LITHUANIAN SPORTS UNIVERSITY

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SKELETAL MUSCLE MASS AND SPECIFIC FORCE: EFFECTS OF GENETIC BACKGROUND AND MYOSTATIN DYSFUNCTION IN RESPONSE TO HYPERTROPHIC AND ATROPHIC STIMULI IN MICE

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ABBREVIATIONS

θ	–	pennation angle
BEH	–	Berlin High growth mouse strain with myostatin dysfuction
BEH+/+	–	Berlin High growth mouse with the wild type myostatin
BM	–	body mass
C57BL/6J	–	C57 black 6 mouse strain from the Jackson laboratory
CON	–	Control group
CSA	–	cross-sectional area
CT	–	twitch contraction time
DUH	–	Dummerstorf High growth mouse strain
EDL	–	extensor digitorum longus muscle
FD	–	food deprivation
FO	–	functional overload
GAS	–	gastrocnemius muscle
HRT	–	twitch half relaxation time
KO	–	knockout gene phenotype
L_0	–	optimal muscle length
L_f	–	optimal fibre length
MM	–	skeletal muscle mass
MND	–	myonuclear domain
MPS	–	muscle protein synthesis
MPD	–	muscle protein degradation
Mstn	–	mice myostatin gene
MSTN	–	human myostatin gene
MyHC	–	myosin heavy chain
P_0	–	peak isometric tetanic force of isolated muscle
P_t	–	peak isometric twitch force of isolated muscle
PCSA	–	physiological cross-sectional area
PL	–	plantaris muscle
RMR	–	resting metabolic rate
sP_0	–	specific tetanic force of isolated muscle
SOL	–	soleus muscle
TA	–	tibialis anterior muscle
WT	–	wild type genotype

INTRODUCTION

Skeletal muscle is an abundant tissue compromising 40–50 % of the body mass in humans (Sakuma et al., 2014). Its main role is to generate force for posture maintenance and performance of daily movements, but it also acts as a metabolically active organ. Muscle mass and function are of particular importance for health and well-being as well as for the athletic performance in sports (Wolfe, 2006). On the contrary, muscle loss is undesired process characterising the consenquence of various diseases and/or catabolic conditions and leads to weakness, frailty and even death (Schiaffino et al., 2013). Thus, investigations of physiological and genetic factors underlying muscle mass and force is crucial for solving and improving health issues of society.

Mouse is the most widely used mammalian species in biomedical research. There are several reasons for its popularity. Firstly, analysis of mouse genome has shown that about 99 % of mouse genes have a homolog in the human genome (Guénet, 2005). Secondly, distinct mouse strains show significant variation in phenotypic traits facilitating identification of genes responsible for those traits. Thirdly, contrary to the inbred mice, humans present an outbred population complicating analysis of association between genes and phenotypic traits. Moreover, genetically engineered mice with mutations of specific genes, as in case of myostatin knockout (KO) mice (McPherron et al., 1997), provide a useful tool in identifying functions of those genes. Finally, studies of mice are cost effective due to small body size and relatively short lifespan which is 30 times shorter than in humans (Chang, 2013).

Physiological factors that influence the relationship between skeletal muscle mass and force are of large interest. Both these traits to a large extent are determined by genetic factors (Pescatello et al., 2013). Mice of distinct genetic backgrounds (strains) vary substantially in body and skeletal muscle mass (Lionikas et al., 2013a). Large body and skeletal muscle mass in the Berlin High (BEH) and Dummerstorf High (DUH) strains compared to other mouse strains, including a “classic” C57BL/6J strain, is a result of the selective breeding for large body mass and/or carcass protein accretion (Bünger et al., 2004). It is, however, unclear how these enlarged skeletal muscles differ from skeletal muscles of other strains. It is often believed that muscle force is proportional to muscle mass and muscle force per cross-sectional area (CSA) or specific force is a constant value

with the exception of pathological conditions as Duchenne muscular dystrophy (Gregorevic et al., 2004). However, this is an oversimplified view. There might be an optimal range of skeletal muscle mass for force production. Beyond this range muscle force might be compromised (Amthor et al., 2007). For instance, changes in an architecture or concentration of contractile proteins in hypertrophied muscles might affect force generation (Degens et al., 2009; Van der Meer et al., 2011; Qaisar et al., 2012). *In vivo* studies on humans do not allow us to examine these factors comprehensively. Methodological limitations such as difficulties in evaluating the actual muscle mass, influences of neural activation and coactivation of antagonistic muscles complicate assessment of specific muscle force (Erskine et al., 2010). Mouse model permits *ex vivo* experimentation where skeletal muscle can be isolated and stimulated electrically, thus avoiding most of those issues. Therefore, an identification of mouse strains that differ in specific force could be an important initial step, which would facilitate search for the relevant genetic factors and physiological mechanisms responsible for variation in specific muscle force.

