (E54 K) dilated cardiomyopathy mouse model by expression of slow skeletal Troponin I

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Dilated cardiomyopathy (DCM) is a heterogeneous group of inherited and acquired disease characterized by decreased contractility and enlargement of cardiac chambers. It is estimated that 30–50 % of DCM has genetic linkage. We have previously shown that mice with E54 K mutation in α-tropomyosin (Tm54) have typical DCM phenotype and reduces myofilament Ca^{2+} sensitivity. Thus, we hypothesized that sensitization of the myofilament to Ca^{2+} in early phase of DCM development would rescue the phenotype of the disease. To sensitize Tm54 myofilament we crossbred Tm54 mice with mice expressing slow skeletal troponin I (ssTnI). ssTnI is a neonatal isoform and its expression results in an increased myofilament Ca^{2+} sensitivity. Four groups of mice were generated: non-transgenic (NTG), Tm54, ssTnI and Tm54/ssTnI. The systolic function was significantly reduced in the Tm54 mice compared to NTG, but restored in Tm54/ssTnI mice. Tm54 mice also showed increased diastolic LV dimension and HW/BW ratios when compared to NTG, which were improved in the Tm54/ssTnI group. Phosphorylation levels of ERK1/2 trended toward a decrease in Tm54 compared to NTG and was restored in the Tm54/ssTnI group. β-myosin heavy chain expression was increased in the Tm54 animals compared to NTG and was partially restored in Tm54/ssTnI group. Analysis by 2D-DIGE indicated a significant decrease in two phosphorylated spots of troponin I in the Tm54/ssTnI animals compared to NTG and Tm54. The decrease in troponin I phosphorylation contributes to the increased myofilament Ca^{2+} sensitivity in the ssTnI transgenic crossed with Tm54 transgenic animals. Analysis by 2D-DIGE also indicated no significant changes in troponin T, regulatory light chain, myosin binding protein C and tropomyosin phosphorylation. Our data indicate that myofilament sensitization to Ca^{2+} may be a useful preventative therapeutic strategy in sarcomere-linked DCM associated with decreased sensitivity.

Angiogenesis-related gene expression profile in skeletal muscle of patients with chronic heart failure after high-intensity interval exercise training

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Introduction: The pathophysiology of chronic heart failure (CHF) involves multiple functional, histological and molecular alterations in skeletal muscle. 1 Capillary density is a peripheral limiting factor of exercise capacity and is significantly decreased in skeletal muscle of CHF patients. Although it is well known that exercise induces skeletal muscle angiogenesis, however there is little information regarding the potential angiogenic effects of exercise training on skeletal muscle of CHF patients. 2 The aim of this study was to examine and compare the effects of two high-intensity interval training (HIIT) programs on the expression profile of angiogenesis-related genes in skeletal muscle of CHF patients.

Methods: Thirteen male, stable CHF patients (age: 51 ± 13 years; BMI: 27 ± 4 kg/m^2) participated, after stratified randomization, in either a HIIT ($n = 6$) or HIIT combined with strength training (CT) program ($n = 7$) consisting of 36 sessions (3/week). The patients of the HIIT program exercised for 3 min at 50 % of peak oxygen uptake (VO2p) followed by 4 cycles alternating 4 min of exercise at 80 % VO2p with 3 min at 50 % VO2p. The patients assigned to the CT program exercised for 3 min at 50 % of VO2p followed by 2 cycles alternating 4 min of exercise at 80 % of VO2p with 3 min at 50 % of VO2p, for a total duration of 17 min followed by 14 min of strength training (60–70 % of the 1-RM). Both exercise regimens were of the same total duration (31 min). All patients performed a cardiopulmonary exercise test and underwent percutaneous needle biopsies of the vastus lateralis muscle before and after the completion of the exercise program. Real Time-PCR was used to measure changes in the mRNA expression of VEGF, VEGFR-2, HIF-1a, Ang-2, Ang-1, Tie-2, ERR-a, ANGPTL-4, and MMP-9.

Results: An increased expression of VEGF, HIF-1a, Ang-2 and ERR-a ($p = 0.028$, 0.012 0.003 and 0.023 respectively) was found in the total number of patients following the exercise training compared with the pre-exercise expression levels. The expression of ANGPTL-4, MMP-9, Ang-1, Tie-2 and VEGFR-2 were also increased, however without reaching statistical significance ($p > 0.05$). Moreover, Ang-2 exhibited a significant increased expression after both exercise protocols (HIIT: $p = 0.028$; CT: $p = 0.043$), while there were not significant differences between the training protocols regarding the expression changes of the genes examined ($p \leq 0.05$). Significant correlations were revealed between the angiogenic factors examined.

Conclusion: The findings of this study suggest that HIIT enhances the expression profile of angiogenic factors in skeletal muscle of CHF patients, possibly driving the angiogenic program in the exercised muscle. Moreover, both HIIT and CT program appear to be capable of promoting angiogenesis and should be further investigated whether they are similarly beneficial as therapeutic regimens for skeletal myopathy of CHF patients.

References:

1. Piepoli, M. (1998) *Cardiologia* **43(9)**: 900 + 917.
2. Bloor, C. M. (2005) *Angiogenesis* **8(3)**: 263–271.

Session 9: Muscle Exercise and Plasticity

Oral presentations

Adaptation of motor unit contractile properties to a strength and endurance training

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The influence of the two kinds of increased physical activity, the strength and the endurance training, on contractile properties of motor units in rat medial gastrocnemius will be presented. The endurance training was performed on a laboratory treadmill (rats were running up to 80 min daily, at 30 m/min). The strength training of animals was performed with the resistance apparatus, in which rats were climbing to reach food against a load put on their shoulders (the resistance progressively increasing up to more than 200 % of rat's body weight). Both types of training resulted in modified proportion of motor units. Two weeks of endurance training increased a proportion of fast resistant motor units, whereas two months of this training increased a proportion of slow units. Five weeks of the strength training increased the proportion of fast resistant units. The two training forms evoked a shortening in the contraction time of fast motor units and the change in the force-frequency relationship. The resistance training had

moderate influence on the force parameters whereas the strength training considerably increased the force of fast motor units, especially of fast resistant type. The twitch-to-tetanus ratio for fast resistant motor units decreased for animals from two trained groups. Finally, the fatigue resistance of fast resistant motor units increased considerably within two weeks of the endurance training whereas the strength training had no influence on this parameter. In conclusion, the training evokes transformative changes of motor units and influences selectively contractile properties mainly of fast motor units.

Acknowledgements The study was supported by National Science Centre grants No 2013/11/B/NZ7/01518 and 2013/09/B/NZ7/02555.

Skeletal muscle deterioration in dilated cardiomyopathy: molecular mechanisms and effects of prolonged endurance training in a mice model

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Dilated cardiomyopathy (DCM) is a progressive disease that often results in death from congestive heart failure or sudden cardiac death. Although the beneficial effects of exercise are recognized in cardiac patients rehabilitation, patients develop exercise intolerance (EI). To clarify the molecular mechanisms underlying EI in DCM we used the transgenic mouse model Tg αq*44 h, characterized by a slow development of the disease. Gastrocnemius muscle was analyzed before and after a protocol of endurance exercise. At 4–6 months of age (before mice develop the disease) the functional performance (time and distance of running) of Tg mice was similar to the control mice (C). Functional performance started to decrease at 10–12 months (when mice develop the disease) and significantly worsened at 14 months (after mice develop the disease). DCM resulted in MHC isoforms shift from the slow to the fast isoforms at all stages of the disease; slow phenotype was restored by exercise.

At 4–6 months of age Tg mice showed (i) lower levels of PGC1α (regulator of mitochondrial biogenesis) and DRP1 (involved in mitochondrial fission) in comparison to C mice suggesting a mitochondrial dysfunction (ii) lower levels of SOD1 (antioxidant defense system protein) and AMPK (energy sensor) in comparison to C mice suggesting the presence of redox imbalance and energy impairment. All these alterations precede the onset of the disease.

At 10–12 months, mitochondrial and energetic alterations persisted and a higher level of protein oxidation was found in Tg mice according with the early SOD1 decrease. In addition, the catabolic pathways basal levels, atrogin1 and MuRF1 (ubiquitine proteasome system), Cathepsin-L, Beclin1 and LC3 (autophagic system) were lower in Tg mice suggesting a degradation processes alteration.

At 14 months (after mice develop the disease), in addition to the previous changes observed at 10–12 months, an increase of Parkin (mitophagy marker) was found suggesting a mitophagy increase in Tg mice.

The early mitochondrial alteration in association with the low catabolic basal levels could be responsible for oxidised proteins and damaged mitochondria accumulation with possible effects on muscle function in Tg mice.