Myostatin dysfunction, which was a main focus of this thesis, is the one of the known genetic factors affecting skeletal muscle mass and force. After myostatin discovery by Se-Jin Lee and colleagues it quickly attracted the attention of scientists from various fields including pharmacologists, physiotherapists and even sport scientists (McPherron et al., 1997). Myostatin is a natural inhibitor of muscle growth and protein synthesis. Mice with a deleted function of the myostatin gene (*Mstn*) display a striking increase in skeletal muscle mass which results a double-muscle phenotype (McPherron et al., 1997). On the other hand, muscle-specific overexpression of myostatin induces a significant reduction in skeletal muscle mass in mice (Reisz-Porszasz et al., 2003). In addition to the prominant muscularity, myostatin-deficient mice show improved bone formation, reduced adiposity as well as increased resistance to weight gain and insulin sensitivity when fed high fat diet (McPherron, Lee, 2002; Hamrick et al., 2006; Wilkes et al., 2009; Elkasrawy, Hamrick, 2010). Altogether, this suggests that myostatin inhibition might be an effective treatment against various muscle wasting conditions including aging-related sarcopenia as well as metabolic dysfunctions such as obesity and diabetes. Indeed myostatin blocking antibodies have a positive effect on muscle mass and function on *mdx* mice representing a model of Duchenne muscular dystrophy (Bogdanovich et al., 2002; Wagner et al., 2002). There is also

some interest in the myostatin inhibition effect on skeletal muscle mass and function of otherwise-normal individuals (e.g. athletes). Large muscles and fast glycolytic fibre profile in myostatin-deficient animals might be beneficial in strength and power events in sports (Girgenrath et al., 2005). Indeed, whippets which are heterozygotes for myostatin mutation are excellent performers in dog races (Mosher et al., 2007). Moreover, the woman who is heterozygous for myostatin mutation and gave birth to the homozygous child is a former sprinter (Schuelke et al., 2004). However, before considering myostatin inhibition as a performance enhancing strategy, more research should be done on physiological effects of myostatin dysfunction using animal models. In fact, studies of myostatin-deficient mice demonstrate that force in isolated skeletal muscles of these animals might be compromised compared to the wild type (WT) animals with the normal myostatin function (Amthor et al., 2007; Qaisar et al., 2012). There is also a gap in knowledge about a plasticity of skeletal muscles in myostatin-deficient animals, because little research has been carried out on adaptability to exercise training of these animals. Several studies examined effect of endurance training on skeletal muscle of myostatin-deficient mice (Savage, McPherron, 2010; Matsakas et al., 2010; Matsakas et al., 2012) but there is a lack of research about adaptations to high resistance training. Thus we carried out a study on compensatory hypertrophy of skeletal muscles in myostatin-deficient animals. Indeed athletes involved in strength training but not endurance training might be those who could benefit from myostatin inhibition.

It is well established that an increase of myostatin expression is a key factor in every type of skeletal muscle atrophy examined to date (Allen et al., 2010). Most of the studies are focused on myostatin inhibition in pathological conditions such as Duchenne muscular dystrophy (Bogdanovich et al., 2002; Wagner et al., 2002). However, the otherwise normal individuals might be subjected to catabolic stimuli as well. For instance, they might undergo skeletal muscle wasting either involuntary due to starvation, bed rest and/or space flight (Hegarty, Kim, 1981; Fitts et al., 2010; Sandonà et al., 2012). Various dietary regimes often involve intermittent fasting which reduces lean body mass (Varady, 2011). Thus, muscle wasting can be a result of conscious manipulations involving caloric restriction in order to achieve cosmetic goals which are associated with body shape and appearance. However, caloric restriction is also associated with improved health and longevity biomarkers in rodents and humans (Heilbronn et al., 2006; Ingram et al., 2006). It

seems that caloric restriction will be popular in near future as new dietary interventions are being developed (Varady et al., 2015). In this scenario myostatin inhibition might be a useful strategy to preserve skeletal muscle mass during caloric restriction. Moreover, myostatin inhibition is a promising target for an enhanced fat loss as well (McPherron, Lee, 2002). To date, few attempts have been made to investigate effects of myostatin deficiency on skeletal muscle mass after food deprivation (FD) (Allen et al., 2010; Collins-Hooper et al., 2015). These studies have produced contradictory results and more research is needed. Effects of FD on contractile properties of skeletal muscle also need to be examined in this regard. It appears that FD causes reduction in muscle mass which is associated with an increase in specific force of fast-twitch skeletal muscles of myostatin-deficient mice (Collins-Hooper et al., 2015). Similar findings have been reported after 5 week caloric restriction study (Matsakas et al., 2013). Based on these evidences, we carried out a study in which we examined effects of myostatin dysfunction on skeletal muscle mass and force in both predominantly slow-twitch and fast-twitch muscles following FD. Slow- and fast-twitch muscles are showing different metabolic and functional properties as well as a content of myostatin-binding ActRIIB receptors (Mendias et al., 2006). Therefore effects of myostatin dysfunction and FD might be muscle specific. Moreover, we also examined how a genetic background affects these parameters following FD.