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Modulation of Muscle IGF-I production alters glucose uptake during exercise

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IGF-I and insulin are intrinsically connected through their actions on the IGF-I and insulin receptors to regulate blood glucose. Reduced circulating IGF-I can be compensated by heightened insulin, but chronically elevated insulin can lead to insulin resistance and ultimately diabetes. Further, increased circulating or local muscle IGF-I may enhance glucose uptake. If IGF-I from muscle and liver is equivalent, then loss of muscle IGF-I should result in a similar pathologic diabetic state. By extension, if muscle IGF-I is elevated, it may serve a protective role in glucose homeostasis, either through increased muscle mass providing a greater glucose sink, or through increased hybrid receptor activation by IGF-I. To address the impact that these factors have on metabolism, we performed local AAV-IGF-I injections into both hindlimbs of adult male mice, and subjected the mice to tests for body composition, glucose uptake, and energy expenditure compared to age-matched controls. It was not surprising that the hindlimb injections boosting IGF-I levels only in a small group of muscles did not alter the whole animal body composition. Further, when mice were subjected to treadmill running for 60 min, there were no significant changes in blood levels of glucose or lactate pre- or post-exercise. While increased muscle mass did not appear to alter basal glucose uptake, increased IGF-I content altered contraction induced glucose uptake in muscle when normalized to mass. To understand the consequences of diminished muscle IGF-I production, we generated mice with inducible muscle specific IGF-I deletion, with induction in adult mice. In these mice, glucose levels following treadmill running increased by 10 %, in contrast to controls where blood glucose decreased by ~40 %, supporting that glucose clearance is mediated in part through muscle IGF-I. Based on these results, we assert that the IGF-I produced by the muscle has an endocrine function, and like IGF-I produced by the liver, modulation of muscle levels of IGF-I will lead to changes in glucose homeostasis.

miR-542: a novel regulator of muscle mass and function

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A high quality of life is dependent on the ability to move freely as it enables us to complete our daily tasks independently. Accordingly, diseases that lead to a loss of muscle mass and a reduction in the endurance of the muscles markedly reduce the quality of life. Muscle wasting is a common co-morbidity of a number of chronic diseases including chronic obstructive pulmonary disease (COPD) as well as occurring in the critically ill and in older people. In these groups a loss of muscle mass and function is associated with a reduction in life expectancy. Consequently, understanding the mechanisms that regulate muscle mass is of significant interest.

In a PCR based screen we found that both miR-542-3p and miR-542-5p were markedly elevated in patients with COPD. Further analysis showed that these miRNAs were inversely correlated with lung function in these patients as well as with fat free mass index (FFMI) and strength. We therefore determined the expression of these miRNAs in patients with established intensive care unit acquired weakness (ICUAW). In patients with ICUAW both miR-542-3p and miR-542-5p were noticeably elevated (more than 20 fold) compared to patients about to undergo aortic surgery. These data suggest that these miRNAs are associated with muscle mass in multiple conditions.

To identify the mechanisms by which miR-542 regulates muscle mass we used bioinformatics analysis to determine the pathways that the miRNAs target. This analysis showed that these miRNAs targeted SMAD7 and SMURF1, inhibitory components of the TGF-β signalling pathway. We therefore determined the effect of miR-542-3p and -5p on the activity of a SMAD-dependent luciferase reporter gene and the localisation of phospho-SMAD2/3 in myoblasts. These analyses showed that in the absence of ligand, both miR-542-3p and -5p increased SMAD dependent gene expression and the accumulation of phospho-SMAD2/3 in the nucleus. *In vitro* the miRNAs suppressed SMAD7 and SMURF1 protein expression. Furthermore, we have previously shown that in ICUAW there is an increase in nuclear phospho-SMAD2/3. Consequently, we analysed the expression of these proteins in patients in ICUAW. Consistent with the in vitro data SMAD7 was suppressed in patients with ICUAW.

Together the data suggest that miR-542-3p and -5p contribute to the loss of muscle mass by increasing the basal activity of the TGF-β signalling pathway.

Posters

Acoustic MyoGraphy (AMG) a non-invasive method for assessing muscle function

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Non-invasive measurement of muscle function during daily activities is important in the general assessment of patients with pain and functional problems.

Recent development of a tested acoustic myography (AMG) unit which can non-invasively detect muscle function (efficiency, spatial and temporal summation) in human subjects (Harrison et al. 2013), enables the real-time assessment of muscle function. The first prototypes of an improved and refined AMG unit (CURO; MyoDynamik ApS, Copenhagen, DK) have now been developed and the data are analyzed using an ESTi^{TM} score that is unique to the CURO. The ESTi^{TM} score combines the three aspects of muscle function to reveal a detailed analysis of muscle performance.

Preliminary trials have shown that this technique is not only sensitive enough to pick up individual muscle group differences in terms of contraction and function, but that it can be used during periods of physical activity. The objective of this work has been to relate the AMG data to muscle fibre recruitment.

AMG data were obtained from the *m. Gastrocnemius* and *m. Latissimus dorsi* muscles of a adult male subject (50 years) taken during a period of physical activity using a Me-Mover, which requires full body balance and activates the core muscles in the body whilst cycling. The data not only reveal the difference between the two muscle groups in terms of fibre recruitment, but also show a weakness in the subjects right leg that affects the overall AMG data. There is evidence of a clear cross-over effect from the leg to the back muscles.

The presented method to assess muscle condition and function is non-invasive and can be applied in more or less any setting, and it is easily transportable. Results are presented on the spot, which is important when used in the clinic where quick correct diagnosis will help to initiate the correct treatment without delay.

References:

Harrison AP et al. (2013) *Physiological Reports* **1**: e00029.

Partial preservation of intrinsic skeletal muscle function in upper- and lower-limbs of oldest-old humans according to in vivo and in vitro evidence

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Loss of skeletal muscle mass and the related decline in muscle strength often contribute to the disability and finally the mortality in the elderly. In this study we investigated the combined effects of ageing and long-term disuse on skeletal muscle exploring muscle function and mass both in vivo and in vitro. In 8 young (YG; 25 ± 6 years) and 8 oldest-old mobile (OM; 87 ± 5 years) and 8 oldest-old immobile (OI; 88 ± 4 years) women upper limb (biceps brachii BC) and lower limb (quadriceps QC) muscles were analysed. *In vivo*, maximal voluntary contraction force (MVC) and force of electrically evoked twitch at rest (RT) were evaluated, whereas muscle volume and physiological cross sectional area (PCSA) were measured from NMR images in QC and BC. Muscle biopsies of vastus lateralis (QC) and BC were taken for in vitro assessment of single fibres to determine the following parameters: cross sectional area (CSA), isometric force (Fo) and tension (Po), and Myosin Heavy Chain (MyHC) isoform composition.

In vivo, compared to the YG, both the OM and OI exhibited a more pronounced loss of MVC in the lower-limb (OM (−60 %) and OI (−75 %)) than the upper-limb (OM = −51 %; OI = −47 %). If the reduction in muscle PCSA (OM = −10 %; OI = −18 %) was taken into account, the voluntary muscle specific force of the lower-limb was more compromised in the OI (−40 %) than the OM (−13 %). However, in vivo, specific tension developed in an electrically evoked twitch (RT) in both upper- and lower-limbs (∼9.8 N m cm^{-2}) was well preserved in OM and OI compared to YG.

The distribution of the myosin isoforms in the biopsy samples showed an increased proportion of MyHC-2X isoform in the upper and lower-limbs of both OM and OI in comparison to the YG, while the proportion of both MyHC-1 and MyHC-2A isoforms were not statistically different. There was, however, a trend for a higher proportion of MyHC-1 isoform in the legs of YG compared to OM and OI.

The analysis of single muscle fibers showed that CSAof fibers from the lower- and upper-limb was similar across the three groups (YG, OM, IM) and in all groups significantly higher in the lower-limbs compared to the corresponding upper-limbs. In OM and OI muscle biopsy samples, however, the relatively thick fibers dissected and studied in mechanical experiments were accompanied by a very abundant population of thin atrophic fibers. Isometric specific tension (Po) was not significantly different among the fibers dissected from three groups (YG, OM, IM).

In conclusion, our results showed that, as expected, a marked loss of muscle mass and voluntary force occurs in the oldest old women and it is more pronounced in the immobile compared to mobile women and in lower limb compared to upper limb muscles. In partial contrast, however, tension in electrically evoked twitch and tension developed by single fibers activated in vitro demonstrated a preservation of function in at least part of the muscle. This suggests that in oldest old the decline is due to loss of muscle fibers and impaired voluntary control.