The aim of the research was to examine effects of genetic background and myostatin dysfunction on specific force and mass of skeletal muscles after muscle hypertrophy and atrophy.

Research objectives:

1. To investigate effects of genetic background on skeletal muscle mass and force.

2. To investigate effects of myostatin dysfunction on skeletal muscle mass and force before and after functional overload.

3. To investigate effects of myostatin dysfunction and genetic background on skeletal muscle atrophy and muscle force during food deprivation.

Research hypotheses:

1. We studied skeletal muscles in mouse strains which differ significantly in body size. On the basis that larger muscle hypertrophy might lead to unfavourable for force production changes in muscle architecture and/or contractile material maintainence (Kawakami et al., 1995; Amthor et al., 2007; Ikegawa et al., 2008), our *hypothesis* was that specific muscle force will be dependent on muscle size and it will decrease with increase in body and muscles mass of the studied mouse strains.

2. Myostatin-deficient mice have increased glycolytic type II fibre content in muscles (Girgenrath et al., 2005; Gentry et al., 2011) which are more prone to hypertrophy (Verdijk et al., 2009). It appears that only muscle mass reduction toward wild type phenotype could restore force production in myostatin-deficient mice (Matsakas et al., 2013; Collins-Hooper et al., 2015). Thus, we *hypothesized* that ablation of the gastrocnemius muscle will be associated with greater compensatory hypertrophy of soleus and its function deterioration in BEH mice with dysfunctional myostatin compared to BEH+/+ mice carrying the wild type myostatin.

3. We studied mass and force of the hindlimb muscles in BEH and BEH+/+ mice as well as in the C57BL/6J background. Our *hypothesis* was that myostatin dysfunction which positively affects a net muscle protein turnover by enhanced protein synthesis (Morrisete et al., 2009; Lipina et al., 2010) will prevent loss of muscle mass and improve force during food deprivation. We also *hypothesized* that there might be differences in loss of muscle mass between genetic backgrounds due to possible changes in a body composition and/or metabolic peculiarities (Konarzewski, Diamond, 1995).

1. REVIEW OF THE LITERATURE

1.1. Factors of specific muscle force

Muscle mass is often referred to as a major predictor of force generation capacity of skeletal muscles (Jones et al., 2008). This means that by overall strength individuals with larger skeletal muscle mass will be stronger than individuals with a smaller muscle mass. Indeed, a large number of studies support this assumption (Fukunaga et al., 2001; Ikegawa et al., 2008). However, specific muscle force or strength which is muscle force normalized to muscle CSA, as measured by magnetic resonance imaging, ultrasonography or computerised tomography, provides a better means of comparison between the skeletal muscles than the absolute strength. Specific strength or specific muscle force varies among individuals (Maughan, Nimmo, 1984; Jones et al., 2008). However, non-muscular factors such as neural activation, recruitment pattern of muscles which are synergists and antagonists as well as tendon compliance may contribute to this variation in specific muscle strength (Degens et al., 2009). This complicates research into muscular factors that influence specific muscle force. It appears that isolated skeletal muscles of mice present a better model for such a research since contribution of neural and other non-muscular factors can be avoided. Thus, all differences in specific force in the experiments of this thesis could be attributed to properties of skeletal muscles. The factors affecting qualitative properties of skeletal muscle are discussed below.

1.1.1. Skeletal muscle fibre composition

Skeletal muscle is comprised of muscle fibres which vary significantly in their physiological properties. Muscle fibre composition can be determined using various methods with histochemical staining for myosin ATPase activity (such fibres are marked by numbers and large letters herein) and immunohistochemical staining for the type of myosin heavy chain (MyHC) (such fibres are marked by numbers and small letters herein) being the most popular. Such assessments suggest that there are three main types of muscle fibres in human skeletal muscles: slow oxidative type I, fast oxidative-glycolytic type IIA or IIa and fast glycolytic type IIX or IIx fibres (Schiaffino, Reggiani, 2011). Type IIB or IIb is not expressed in human muscles, but is found in skeletal muscles of other mammals such as rodents (Smerdu et al., 1994). However, skeletal muscles might also contain some

fibres that belong to various subtypes (e.g. IC or Iβ>IIa, IIC or Iβ<IIa) and/or hybrid fibres (e.g. IIAX or IIax) containing myosin isoforms of two or several distinct fibre types (Smerdu, Erzen, 2001). Type I and Type II fibres have distinct metabolic and contractile properties. It is well established that velocity of unloaded shortening or maximal contraction velocity (V_{max}) and power output, are greater in type II fibres than type I fibres (Close, 1972). V_{max} increases progressively from slow type I fibers to fast IIa, IIx, and eventually IIb fibers which exhibit the highest values of V_{max} in all mammalian species (Bottinelli et al., 1991). The findings on differences in specific force between fibre types are controversial. Some studies of chemically skinned muscle fibres have not found differences in specific force between type I and type II fibres (Lucas et al., 1987; Ruff, Whittlesey, 1991). However, there are studies which demonstrate lower specific force generation of type I fibres than type II fibres (Young et al., 1984; Bottinelli et al., 1991; Stienen et al., 1996; Krivickas et al., 2011). Although it is impossible to draw a conclusion about differences in specific muscle force between fibre types, type II fibres tend to be stronger rather than weaker compared to slow type I fibres. As skeletal muscle is made up of a large number of fibres therefore interindividual variability in fibre composition might be one of the factors affecting specific force. For instance, individuals with a greater number of fast type II fibres in muscle of interest might have a greater specific force of that muscle than individuals with prevailing slow type I fibres. However, studies demonstrated no correlation or weak correlation between specific strength and the perecentage of type II fibres in human quadriceps but rather dependence between specific force and CSA in human quadriceps (Maughan, Nimmo, 1984; Young, 1984).

1.1.2. Muscle architecture

Skeletal muscles differ in their architecture and can be divided into two main types: parallel and pennate (see Fig. 1). Muscle fibres run along the length of muscles in the parallel muscles. In the pennate muscles muscle fibres are at an angle to the longitudinal direction of the muscles (Narici, 1999). Paralell muscles can be further divided into strap, fusiform or fan-shaped while pennate muscles can be further divided into uni-, bi- or multipennate. We studied soleus (SOL) and extensor digitorum longus (EDL) muscles which can be classified as the slightly unipennate muscles (Bukholder et al., 1994). Pennation angle (θ) is the angle at which muscle fibres insert into the aponeurosis in the pennate muscles. θ influences

both force generation and shortening velocity of skeletal muscle (Narici, 1999). The force exerted on the tendon by fibres can be calculated as the product of force generated by all fibres and the cosine of θ, assuming that the aponeurosis is parallel with the tendon (Degens et al., 2009). For the same muscle volume or mass, the pennate muscles would produce more force at the aponeurosis than the parallel muscle (Fig. 1). This is due to the fact that pennate muscles accommodate larger numbers of muscle fibres at the aponeurosis compared to parallel muscles, i.e. the pennate muscles have greater physiological cross-section area (PCSA) than the parallel muscles. The force produced by a given muscle is likely to be proportional to its CSA, or the number of parallel sarcomeres (Narici, 1999). Since pennate fibres are at an angle, the anatomical CSA (ACSA) does not represent PCSA, which is thought to be a key determinant of specific muscle force, and should not be used in calculations. In the parallel muscles the ACSA and PCSA coincide.

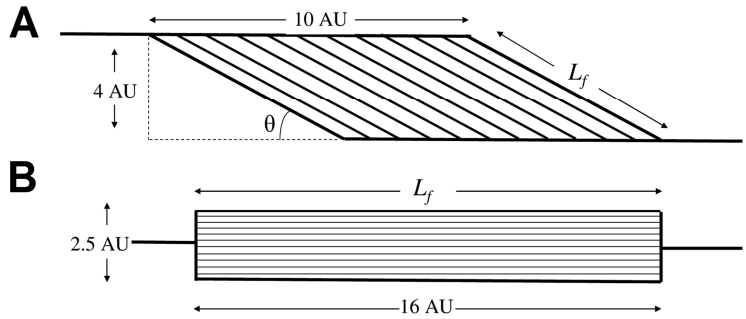

Fig. 1. *Force generation by pennate (A) and parallel (B) muscles of the same volume (adapted from Degens et al., 2009). Muscle fibre pennation angle θ is 30^0 in the pennate muscle. The volume of both muscles is 40 arbitrary units (AU) with the depth of 1 AU. The thickness of the pennate muscle is 4 AU and the length of the aponeurosis with fibres attached is 10 AU. The fascicle length (L_f) of the parallel muscle is 16 AU and the thickness or physiological cross-sectional area (PCSA) is 2.5 AU in this case. For the pennate muscle, L_f = 4 AU * $sin30^0$ = 8 AU and PCSA = muscle volume / Lf = 40 AU / 8 AU = 5 AU. Force generation (F): pennate muscle F = 5 AU * $cos30^0$ = 4.33 AU; parallel muscle F = 2.5 AU * $cos0^0$ = 2.5 AU. Hence, the pennate muscle produces 1.73 times greater force than the parallel muscle*