Uneven distribution of two human Ankrd2 isoforms between skeletal and cardiac muscle

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Muscle ankyrin repeat proteins (MARPs) Ankrd1 and Ankrd2 are components of a titin associated stretch-sensing complex in the myofibril. They are active participants in muscle cell signaling and play a role in regulation of skeletal muscle differentiation, growth and remodeling. Upon different stress stimuli they re-localize to the nucleus and regulate transcription as co-factors. MARPs are differentially expressed in cardiac and skeletal muscle tissue; Ankrd1 is mainly expressed in the heart, while the major site of Ankrd2 expression is skeletal muscle. Although predominant in skeletal muscle, Ankrd2 protein is also detected in healthy adult heart. On the other hand, several isoforms of Ankrd2 have been reported in the protein databases. The mainly investigated ones are canonical, also called cardiac form of 360 aa (UniProtKB/Swiss-Prot: Q9GZV1.3) and form of 333 aa (S-Ankrd2, GenBank: CAC19412.1). These isoforms are products of translation from alternative ATG codons; S-Ankrd2 is translated from distal one, giving rise to the protein that is lacking the N-terminal region of the canonical Ankrd2 protein. Using transcriptome profiling and RNAseq of healthy adult cardiac and skeletal muscle we obtained strong evidence of their uneven distribution between skeletal and cardiac muscle tissues. Reads for longer exon 1 of Ankrd2 were gained only for cardiac muscle, while reads for both shorter and longer exon 1 were detected in both tissues. Since antibodies to different regions of S-Ankrd2 were able to detect both forms, we produced specific antibody to distinguish the canonical Ankrd2 from the S-Ankrd2. It was raised against a peptide specific for the N-terminal extension of Ankrd2 which is not present in S-Ankrd2. Specificity of the antibody for the canonical form was tested and confirmed by immuno-adsorption assay and Western blot. Results obtained by transcriptome profiling were further verified on protein extracts of human cardiac and skeletal muscle tissue, showing preferential distribution of isoforms in different types of striated muscle. Intracellular localization of Ankrd2 isoforms was studied by immunohistochemistry in human skeletal and cardiac muscle sections. We observed preferred nuclear localization of Ankrd2 that was further corroborated by exclusive nuclear targeting of human Ankrd2-GFP protein overexpressed in neonatal rat cardiomyocytes, while S-Ankrd2 was redistributed between the cytoplasm and nucleus. In conclusion, our results further extend the spectrum of regulatory mechanisms governing muscle tissue specific expression of MARP proteins.

Strength training evokes adaptive changes in motoneuron properties

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Different forms of an increased or a decreased muscular activity induce adaptations in contractile properties of motor units as well as in electrophysiological properties of motoneurons (MNs). Long-lasting strength training, with repeated short-term and high-intensity exercises, is responsible for an increase of muscle mass and force, however particular types of motor units respond differently to this kind of training. The aim of this study was to determine whether the strength training induces adaptations in electrophysiological properties of motoneurons (MNs) innervating the trained muscles. Adult Wistar rats were randomly assigned to the training or the control groups. Animals from the training group were nutritionally conditioned in order to make weightlifting put on their shoulders, in a special apparatus, with progressively increasing load, for 5 weeks. Acute electrophysiological experiments were performed on deeply anesthetized animals from both groups, using intracellular microelectrode recordings from motoneurons innervating hind limb muscles. Passive and threshold membrane properties were measured, and rhythmic firing of MNs was analyzed. It was demonstrated that 5-week strength training evoked adaptive changes in both fast and slow types of motoneurons: a shortening of the rise time of action potentials, an increase of the maximum frequencies of rhythmic firing, and an increase in the slope of the frequency-current relationship, which suggests higher susceptibility of motoneurons to an increased or decreased intensity of stimulation. Moreover, a decrease in rheobase currents, and a decrease in the minimum currents required to evoke rhythmic firing was observed in fast-type motoneurons only, suggesting their higher excitability.

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Adaptive changes of motor unit contractile properties to chronic compensatory muscle overload

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The study aimed at examining the influence of long-term muscle overload on the force regulation and contractile properties of motor units (MUs) in the rat medial gastrocnemius muscle (MG). The compensatory overload was induced by bilateral tenotomy of all MG's synergists (lateral gastrocnemius, plantaris and soleus muscles). As a result the MG was the only muscle able to perform the foot plantar flexion. To prevent the excessive stretch of the MG muscle,

and to ensure its regular activity, animals were kept in wheel-equipped cages and performed low-level exercise on a treadmill. Electrophysiological experiments were performed on 109 functionally isolated MUs in overloaded (O) and 148 in control (C) groups, 3 months after the surgery. Hypertrophy of the overloaded muscle was observed in comparison to C group. Higher percentage of slow (S) and fast fatigable (FF) MUs was noted in the O group, together with lower contribution of fast resistant (FR) MUs. Eight MUs in the O group could not be classified unequivocally, since they presented properties typical either for the S or FR type. MUs after overload were able to produce higher absolute tetanic forces, but achieved the same relative force level at higher frequencies than control. Increase in motoneuronal stimulation frequency in fast MUs evoked considerably higher force increase in comparison to control, while the reverse effect could be observed in S MUs. The optimal tetanic contraction of overloaded FR and S units were developed at higher stimulation frequencies and had higher fusion indexes in comparison to the C group. Additionally, S MUs in the O group were more resistant to fatigue, while FF MUs were more fatigable. In conclusion, all three MU types respond to the compensatory muscle overload unequally, and changes in the contractile properties of MU are probably accompanied by transformational processes of muscle fibers. Changes in mechanisms of force regulation and development, both for the whole muscle and for its MUs, likely correspond with altered activity of motoneurons.

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Effect of mechanical loading on the myogenic differentiation of C2C12 cells

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Introduction: Mechanical loading of differentiated myotubes mimics the loading pattern of mature skeletal muscle (Soltow et al., 2013) and alterations in signaling and gene expression responses have been reported upon various protocols of mechanical loading applied on myoblasts (Kook et al., 2008). The purpose of the present study was to investigate the effects of passive stretching of C2C12 cells on signaling and gene expression responses associated with their myogenic differentiation program.

Methods: C2C12 cells, a cell line derived from murine fast-twitch skeletal muscle, were cultured on elastic membranes and mechanically loaded under the same cyclic stretching protocol (i.e., 10 % elongation, at a frequency of 1 Hz, for 1 h) every either 24 or 48 h for 7 consecutive days during their differentiation. Cells were harvested 1 h after the completion of their stretching on the day 7 of differentiation. Phosphorylation of signaling proteins Akt and ERK1/2 was determined by immunoblotting of lysates from stretched and non-stretched myotubes. Real Time-PCR was used to measure changes in the expression levels of the myogenic regulatory factors myoD and myogenin, and of the muscle-specific enzyme creatine kinase (CK) in response to mechanic loading of the differentiating cells.

Results: Passive stretching of the C2C12 myotubes resulted in reduced phosphorylation of Akt without significant changes in ERK1/2 phosphorylation in both cell stretching protocols. Moreover, the mechanical loading of the myotubes decreased the expression of the early differentiation factor myoD as well as of the intermediate myogenic differentiation factor myogenin, while increased the expression levels of CK. No significant differences were revealed between the stretching protocols either in signaling or gene expression responses.

Conclusion: Akt is primarily involved in the myogenic differentiation process and is especially responsive to mechanical stretching of the fast-twitch muscle cells (Sakamoto et al., 2003). Hence, the decreased activation of Akt found in the present study, along with the down-regulation of myoD and myogenin in response to mechanical stretching, might indicate a specific drive towards a delay in the myogenic differentiation program of the stretched C2C12 myoblasts.

References:

Soltow et al. (2013) *Biochem Biophys Res Commun* **3**: 316–321.
Kook et al. (2008) *Mol Cell Biochem* **309**: 133–141.
Sakamoto et al. (2003) *Am J Physiol Endocrinol Metab* **285**: 1081–1088.

The liver X receptors α and β determine skeletal muscle hypertrophy upon chronic muscle contractions

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The liver X receptors alpha and beta (LXRa/LXRb) constitute a family of ligand-activated nuclear receptors that function as transcription factors. LXRs have been identified as fatty acid regulators. Recently, different reports demonstrated the involvement of LXRs in cardiac and chondrocyte hypertrophy adaptations. The role of LXRs in skeletal muscle is, however, still unaddressed, why we studied skeletal muscle hypertrophic responses upon chronic endurance exercise training regimes in LXRa and LXRb knockouts (LXRa-KO/LXRb-KO) compared to wild type (WT) littermates.

LXRa/LXRb-KOs and age-matched WT littermates ($n = 6$ per genotype) were subjected to a standardized and forced chronic treadmill endurance training regime: 4 weeks, 5 days/week, 60 min/day, 18 m/min, 10° incline. After training regime, the M. gastrocnemius (Gas) was dissected from both hindlimbs. Gas muscle were either fixed in 4 % PFA or frozen in ice-cold isopentane and stored at −80 °C until further analyses. PFA-fixed Gas muscles were used for histological and ultrastructural analysis. The Axiovision software (Zeiss, Germany) was used for histological examinations of muscle cross-sectional areas (CSA, μm^2), capillarization, and nuclei content/localization (periphery *vs.* centralized).