The pennate muscle might exhibit a deterioration of force generating capacity when muscle hypertrophy is induced (e.g. after strength training) (Degens et al., 2009). Muscle hypertrophy is associated with an enlargement of single muscle fibres which in need to attach to anatomically unaltered aponeurosis would have to increase θ. An increase in θ would result in less force generated in the longitudinal

direction of skeletal muscles, and this reduction would be proportional to the cosine of θ (Degens et al., 2009). In spite of greater force output by seperate muscle fibres, the specific force of the whole muscle might decrease as a result. For instance, any increase in θ beyond 45^{o} will result in a reduction in the force of the muscle even when muscle volume or mass increases. Thus, individuals with a large degree of muscle hypertrophy might generate muscle forces which are lower than expected from their muscle mass. The comparison of bodybuilders and Olympic weightlifters showed that bodybuilders had greater CSA of the triceps brachii and larger isometric elbow extension force (F), but lower ratio of force to CSA (F/CSA) compared to the weightlifters (Ikegawa et al., 2008). Moreover, there was a significant positive correlation between CSA and θ in these athletes. In addition, the F/CSA was negatively correlated with θ both for bodybuilders and weightlifters. Other studies have observed that θ vary substantially among humans, in the range of 15–53^{o} for the long head and 9–26^{o} for the medial head of triceps brachii, and highest values are in hypertrophied individuals such as bodybuilders (Kawakami et al., 1993). Authors observed significant correlations between muscle thickness and θ for both long and medial heads of triceps brachii. It seems that muscle hypertrophy after strength training is associated with reduction in specific force of the pennate muscles, especially in case of excessive muscle hypertrophy which is often observed in strength athletes (Kawakami et al., 1995). After short and long periods of resistance training specific force of individual muscle fibers remains unchanged or increases in bodybuilders or trained individuals (D'Antona et al., 2006; Erskine et al., 2011). Thus, increased θ is one of the main candidates for a reduction of whole muscle specific strength during muscle hypertrophy.

1.1.3. Myonuclear domain

Skeletal muscle fibres are multinucleated cells. The nucleus contains most of the cell's genetic material and plays critical role in maintaining integrity of the genes and the cell. The nucleus allows to control gene expression, including expression of the genes coding for muscle proteins. The volume of cytoplasm which is taken care of by an individual nucleus or myoneucleus is called the myonuclear domain (MND). The MND size is inversely related to the oxidative capacity of the fibre where it increases from type I to IIA to type IIB/X fibres (Jaspers et al., 2006; Van der Meer et al., 2011). It is hypothesized that MND size might play an important role in specific force generation of mammals (Qaisar et al.,

2012). Myonuclei are usually located in the periphery of muscle fibres, and there might be limitations in supplying the center of fibre with the sufficient amounts of the ribonucleic acids (RNA) required for direct synthesis of specific proteins (Degens, 2012). This might be even more challenging when MND increases. MND does not have a fixed size, and it is likely that MND might increase in the initial phase of muscle hypertrophy (Van der Meer et al., 2011). However, muscles fibres are able to supplement their pool of myonuclei by fusion with activated satellite cells (Zammit et al., 2004). Activated satellite cells act as donors of myonuclei to the muscle fibre undergoing skeletal muscle hypertrophy in response to functional overload. However, skeletal muscle hypertrophy can also occur in the absence of satellite cells activation and without addition of new myonuclei (McCarthy et al., 2011). Under such circumstances muscle enlargement might lead to a decrease in relative concentration of contractile proteins and reduction of specific muscle force. However, myonucleus activity should also be considering. A higher rate of transcription might compensate for enlarged MND during muscle hypertrophy. There is a lack of reliable evidences about the optimal MND size beyond which specific force of skeletal muscle fibres would be impaired. R. Qaisar et al. (2012) studied skeletal muscle hypertrophy in mice with an aim to investigate importance of MND size on specific muscle force at a single fibre level. Overexpression of insulin-like growth factor-1 (IGF-1) and dysfunction of myostatin protein were compared in this study. Changes in MND size were different in these two models of muscle hypertrophy (Fig. 2). Fast-twitch fibres from EDL of myostatin-deficient mice showed reduction in specific force and myosin content which were concomitant with an enlargement of the MND, whereas specific force and MND size in IGF-1 overexpressing mice remained constant. It is worth mentioning that skeletal muscle hypertrophy was ~40 % greater in myostatin-deficient mice compared to IGF-1 overexpressing mice. The authors concluded that there is a critical volume of cell individual myonucleus can support efficiently.

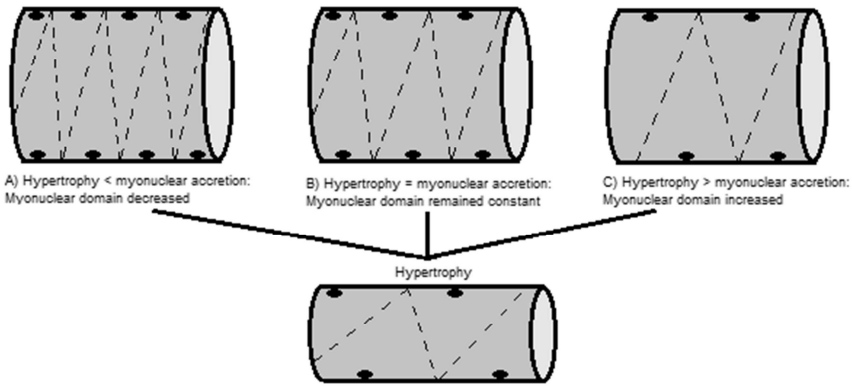

Fig. 2. *Three possible scenarios of changes in myonuclear domain (MND) size in response to the hypertrophic stimulus of skeletal muscle (modified from Van der Meer et al., 2011). The scenario A is the least likely because should be provided by high activation of satellite cells with modest skeletal muscle fibre hypertrophy. The scenario B is probably the most common providing comparable alterations in content of myonuclei and fibre hypertrophy. The scenario C is likely when exceptionally large fibre hypertrophy is induced without a significant activation of satellite cells (Qaisar et al., 2012)*

1.1.4. Lateral force transmission

It is well known that high resistance training leads to a concomitant increase in mass and force generating capacity of skeletal muscles (Narici et al., 1989). Interestingly, *in vivo* studies of humans often show that resistance training leads to a greater increase in force generation compared to skeletal muscle mass (Degens et al., 2009; Erskine et al., 2010). It is likely that neural factors might be of importance here. Indeed, resistance training improves neural activation, but this improvement alone could not contribute to such high increase in specific force (Erskine et al., 2011). Moreover, an increased θ would rather reduce but not improve specific force, thus its contribution to this phenomenon is fully excluded. Force changes of individual fibres can be another candidate to explain this resistance training-induced phenomenon. Whilst an increased specific force of fibres seen in long term trained bodybuilders (D'Antona et al., 2006) supports this idea, the short term training seems does not induce alterations in specific force at single fibre level though changes at whole-muscle level are already prominent. For instance, R. M. Erskine et al. (2011) demonstrated that neither alterations in fibre type composition nor in specific force of individual muscle fibres is associated with 17 % improvement of the whole muscle specific force in young men after the 9

weeks session of high resistance training. In this study, peak power output of the hypertrophied skeletal muscles remained unchanged, thus suggesting reduction in contraction velocity of these muscles by 17 %. There was no relationship between either a peak power output or maximal power per unit fibre volume and isoform composition either before or after training, or between the training-induced changes in these variables. Taken together, this suggests that the quality of the muscle fibres remained unchanged and that the increase in whole-muscle specific tension must be explained by some other factors than changes in the contractile properties of the muscle fibres. The authors suggested that an increase in lateral force transmission might be associated with increased specific force of skeletal muscle. In fact, previous studies confirmed that a lateral force transmission might occur during the onset of resistance training-induced hypertrophy of skeletal muscle (Jones et al., 1989). The lateral force transmission is generated by emerging lateral attachments between intermediate sarcomeres and the extracellular matrix (Fig. 3). The origin of these attachments is not clear but it might be a connective tissue-related and/or protein-related such as as desmin and dystrophin that are associated with structural complexes known as costameres (Miller et al., 2005; Woolstenhulme et al., 2006; Kosek, Bamman, 2008). These changes in the connective tissue attachments between muscle fibres and the tendons result in a greater isometric force with concomitant reduction in the effective length of the muscle fibres (Jones et al., 1989). Generally, it is assumed that tension is transmitted longitudinally in a muscle fibre through serial sarcomeres so that the force is proportional only to the CSA and is independent of the length. However, the formation of attachments would serve to effectively increase the number of parallel sarcomeres per PCSA, and, as consequence, the specific tension of the muscle fibres would increase. On the other hand, it would result in a decrease of the maximal shortening velocity of the muscle by reducing the functional length of the skeletal muscle (Jones et al., 1989; Erskine et al., 2011). Summing up, a power output normalized to muscle volume would be expected to remain unchanged in such circumstances because maximal muscle power is a product of force and contraction velocity. These assumptions agree well with evidences that muscle specific force increases and power generation remains unchanged following resistance training (Erskine et al., 2011).

Of interest, a lateral force transmission might be limited exclusively to humans. For instance, studies with rodents have not shown an increased specific force of skeletal muscles subjected to functional overload (Roy et al., 1985; Degens

et al., 1995). Moreover, plantaris (PL) and soleus (SOL) muscles of rats even exhibited decrease in specific force following compensatory muscle hypertrophy in several studies (Kandarian, White, 1989, 1990). Thus, rodents' studies do not support a hypothesis about an enhanced lateral force transmission following skeletal muscle hypertrophy, at least in those animal models.