Gas CSA were significantly (p0.001) reduced upon chronic ndurance exercise training in both LXRa- and LXRb-KO compared to their sedentary counterparts. In contrast, trained WT Gas muscle adapted with significantly ($p < 0.001$) increased CSA upon the chronic training intervention. Interestingly, the capillarization was unaffected, but adapted in physiological manner in both LXR-KOs, as the capillarization was significantly ($p < 0.001$) increased upon the training regime compared to sedentary KOs. Trained WTs showed a similar adaptation compared to sedentary WTs ($p < 0.001$). Centralized nuclei were unchanged between sedentary genotype, but significantly increased upon chronic training in both LXRa and LXRb-KO ($p < 0.05$ for both) compared to WT.

Our data show for the first time that both LXR family members possess critical roles in skeletal muscle hypertrophy adaptations towards physiological stimuli. Both LXR-KOs lack the ability to induce skeletal muscle hypertrophy upon chronic muscle

contractions. Currently, we are exploring the underlying mechanisms of this clinically-relevant phenomenon. Overall, these data shed new light on skeletal muscle hypertrophy mechanisms by identifying a potentially new therapeutic target to fight muscle loss.

Force enhancement after stretch in mouse soleus muscles: no evidence for contribution of mechanosensitive TRPV4 cation channels

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Transient receptor potential (TRP) channels are widely expressed in different organs and cell types including skeletal muscle fibres. The TRP family of cation channels is responsible not only for calcium homeostasis but also for mechanical and chemical sensation. TRPV4 has been reported to respond to mechanical and osmotic stimulation in osmosensory neurons and skeletal muscle fibres. A recent investigation suggested that TRPV4 is an important constituent or organizer of mechanosensitive ion channels in mouse skeletal muscle fibers. Furthermore TRP channels became candidates to be involved in increased Ca^{2+} influx and secondary pathological changing in X-linked muscular dystrophies. Indeed, muscle fibres of *mdx* mice are more sensitive to eccentric contractions and gain increased membrane permeability than wildtype mice. In the current study we investigated whether moderate passive stretching causes increases of isometric force in mouse soleus muscles and whether TRPV4 channels contribute to the phenomenon. For this, we used muscles from TRPV4 deficient (knockout) mice and from WT mice. We further tested whether *mdx* muscles respond differently from their corresponding controls. We recorded twitches from isolated soleus muscles in response to single electrical stimuli and 50-Hz tetani before, during and after phases of cyclic stretching. For this, muscles were adjusted to 95 % of their optimal length and subsequently stretched and relaxed by ± 10 % at a 1-Hz sinus rhythm. Twitches and tetani were recorded before, after 3, 6 and 9 min of stretching and again after 3, 6, and 9 min of resting. Amplitudes of twitches from WT mice were significantly increased after cyclic stretching (22.5 ± 1.2 *vs.* 21.0 ± 1.1 mN, mean ± SEM, $p < 0.01$, $n = 19$) compared to initial values before stretching. Interestingly, muscles from TRPV4 knockout mice showed similar levels of increase after stretching (19.4 ± 1.1 *vs.* 18.2 ± 1.1 mN, $p < 0.01$, $n = 19$). The increase of isometric twitch force was almost reversible after 9 min of rest without stretching. Not only TRPV4 deficient mice but also *mdx* mice showed increases of twitch amplitudes after stretching. Tetanic forces of all four tested strains were not significantly affected by the stretch protocol. We conclude that mouse soleus muscles show increased twitch forces in response to passive stretching. However, the cation channel TRPV4 does not seem to play a role in this process. It is still questionable, if the force enhancement in response to mechanical stimulation is related to the activity of mechanosensitive ion channels of the sarcolemma in the muscle fibres.

Chronic neck pain assessment using mfBIA

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Chronic neck pain is common, and it affects more than 30,000 individuals in Scandinavia (Pohjola, Finland). The 12 months prevalence of activity limiting pain occurs in up to 11.5 % of cases, whilst 30–50 % of individuals report pain to some degree (Hogg-Johnson et al. 2008, Spine 33: S39–S51). Typically such injuries are associated with a rapid deceleration of the head and neck e.g. car crash, cycle accident etc., and it affects mostly subjects in full-time employment.

Bioimpedance is a non-invasive technique used to assess muscle mass and health. Multi-frequency bioimpedance has recently been shown to provide details regarding muscle resting tension, cellular activity as well as training level and recovery rate following muscle injury (Nescolarde et al. 2013, Phys. Meas. 34: 237–245; Bartels et al. 2015, Phys. Reports 3(3) e12354). In a female patient, aged 53 years, height 165 cm, weight 70 kg (BMI 26) who suffered a fall from a horse in 2011 (3 years prior), we assessed the neck muscle sternocleidomastoid. Treatment (AtlasBalans; SE) was applied directly to the neck region for a period of 30 min. mfBIA measurements were carried out prior to and following treatment using a multi-frequency BioImpedance Analysis unit (SFB7, Impedimed, AUS).

Prior to treatment, the sternocleidomastoid muscle exhibited a 2 times normal resting tension (assessed by centre frequency, fc) and was slightly more tense on the right *vs.* left hand side. AtlasBalans treatment induced a 20 % relaxation in the sternocleidomastoid muscle.

These findings are in accordance with other observations that the sternocleidomastoid muscle is principally involved in head deceleration type injuries. We suggest that a high level of resting tension is not only measureable in these patients, but that it can be relieved with massage forms of treatment like AtlasBalans.

References:

Hogg-Johnson S et al. (2008) *Spine* **33**: S39–S51
Nescolarde L et al. (2013) *Phys. Meas.* **34**: 237–245
Bartels EM et al. (2015) *Phys. Reports* **3**(3): e12354

Session 10: Muscle Metabolism and Bioenergetics

Oral presentations

Work-loop microcalorimetry informs the thermodynamics of contraction of isolated rat trabeculae carneae

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The heart is a thermodynamic machine. With each beat, it develops force, shortens and performs pressure–volume work (W). We mimic these three-dimensional events, which take place in the macroscopic domain, by studying the behaviour of 1-D ventricular trabeculae undergoing force–length work-loops. In either case, mechanical contraction is achieved, in the microscopic domain, by the relative sliding of the contractile filaments, funded by the Gibbs Free Energy (ΔG_{ATP}) of hydrolysis of ATP by the Ca^{2+}-triggered, actin-activated myosin ATPase of the cross-bridges. The thermodynamic efficiency of these events is imperfect. Hence, the performance of microscopic work is accompanied by the liberation of heat (Q). By summing the work performed and the heat evolved we obtain a measure of enthalpy

generation by the muscle: $\Delta H = W + Q$. Enthalpy is a function of after-load; thus, so is mechanical efficiency: $\varepsilon = W/\Delta H$.

We can achieve this thermodynamic characterisation of muscle contraction because we have developed a unique 'work-loop calorimeter' in which, using custom-written control software, a rat trabecula undergoes a quasi-realistic work-loop, while its heat output is measured simultaneously. Heat production is inferred from the increase in temperature between upstream and downstream thermoelectric sensors, as the superperfusate flows over the muscle. At a flow rate of 0.5 mL/s, thermal noise is of the order of 1 mK, thereby allowing heat to be resolved to approximately 10 nW.

We have used the work-loop calorimeter to characterise the mechano-energetics of trabeculae from both healthy and pathologically hypertrophied hearts. A particularly revealing result presents in diabetic cardiomyopathy. Whereas the peak pumping efficiency of the rat whole heart (approximately 15 %) is shifted to a reduced afterload [1], that of its isolated trabeculae [2] remains unchanged.

References:

1. Han J-C et al. (2014) *Cardiavascular Diabetology* **13**: 1–12.
2. Han J-C et al. (2014) *Cardiovascular Diabetology* **13**: 1–16.

Heat Shock Protein 72 protects against lipid-induced insulin resistance in skeletal muscle via AMPK and CaMKK upregulation

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Insulin resistance, a condition in which cells fail to respond to insulin, is associated with many health related complications, including type 2 diabetes and heart disease. Recently it was shown that expression of heat shock protein 72 (Hsp72) in skeletal muscle is positively correlated with insulin sensitivity and negatively correlated with body fat percentage in humans. Moreover it was observed that increased level of Hsp72 in muscle can protect against obesity-induced insulin resistance, but the underlying molecular mechanisms remain poorly understood. Therefore, the aim of this study was to investigate the molecular mechanisms involved in Hsp72-associated regulation of insulin sensitivity in skeletal muscle and to define the roles of Hsp72 domains in its effect on insulin signaling.

Herein we show that Hsp72 decreased palmitic acid-induced insulin resistance and lipid accumulation in C2C12 myotubes. Additionally, C2C12 cells overexpressing Hsp72 were characterized by upregulation of 5′AMP-activated protein kinase (AMPK), what was attenuated by Ca2 +/calmodulin-dependent protein kinase inhibitor, STO609. Moreover inhibition of AMPK with compound C attenuated the Hsp72-induced improvement in insulin sensitivity in C2C12 myotubes. We also observed that overexpression of Hsp72 with an inactive ATPase domain (Hsp72 K71E) did not affect insulin sensitivity nor lipid accumulation in C2C12 myotubes, but AMPK in these cells was significantly downregulated. Interestingly Hsp72 without C-terminal EEVD domain (Hsp72 ΔEEVD) increased insulin sensitivity, decreased lipid content and also upregulated AMPK in C2C12 cells. Furthermore, the effect of Hsp72 ΔEEVD on insulin pathway was more evident in comparison to wild type Hsp72.

Overall, this study showed that Hsp72 decreases lipid accumulation and improves insulin sensitivity via upregulation of AMPK pathway in C2C12 cells, in CaMKK-dependent manner. Furthermore, active ATPase domain in Hsp72 is required to increase insulin sensitivity and AMPK phosphorylation in myotubes, while C-terminal EEVD domain of Hsp72 is dispensable for its action on insulin signaling.

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Disturbed mitochondrial dynamics and network in diaphragm muscle fibers of mechanically ventilated critically ill patients

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Background: In mechanically ventilated critically ill patients, weakness of the main inspiratory muscle, the diaphragm, may delay weaning and increase the duration of hospitalization and morbidity. This weakness is largely caused by contractile weakness and atrophy of individual diaphragm fibers and activation of the ubiquitin–proteasome pathway. The mechanisms that activate proteolysis in the diaphragm of critically ill patients are unknown, but might involve changes in mitochondrial function.

Aims and Objectives. To investigate mitochondrial dynamics, morphology, and function in diaphragm fibers of critically ill patients.

Methods: Muscle fibers obtained by diaphragm biopsies of mechanical ventilated critically ill patients ($n = 28$) and control subjects ($n = 27$) were analyzed by electron microscopy, Western blotting, immunohistochemistry and respirometry.

Results: Control and critically ill patients were mechanically ventilated for 1.4 ± 0.1 h and 153 ± 32.0 h respectively, ($p < 0.0001$). Electron microscoy showed abnormal general impression of mitochondria in 6/7 critically ill and 3/8 control patients. Compared to controls, PGC1-α—involved in mitochondrial biogenesis—was decreased by 23.81 % ($p = 0.016$) in the critically ill, whereas the ratio of phosphorylated AMPK/total AMPK—an indicator of metabolic stress—was increased by 62.75 %. Compared to controls, mitochondrial fusion proteins Mfn1, Mfn2 and OPA1 were decreased in the critically ill, respectively 16.67 % ($p = 0.046$), 12.50 % ($p = 0.008$) and 12.50 % ($p = 0.010$), whereas mitochondrial fission protein DRP1 was increased by 20.83 % ($p = 0.04$) in the critically ill. The contents of mitochondrial complex III-IV were significant lower in critically ill patients. Respirometry did not differ between control and critically ill patients.

Conclusions: These data suggest a disturbed mitochondrial network, a lower content of mitochondrial complexes supported by altered mitochondrial dynamics with a shift towards mitochondrial fission in

diaphragm muscle fibers of mechanically ventilated critical ill patients. These alterations may contribute to diaphragm weakness and to weaning failure in critically ill patients.

Hacd1-knockout mice are protected against high-fat-diet-induced obesity and insulin resistance

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While congenital myopathies constitute a group of rare disorders, the number of mutations and genes implicated in these myopathies increases. We identified that a loss of function mutation in HACD1/PTPLA gene causes a myopathy characterized by muscle weakness and exercise intolerance in Labrador retrievers. Within the endoplasmic reticulum, HACD1 participates to the elongation of very long chain fatty acids ($C \geq 18$). Using animal and cellular models for HACD1 deficiency, we proved that HACD1 is specifically and dynamically regulated in differentiating myoblasts, where it regulates cell membrane composition and fluidity. As a consequence, HACD1 loss of function in mouse and dog impairs myoblast fusion during development and has likely consequences on membrane and organite dynamics during adulthood. Skeletal muscles represent 40 % of the body mass and besides their essential roles in locomotion and breathing, they constitute the main glucose sink of the organism in response to insulin. We thus hypothesized that the reduced muscle mass and spontaneous locomotion of Hacd1-knockout (KO) mice would lead to an altered sensitivity to insulin. Counter intuitively, Hacd1-KO mice exhibited higher glucose tolerance and insulin sensitivity, both in normal and high fat diet. Moreover, during high fat diet, despite eating the same quantity of food, Hacd1-KO mice exhibited a resistance to obesity, with reduced weight gain and fat accumulation. To further understand the fate of the increased internalized glucose, we performed histological staining of muscle biopsies and revealed that Hacd1-KO mice did not accumulate glycogen but exhibited a higher level of oxidative activity. On skinned muscle fibers, we showed that mitochondrial beta oxidation and uncoupled respiration was elevated in Hacd1-KO mice, suggesting that this increased catabolic activity consumed increased levels of lipids and glucose and partially compensated the over ingested fat during high fat diet. We now aim at exploring the structure and lipid composition of mitochondria membranes.

Taken together, our results demonstrate for the first time that HACD1 plays a major role in muscle metabolism. Moreover, the protective effect of HACD1 deficiency towards diet-induced obesity and insulin resistance may provide grounds for the development of new therapies based on candidate lipids to counteract the morbid evolution of metabolic syndrome.

Posters

Capric acid up-regulates UCP3 expression without PDK4 induction in mouse C2C12 myotubes

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Uncoupling protein 3 (UCP3) and pyruvate dehydrogenase kinase 4 (PDK4) in skeletal muscle are key regulators of the glucose and lipid metabolic processes that are involved in insulin resistance. Medium-chain fatty acids (MCFAs) have anti-obesogenic effects inrodents and humans, while long-chain fatty acids (LCFAs) cause increases in body weight and insulin resistance. To clarify the beneficial effects of MCFAs, we examined *UCP3* and *PDK4* expression in skeletal muscles of mice fed the MCFA- or LCFA-enriched high-fat diet (HFD). Five-week-feeding of the LCFA-enriched HFD gained high body weight and induced glucose intolerancein mice, compared with those in mice fed the MCFA-enriched HFD. However, the amounts of *UCP3* and *PDK4* transcripts in the skeletal muscle of mice fed the MCFA- or LCFA-enriched HFD were similar. To further elucidate the specific effects of MCFAs, such as capric acid (C10:0), on lipid metabolism in skeletal muscles, we examined the effects of various FAs on expression of *UCP3* and *PDK4*, in mouse C2C12 myocytes. Although palmitic acid (C16:0) and lauric acid (C12:0) significantly induced both expression of *UCP3* and *PDK4*, capric acid (C10:0) upregulated only *UCP3* expression via activation of peroxisome proliferator-activatedreceptor-d. Furthermore, palmitic acid (C16:0) disturbed the insulin-induced phosphorylation of Akt, while MCFAs, including lauric (C12:0), capric (C10:0), and caprylic acid (C12:0), did not. These results suggest that capric acid (C10:0) increase the capacity for fatty acid oxidation without inhibiting glycolysis in skeletal muscle.

Sleep debt induces muscle atrophy in rats through activation in autophagy system

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Studies have shown that rodents submitted to paradoxical sleep deprivation (PSD) or paradoxical sleep restriction (PSR) protocols suffer high loss of skeletal muscle tissues. A catabolic profile has been observed, with a reduction in anabolic hormones and growth factors, concomitant with increased corticosterone (C) levels; however, the molecular mechanisms involved in this process is unknown.

Aim: Evaluate the molecular signalling pathways related with maintenance of skeletal muscle in sleep-deprived and chronic sleep-restriction rats.

Methods: Forty 90-day old Wistar male rats were randomly allocated into one of four groups: control sleep deprivation (CTRL-PSD), PSD for 96-h (PSD-96), control sleep restriction (CTRL-PSR), PSR for 21 consecutive days (PSR-21). The PSD and PSR protocols were performed using the modified multiple platform method, which consisted of placing 10 rats inside a tiled water tank containing 15 circular platforms, 6.5 cm in diameter, with water up to 1 cm of their upper surface. Thus, the rats could move around from one platform to another by jumping inside the tank, avoiding immobilization. When reaching the paradoxical phase of sleep, they fall into the water due to muscle atonia and awake. After the PSD or PSR periods, the rats were decapitated for blood and muscle extraction. It was analyzed muscle fiber cross-sectional area (CSA) of *Plantaris* muscle, body weight, C, testosterone (T), and IGF-1 levels, as well the abundance of selected proteins involved in autophagy: ubiquitinated proteins, LC3 and p62/

SQSTM1; protein synthesis: Akt, mTOR, and p70S6 K by western blot.
Results: Both sleep debt protocols (PSD-96 and PSR-21) presented reductions in body weight, CSA muscle fiber, and T and IGF-1 blood levels, while C was significantly increased ($P < 0.05$). The abundance of p62/SQSTM1 and ubiquitinated proteins were increased in PSD-96 and LC3 were increased in PSR-21 when compared to respective control groups.
Conclusion: Hormonal alterations associated to sleep debt-induced muscle atrophy is, in part, associated with activation of autophagy system.
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The influence of adenylate kinase and hexokinase on respiration in creatine-deficient GAMT −/− and AGAT −/− mouse cardiomyocytes

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Creatine kinase (CK) is considered an important spatial and temporal energy buffer in the heart. It catalyzes the transfer of a phosphoryl group between creatine (Cr) and ATP. Cr can be obtained from the diet or synthesized in the body by two enzymatic reactions involving L-arginine:glycine amidinotransferase (AGAT) yielding guanidinoacetate (GAA), and guanidinoacetate methyltranferase (GAMT) synthetizing Cr from GAA. Both GAMT −/− and AGAT −/− mice lack a functional creatine kinase (CK) phosphotransfer system due to their deficiency of GAMT and AGAT enzymes, respectively. As we have shown earlier, GAMT-deficiency is not associated with any changes in cardiomyocyte mitochondrial organization and both GAMT and AGAT-deficiencies do not alter intracellular compartmentation in relaxed cardiomyocytes.