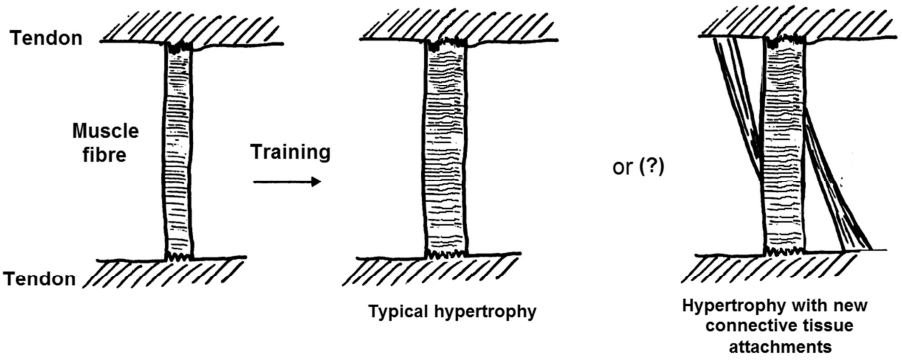

Fig. 3. *An example of the formation of connective tissue attachments between a muscle fibre and tendon during a resistance training which might cause a lateral force transmission in addition to a standart longitudinal force transmission along a fibre membrane to a tendon (adapted and modified from Jones et al., 1989)*

1.2. Myostatin

1.2.1. Discovery of myostatin

Five decades ago W. S. Bullough (1962, 1965) hypothesized that there are biologically active molecules controlling growth and development of all tissues. According to this hypothesis, these molecules should be produced within a tissue and act systemically as well as on the tissue itself. These molecules were named 'chalones'. Nevertheless, this interesting theory was abandoned since there was no clear experimental evidence in support of it. Discovery of myostatin and other growth factors have breathed a new life into this theory.

Myostatin which is also referred to as growth differentiation factor 8 (GDF-8) was discovered in 1997 (McPherron et al., 1997). It is a protein which belongs to transforming growth factor beta (TGF-β) superfamily of secreted growth factors and is coded by MSTN gene in humans and other mammals. Myostatin is primarily expressed in developing and adult skeletal muscle albeit small expression levels of myostatin have been detected in the heart, adipose tissue and mammary gland of mice (McPherron et al., 1997; Ji et al., 1998; Sharma et al., 1999). Studies of

myostatin deficient mice showed that myostatin is a key regulator of skeletal muscle mass. Myostatin knockout (KO) mice demonstrated a striking muscular phenotype as skeletal muscles were two to three times greater than in the wild type (WT) mice (McPherron et al., 1997; Gentry et al., 2011). This increase in muscle mass was due to both increase in CSA and number of muscle fibres. Moreover, these effects of myostatin were dose-dependent as heterozygous mutant mice have a milder increase in muscle mass than homozygous mutants (Mendias et al., 2006). As expected, transgenic mice overexpressing myostatin displayed reduced muscle mass and decreased fiber size compared to WT animals (Reisz-Porszasz et al., 2003). Publication of the paper by A. C. McPherron et al. (1997) about myostatin spurred an interest of researchers in application of myostatin inhibition in various areas, such as prevention of muscle wasting in various conditions and disease states, enhancement of meat production in agriculture, performance enhancement in sports, just to name a few. In addition to increased muscle mass, myostatin-deficiency reduces fat mass, increases resistance to weight gain, improves insulin sensitivity and stimulates bone formation in mice (McPherron, Lee, 2002; Hamrick et al., 2006; Wilkes et al., 2009; Elkasrawy, Hamrick, 2010).

MSTN gene has been extensively studied in various species (McPherron, Lee, 1997). Studies revealed that myostatin sequence is highly conserved through evolution across most species suggesting a similarity of myostatin functions across the species. Firstly, it is worth to mention that a MSTN gene is encoding a sequence of the myostatin precursor, which consists of the 3 distinct regions: an initation region, N-terminal region (propeptide) and C-terminal region. The only C-terminal region is considered as a mature (active) protein when it gets rid of the initiation region and propeptide (Lee, 2004). In fact, a sequence of mature myostatin is identical in most vertebrate species including humans, mice, rats, pigs, chicken and turkey (McPherron, Lee, 1997). In addition, the sequences of bovine, ovine and baboon contain only 1–3 amino acid difference in the C-terminal region compared to aforementioned species. From all species, fishes have the most divergent sequence of mature myostatin, as an example is zebrafish with only 88 % identical to the others in this region.