The aim of this study was to examine whether alternative energy transfer systems are up-regulated in GAMT −/− and AGAT −/− cardiomyocytes to compensate for the lack of CK phosphotransfer. The experiments were performed on freshly isolated permeabilized adult cardiomyocytes. We measured ATP-stimulated respiration and the influence of activating adenylate kinase (AK) with AMP and hexokinase (HK) with glucose, and compared to the influence of activating CK with creatine. Their coupling was assessed using the competitive ADP-trapping PEP/PK assay. The respiration data were complemented with spectrophotometric recordings of total enzyme activities of CK, AK and HK using coupled enzyme assays.

As expected on the basis of CK activity distribution between mitochondria and cytosol, the competitive PEP/PK consumed 70–75 % of the ADP produced by CK before it reached the mitochondria in both GAMT −/− and AGAT −/− mice. In comparison, PEP/PK consumed 80–85 % of the ADP produced by hexokinase and almost all ADP produced by AK in both mouse models. Our results confirm the coupling of CK to respiration in both GAMT −/− and AGAT −/− and their respective wildtype littermates. The coupling of HK is close to that of CK, consistent with some of HK binding to the mitochondria. In contrast, AK seems to be mainly cytosolic. Our results suggest no compensatory changes in the compartmentation of HK, AK or CK in GAMT −/− and AGAT −/− mice.

Local capillary supply in muscle is not determined by local oxidative capacity

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Current dogma suggests that the prime determinant of global capillary density in a muscle is the oxidative capacity. However, feedback control during ontogenetic growth or adaptive remodelling is likely more complex than simply matching supply and demand in response to integrated tissue function. For example, fibre size is a more important determinant of the local capillary supply than fibre type and fibre oxidative capacity, although local control mechanisms may still require each capillary to serve a similar maximal demand for oxygen. Here we explore this question from the perspective of individual capillaries, and tested the hypothesis that the maximal oxygen consumption (MO_{2max}), or total mitochondrial volume (V_{mito}), served by an individual capillary is relatively constant, and independent of the volume of tissue associated with an individual capillary (capillary domain area). We demonstrate that (1) local MO_{2max} varies more than 10-fold between capillaries supporting this demand and (2) local MO2max was positively correlated to capillary domain area in both vastus lateralis ($R = 0.750$, $P < 0.001$) and soleus ($R = 0.697$, $P < 0.001$) muscles. Thus, capilles with larger domains supply a larger volume of mitochondria, and hence a potentially larger maximum oxygen flux. Ti suggests that, in contrast to common assumptions, capillary distribution at the cellular scale is not primarily dictated by local oxidative capacity, but rather by factors such as fibre size, or consequences of this such as substrate delivery/metabolite removal.

Muscle-specific overexpression of *Scd1* affects energy homeostasis and lipid/glucose metabolism in skeletal muscle

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Recent studies showed that SCD1 activity is essential for the regulation of lipid-mediated metabolic pathways and energy metabolism. Global *Scd1* knockout leads to improved insulin sensitivity and protects from diet- and leptin deficiency-induced obesity in mice. In present study we show that mice with muscle-targeted overexpression of Scd1 (Scd1 mTg) are susceptible to high-fat diet insulin resistance development associated with impaired lipid metabolism. The molecular mechanisms of such phenomenon are still being investigated. As skeletal muscle is main site of insulin resistance development, we decided to use whole-genome approach to examine muscle transcriptome changes upon *Scd1* knockout/overexpression in mice skeletal muscle. Microarray data were integrated and interpreted with Ingenuity Pathway Analysis platform. The expression pattern of genes involved in lipid metabolism shifts towards lipid accumulation in Scd1 mTg muscle. Also the risk of obesity development is higher in Scd1mTg mice because of expression of *Ppargc1a*, *Lipe*, *Pparg*, *Cebpb*, *Rfrg*, and *Med1* genes is reduced in Scd1mTg muscle comparing to wild type mice. On the other hand, skeletal muscle of Scd1

KO mice have higher metabolic rate due to higher expression of genes involved in lipid oxidation and oxygen consumption. Because of lower expression of genes related to ROS synthesis, Scd1 KO muscle might be protected from oxidative stress. In contrast, the ATP synthesis tends to be lower in Scd1 mTg muscle. Moreover we have identified several genes that are involved in maintenance of skeletal muscle insulin sensitivity in Scd1KO mice and 11 genes of susceptibility to insulin resistance development in Scd1 mTg mice. Our data clearly indicate, that Scd1 expression in skeletal muscle is crucial for skeletal muscle homeostasis.

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Urocortin 2 reduces overall proteolysis and stimulates cAMP/PKA/CREB and Akt/Foxo1 signaling pathways in C2C12 muscle cells

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Introduction: Studies reported that Urocortin 2 (Ucn2) treatment presents anti-atrophic and hypertrophic effects in rodent skeletal muscle. However, the intracellular mechanisms responsible for these results remain unclear.

Objectives: Our aim was to evaluate in vitro the effects of Ucn2 in the overall proteolysis in skeletal muscle and the intracellular mechanisms involved in the control of muscle protein breakdown by Ucn2 in C2C12 muscle cells.

Methods: All experiments and protocols were approved by the Ethical Commission of Ethics in Animal Research from Ribeirão Preto Medical School of the University of São Paulo (protocol: 063/2014). EDL and soleus muscles from male Wistar juvenile rats ($\sim$90 g) were harvested and incubated with Ucn2 (10^{-8}, 7×10^{-8}, 10^{-7}, 5×10^{-7} M) for 2 h to investigate the rates of protein degradation by measuring the tyrosine release in the incubation medium. Four-day differentiated C2C12 myoutubes were incubated with Ucn2 (10 nM) for 5 min, 30 min and 3 h. C2C12 myoblasts were transfected with human (h) or mouse (m) Ucn2 plasmids for 24 h. The phosphorylation levels of PKA substrates, CREB, Akt and Foxo1 were analyzed by western blot in lysed muscle cells.

Results: Ucn2 in vitro decreased overall proteolysis in EDL muscles of normal rats by 13 % in 7×10^{-8} M Ucn2 concentration (nmol tyrosine mg muscle^{-1} 2 h^{-1}: 0.230 ± 0.006 *vs.* 0.265 ± 0.022, control group) and 19 % in 5×10^{-7} M Ucn2 (0.210 ± 0.014 *vs.* 0.259 ± 0.007, control group). The simultaneous incubation with Ucn2 (5×10^{-7} M) and IBMX (10^{-4} M), a non-selective inhibitor of cAMP phosphodiesterases, did not promote any additional reduction in overall proteolysis in EDL muscles. C2C12 myotubes incubated with Ucn2 (10 nM) for 30 min showed increased levels of phosphorylated PKA ($\sim$2x) with no change in the phosphorylation levels of Akt. The overexpression of both hUcn2 and mUcn2 in C2C12 myoblasts increased the phosphorylation levels of Akt (56 and 69 %, respectively), Foxo1 ($\sim$3.7x and 2.5x, respectively) and CREB (64 and 56 %, respectively).

Conclusion: These results suggest that the anti-proteolytic effects of Ucn2 in skeletal muscle may be mediated by the activation of cAMP/PKA/CREB and Akt/Foxo1 signaling pathways that inhibits protein degradation.

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Fish-oil supplementation on Ser211 glucocorticoid receptor phosphorylation during dexamethasone-induced muscle atrophy

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Many conditions are related with muscle atrophy, such as inactivity, aging, sepsis, diabetes, cancer and corticosteroid therapy, leading to muscle atrophy due to increase of protein degradation and/or reduction of protein synthesis involving at least five systems: lysosomal, calpains, caspases, metalloproteinase, and ubiquitin–proteasome. Steroid is one of the most prescribed drugs and its long-term use is related to muscle atrophy. Many studies have been searching for supplements in order to prevent this side effect; however, previous study has shown that fish-oil can aggravate the dexamethasone-induced muscle atrophy. The goal of this study was to assess whether fish-oil supplementation (EPA + DHA) during dexamethasone-induced muscle atrophy can impact the glucocorticoid receptor phosphorylation (P-GRser211), knowing that this activated site is in turn correspondent to the catabolic activity of the muscle. Methods: Treated and non-treated rats with fish-oil (40 days) were subjected to dexamethasone administration, forming 4 groups: CT (control); DX 2.5 (dexa 2.5); FO (fish-oil) and DX + FO (dexa 2.5 + fish-oil). Muscles were extracted to cross sectional areas evaluation and P-GR (ser211) western blot analysis. Dexa administration led to a reduction around 47 % on cross sectional area of 2B muscle fibers type (DX 2.5 and DX + F-O groups). DX + FO group showed a significant muscle atrophy on 1 an 2A fiber types, not observed in other groups, including DX 2.5 group. P-GR evaluation showed a higher relative expression on DX 2.5 group compared to CT or FO groups but lower than DX 2.5 + FO group, which showed higher relative expression than any other groups. Previous and concomitant administration of FO with DX 2.5 caused aggravation of muscle atrophy (mainly in 1 and 2A fiber types) associated to increased glucocorticoid receptor activity, however, further tests would be useful to better understand the aggravation related to fish-oil supplementation. In conclusion, while the fish-oil is known to be effective in attenuating the muscle atrophy induced by sepsis and cancer, its concomitance with glucocorticoid can aggravate its side effects to skeletal muscle, probably GR-mediated. The identification of nutritional supplements able to alleviate the side effects of corticosteroids on skeletal muscle and the potential molecular pathways involved in this process, would be very important in the medical practice.

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Composition of cell culture media modulates energy- and nutrient-sensing signalling pathways in cultured skeletal muscle cells

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Cultured skeletal muscle cells are widely used to study signalling pathways in skeletal muscle. Advantages of using cultured cells include easily controllable experimental conditions and absence of systemic feedback effects that might obscure signalling responses to experimental stimuli. To reduce basal signalling activity, experiments are usually performed in basal media without serum. However, whether cells cultured in pure basal medium really have reduced signalling activity has not been rigorously tested. To address this salient issue we studied signalling activity in primary human myotubes, rat L6 myotubes and HEK-293 cells during 24-h serum starvation. We measured phosphorylation of 7 signalling proteins involved in AMP-activated protein kinase (AMPK), the mammalian target of rapamycin (mTOR), and the extracellular signal-regulated kinase (ERK1/2) pathway, across 6 time points and a total of 6 different experimental conditions. We found that serum starvation induces rapid, dynamic and time-dependent fluctuations in signalling activity. The pattern of signalling responses was dissimilar between different cell types and between different signalling pathways in the same cell type. Unlike serum, whose changeable composition is a major source of variability, basal media have fixed composition and are often thought to guarantee optimal environment for conducting signalling experiments. Nevertheless, basal media are replete with various nutrients that may impact on signalling pathways. Notably, some standard media used for growing muscle cells contain high concentrations of nucleosides which could affect the nucleotide-sensitive AMPK pathway. To test this hypothesis, we treated L6 myotubes with AMPK activator AICAR in the presence or absence of nucleosides. We found that nucleoside-free medium augmented AICAR-stimulated AMPK activation in L6 myotubes. Our results suggest that enhanced AICAR action might be due to disinhibition of AICAR uptake in nucleoside-free medium. Alternatively, up-regulation of purine transporters or alterations in purine metabolic pathways may also explain augmented AMPK activation. Collectively, our results show that serum starvation does not induce uniform reduction in basal signalling activity and that choice of basal medium might importantly determine the result of signalling experiments. Physiological extrapolations of results obtained from serum-starved cells grown in pure basal media should be subject to constant scrutiny.

Regulation of insulin sensitivity by endogenously synthesized 2-AG in skeletal muscle is associated with SCD1 gene expression

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The endocannabinoid system (ECS) is involved in regulation of energy metabolism, e.g. lipogenesis in adipose tissue and liver as well as glucose uptake into skeletal muscle. One of the most abundant endocannabinoid is 2-Arachidonyloglicerol (2-AG). In a condition of obesity, ECS becomes overactivated and might be involved in the pathogenesis of type 2 diabetes. It has been already shown that key components of the ECS, cannabinoid receptors (CB1R and CB2R) and enzymes that synthesize and degrade 2-AG, diacylglycerol lipase (DAGLα and DAGLβ) and monoacylglycerol lipase (MAGL), respectively, are present in human and rodent skeletal muscles. However, it is still unknown what is the role of endogenously synthesized 2-AG in the modulation of insulin sensitivity in skeletal muscle. In our studies, we showed that inhibition of MAGL with specific inhibitor (JZL 184) improves response to insulin in differentiated C2C12 myotubes. Interestingly, this effect is not mediated by CB1R activation. Moreover, mice injected with JZL 184 show better insulin sensitivity, which is accompanied with significantly lower weight gain and reduced fasting glucose level when compared to control. Higher level of monoacylglycerols, including 2-AG, leads to increase in free fatty acids content and changes in proteins involved in lipid metabolism, including AMP-activated protein kinase, hormone sensitive lipase as well as fatty acid synthase. Furthermore, we noticed that after JZL 184 injections the level of stearoyl-CoA desaturase-1 (SCD1) protein, which is a key regulator of fatty acid metabolism is higher. Interestingly, we showed that in Gastrocnemius of transgenic mice with muscle specific overexpression of SCD1 the level of DAGLb is significantly increased. Moreover, in Gastrocnemius of SCD1 −/− mice the level of DAGLb is lower. These results suggest that endogenously synthesized 2-AG might play an important role in enhancing insulin action by changing lipid metabolism in skeletal muscle.

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Session 11: Neuromuscular Interactions

Oral presentations

The molecular machinery for pretzel formation at the neuromuscular junction

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Mammalian neuromuscular junctions (NMJs) undergo a postnatal topological transformation from a simple oval plaque to a complex branch-shaped structure often called a "pretzel". Although abnormalities in NMJ maturation and/or maintenance are frequently observed in neuromuscular disorders, such as congenital myasthenic syndromes (CMSs), the mechanisms that govern synaptic developmental remodeling are poorly understood. It was reported that myotubes, when cultured aneurally on laminin-coated surfaces, form complex postsynaptic machinery, which resembles that at the NMJ. Interestingly, these assemblies of postsynaptic machinery undergo similar stages in developmental remodeling from "plaques" to "pretzels" as those formed in vivo. We have recently demonstrated that podosomes, actin-rich adhesive organelles, promote the remodeling process in cultured myotubes and showed a key role of one podosome component, Amotl2.

We now provide evidence that several other known podosome-associated proteins are present at the NMJ in vivo and are located to the sites of synaptic remodeling. Additionally, we identified proteins that interact with Amotl2 in muscle cells. We show that two of them: Rassf8 and Homer1, together with other podosome components, are concentrated at postsynaptic areas of NMJs in the indentations between the AChR-rich branches. Our results provide further support for the hypothesis that podosome-like organelles are involved in synapse remodeling and that Rassf8 and Homer1 may regulate this process.

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A quest to identify cytoskeletal proteins involved in neuromuscular synapse formation

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The formation and maintenance of the neuromuscular synapse (NMS) are crucially linked to signal transduction events induced by the receptor tyrosine kinase MuSK. MuSK becomes autophosphorylated and initiates its kinase activity in response to motoneuron-derived agrin. Activated MuSK phosphorylates downstream targets to induce a signaling cascade driving presynaptic and postsynaptic differentiation characterized by the clustering of acetylcholine receptors (AChRs). Impaired MuSK function results in acute neuromuscular deficiencies as shown during myasthenia gravis or to perinatal death in MuSK deficient mice due to respiratory failure.

To identify and investigate the phosphoproteomic map of MuSK signaling, we performed a global and quantitative mass spectrometry approach using a muscle cell culture system modeling postsynaptic differentiation. We identified a total of 10183 phosphopeptides of which 203 were at least 2-fold up/down regulated. Regulated phosphopeptides were classified into four different clusters according to their temporal profiles. Within these clusters we found an overrepresentation of specific protein classes associated with different cellular functions. Particularly, we detected an enrichment of regulated phosphoproteins involved in cytoskeletal rearrangements. Due to the indispensable role of the cytoskeleton in NMS formation, we have focused our efforts on regulated phosphotargets with known cytoskeletal functions such as Palladin, Paxillin, and focal adhesion kinase. Our aim has been to determine the role of phosphotargets during MuSK signaling, AChR clustering and NMS formation by RNAi-mediated silencing, specially in differentiated myotubes. For that, we generated TET-ON muscle cell lines for subsequent doxycycline-inducible miRNA expression.

The cytoskeletal regulator Palladin exists in multiple isoforms and is expressed ubiquitously. Recently it has been shown that isoforms are specifically up- or downregulated during muscle differentiation. In addition, knock-down of Palladin modulates muscle cell differentiation in vitro. In preliminary experiments we find that silencing of Palladin affects the size of myotubes and the number of AChR clusters in an isoform-dependent matter. In ongoing experiments we are addressing the exact effect of Palladin on AChR cluster formation, stability and on myotubes differentiation. Furthermore, similar experiments will be performed for other phosphotargets. With these studies we expect to discover novel regulatory factors of neuromuscular synapse formation.

Protein kinase CK2 interacts at the neuromuscular synapse with Rapsyn, Rac1, 14-3-3γ and Dok-7, and phosphorylates the latter two

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Previously, we demonstrated that the protein kinase CK2 associates with and phosphorylates the receptor tyrosine kinase MuSK at the neuromuscular junction (NMJ), thereby preventing fragmentation of the NMJs (Cheusova et al., *Genes & Development*, 2006). Here, we asked whether CK2 interacts with other proteins involved in processes at the NMJ, which would be consistent with the previous observation that CK2 appears enriched at the NMJ. We identified the following proteins to interact with protein kinase CK2, (a) weakly the α and β subunits of the nicotinic acetylcholine receptors (AChR), (b) dishevelled (dsh), and (c) another four proteins with strong interaction: Rapsyn, Rac1, 14-3-3γ and Dok-7. It turned out that CK2 phosphorylates 14-3-3γ at serine residue 235 and Dok-7 at several serine residues, but neither Rapsyn nor Rac1. Further, phosphomimetic Dok-7 mutants aggregate AChRs in C2C12 myotubes with significantly higher frequency than wildtype Dok-7. Additionally, we mapped the interacting epitopes of all four binding partners to CK2 and gained thereby insights to surmise on the potential role of the CK2–Rapsyn interaction.

Posters

Adaptor protein SH3BP2 regulates organization of the neuromuscular junction postsynaptic machinery

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The dystrophin-associated glycoprotein complex (DGC) is a multiprotein transmembrane complex responsible for stabilization of the postsynaptic machinery by crosslinking it to extracellular matrix and actin cytoskeleton. In addition the DGC acts as a scaffold for the assembly of signaling molecules. Mutations in several components of the DGC are known to cause muscular dystrophies, a group of muscle diseases that are characterized by progressive skeletal muscle weakness. One of the cytoplasmic components of DGC, alpha-Dystrobrevin1 (αDB1) was shown to play important role in the organization of the NMJ postsynaptic machinery in vivo and AChR clusters in cultured myotubes. Alpha-DB1 has C-terminal fragment that is phosphorylated at three tyrosine residues and this phosphorylation is important for αDB1 activity at the NMJ. To gain insight into the molecular machinery that orchestrate synaptic organization we performed biochemical screen for αDB1 phospho-specific interacting proteins and identified SH3BP2 as a binding partner.

SH3BP2 is a scaffold protein which contains three domains (PH, Proline-Rich Region and SH2) and recruits proteins like guanine nucleotide exchange factor (VAV), phosphoinositide phospholipase C (PLCγ) and growth factor receptor-bound protein 2 (Grb2). Our studies confirmed that SH3BP2 binds specifically to phosphorylated αDB1 and is concentrated at the NMJ postsynaptic machinery. Overexpression of full length and truncated mutant proteins demonstrated that every domain of SH3BP2 has a capability to localize at the NMJ. Cultured myotubes depleted of SH3BP2 had impaired ability to cluster AChRs. Our results suggest that SH3BP2 acts as a scaffold protein involved in the organization of the NMJ postsynaptic machinery.

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Sex differences in motor unit contractile properties and in the decomposition of motor unit tetanic contractions of rat soleus muscle

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Sex-related differences in contractile properties of motor units (MUs) and parameters of their action potentials were studied in the soleus muscle. Experiments were performed on adult Wistar rats (males and females) under general anesthesia. Functional isolation of a MU was achieved by electrical stimulation of single axons from L4–L5 ventral roots of spinal nerves. The contraction and the half-relaxation times were significantly longer in female MUs, which might be due to differences in muscle architecture. On the other hand, no differences were observed in twitch forces but tetanic forces were twice higher in males. The steep part of the force-frequency curve was rightward located for males and in females the same relative levels of tetanic forces could be achieved at lower stimulation frequencies than in males. The amplitudes of MU action potentials were four times higher in male rats, whereas the time parameters were similar. Analysis of long-lasting activity of slow MUs revealed potentiation of the twitch and unfused tetanic contractions recorded after 4 min of repeated tetanic activity within the standard fatigue test. Moreover, it was found that 85 % of MUs in males and only 13 % MUs in females displayed sag in unfused tetani, a phenomenon typical for fast MUs, however only after, and not before the standard fatigue test. This observation corresponds with higher content of IIa myosin and faster contraction of MUs in male soleus muscle. Mechanisms of force development were additionally compared between sexes with the method of decomposition of unfused tetanic contractions evoked by stimulation at variable interpulse intervals into twitch-shape responses to individual stimuli. Significantly higher variability between parameters of the decomposed responses was observed for males: the mean ratio of forces of the strongest decomposed twitch to the first (the weakest) twitch amounted to 3.8 (*vs*. 2.8 for female MUs), and the mean ratio of the force–time area for the strongest decomposed to the first twitch was 7.35 (*vs*. 5.07 for female MUs). In conclusion, sexual dimorphism of rat soleus implies several variations in motor control processes that should be taken into consideration while interpreting results of studies performed on males or females.

Novel Alpha-Dystrobrevin-binding partners involved in the organization of neuromuscular junction postsynaptic machinery

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Neuromuscular junctions (NMJs) are synapses formed between motor neurons and muscle fibers. Abnormalities in NMJ development lead to various severe neuromuscular disorders, such as limb-girdle myasthenia. Despite their crucial role, the mechanisms that orchestrate NMJ development are poorly understood. The dystrophin-associated glycoprotein complex (DGC) is a major laminin receptor in the muscle required for developmental remodeling of the NMJ. Its functions include stabilization of the postsynaptic machinery by crosslinking it to the extracellular matrix (ECM) and the cytoskeleton as well as serving as a platform for the assembly of signaling molecules. Alpha-Dystrobrevin-1 (aDB1) is a cytoplasmic component of DGC, which was previously shown to be critical for proper synaptic organization. The goal of our research was to gain insight into the mechanism of aDB1 function at the NMJ, especially into the role of aDB1 phosphorylation, which is crucial for the activity of this protein at NMJ.

Our biochemical screen resulted in identification of several novel aDB1-interacting proteins, including Liprin-α1, α-Catulin and Usp9x. We showed that Liprin-α1 and α-Catulin are both enriched at the NMJ postsynaptic machinery and α-Catulin is indispensable for clustering of postsynaptic acetylcholine receptors (AChR) in myotubes. Subsequently, we assessed the importance of aDB1 phosphorylation in the organization of the NMJ postsynaptic machinery. Overexpression of mutant proteins demonstrated that tyrosines Y713 and Y730 are the most important for AChR cluster formation. We showed that Y713 phosphorylation is dynamically regulated during NMJ development and after denervation. Our experiments revealed that aDB1 phosphorylation at Y713 serves as an additional binding site for the recruitment of signaling proteins including Grb2, SH3BP2, Arhgef5 and PI3 K. Finally, we demonstrated that Grb2 is concentrated at the NMJ postsynaptic machinery and that myotubes depleted of Grb2 have compromised ability to form complex assemblies of AChRs. Our results indicate that at the NMJ aDB1 may act as a scaffold protein for other effector proteins that orchestrate the postsynaptic machinery.

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Altered neuromuscular junction morphology caused by tissue specific Hsp67Bc knock-down in *D. melanogaster*

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Small heat shock proteins (sHSPs) are molecular chaperones, widely spread among all leaving organisms. They play substantial roles both in organisms development and protein homeostasis. Also, mutations in sHSPs coding genes underlie a number of inherited diseases affecting the function of muscle and nervous tissue.

Drosophila melanogaster Hsp67Bc gene encodes sHSP identified as a nearest functional orthologue of human HSPB8. Immunostainings confirmed neural localization of the protein in embryos, predicted from previously showed in situ hybridization. The strongest signal is visible in the ventral cord (central nervous system) of late-stage embryos, in centrally localized cell clusters. In larval stages, Hsp67Bc shows both muscle and neural localization, with a slight increase at neuromuscular junctions (NMJ). Both muscle and neural knock-down did not show any major defects in tissue morphology. Detailed analysis in both cases revealed the altered morphology of NMJ. Hsp67Bc knock-down showed increased complexity of arborization

and the elevated number of synaptic buttons. Moreover, muscle specific knock-down displayed a decrease in synaptic button size. Here we show localization of few neural and muscle markers at NMJs with an altered morphology. Our data proves the involvement of Hsp67Bc in NMJ formation and development, especially in muscle-dependent NMJ growth.

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