Fig. 4. *The Belgian Blue cattle are carrying a mutation in the MSTN gene resulting in a double-muscle phenotype (adapted from McPherron, Lee, 1997)*

1.2.2. Mutations of the myostatin gene

It appears that incidences of spontaneous mutations within MSTN gene are relatively rare. All so far identified mutations were of different type in different species. The best representatives of mutation in MSTN gene are so-called double-muscled breeds of cattle as Belgian Blue and Piedmontese. Belgian Blue cattle are carrying an 11 nucleotide deletion causing a frameshift and Piedmontese cattle are carrying a 2 nucleotide missense mutation in mature myostatin region compared to the classical Holstein sequence, respectively (McPherron, Lee, 1997). The Belgian Blue breed shows 20–25 % increase in muscles mass and a decrease in mass of other organs, including connective tissue and intramuscular fat (Fig. 4). Owners of whippets, the dog breed known for its running speed in racing events, have reported that there are dogs, which differ substantially in body conformation from other dogs (Mosher et al., 2007). Such dogs, named as “bully” dogs, are heavier, have particularly well-developed musculature of legs, neck and chest. D. S. Mosher et al. (2007) examined this breed and established three possible genotypes (+/+, *mh*/+ and *mh/mh*) regarding to the MSTN gene. The *mh* allele is carrying 2-bp deletion in the third exon of MSTN causing a premature truncation of the protein by 17 %.

Although myostatin KO mice have been useful in studying myostatin functions, these mice were generated using techniques of genetic engineering (McPherron et al., 1997). However, there are also natural *Mstn* mutant mice. The mutation, termed *Compact* (*Cmpt*), is associated with the 12-bp deletion in the

Mstn gene encoding propeptide sequence (Szabo et al., 1998). The mice carrying *Cmpt* mutation display the hypermuscular phenotype similar to that observed in *Mstn* KO mice (Amthor et al., 2007). The mutated region in *Cmpt* mice is not encoding a mature myostatin and might be involved in a proper folding of mature myostatin and participate in the proteolitic cleavage during the production of the mature myostatin protein (Lee, 2004). Interestingly, this *Cmpt* mutation was simultaneously identified by two separate groups of scientists working in two separate places – Hungary and Germany (Varga et al., 1997). The Berlin High (BEH) strain, which we used in our experiments, was developed at the Technical University of Berlin by a long-term selection for high carcass protein content and inbreeding such individuals to each other (Bünger et al., 2004). Another known mutation in a non-coding region of MSTN gene has been established in Belgian Texel sheeps renowned for their exceptional meatiness (Clop et al., 2006). Genetic assays revealed that, despite the production of myostatin mRNR in these sheeps, a very little amount of protein is generated due to G to A nucleotide substitution in the 3' UTR that creates a target site for miR-1 and miR-206, microRNAs (miRNAs) that are highly expressed in skeletal muscle.

Incidences of myostatin dysfunction among humans are of particular interest regarding the application of myostatin dysfunction in treatment of muscle wasting diseases or search for enhancers of the atlethic performance. To date, only one case of MSTN gene disruption has been reported in humans. M. Schuelke et al. (2004) reported G to A nucleotide substitution in non-coding region of 1 intron in both alleles of MSTN gene of a child. It appears that this substitution results in missplicing and consequently severely truncated protein. The newborn boy with this MSTN mutation showed excessive musculature compared to the infants of the same age, sex and weight. The boy did not experience any health problems during a 4.5 year period of observation. The mother of the child was a carrier of the one defected allele and appeared muscular as well. Except for the mother, no other family members were available to provide samples for genetic analysis.

1.2.3. Molecular mechanisms of myostatin

MSTN gene structure. MSTN gene is a relatively small gene (7.7 kb) located on chromosome 2 in humans (Gonzalez-Cadavid et al., 1998). The full-length cDNA cloning assay revealed that it is made up of three exons with two introns intervened between them. The sequence of the MSTN gene is transcribed to a

3.1 kb mRNA that encodes a 375 aminoacid precursor protein (Patel, Amthor, 2005) (Fig. 5). Following the translation the precursor protein of myostatin is a non-functioning molecule, which has to undergo several proteolytic processes in order to become a biologically active molecule (McPherron et al., 1997).

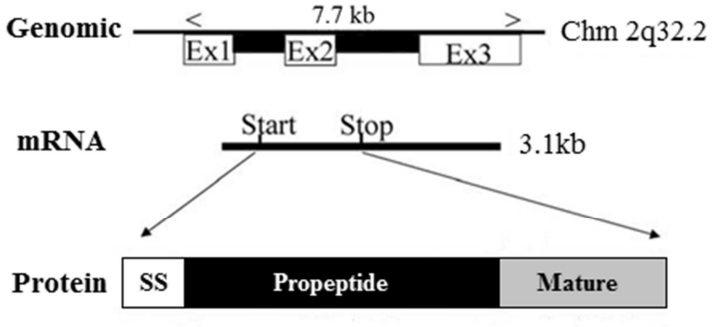

Fig. 5. *The molecular organization of the human MSTN gene and its downstream products of the transcription (mRNR) and translation (protein) processes (modified Patel, Amthor, 2005)*

Precursor protein processing. The processing of myostatin precursor protein is shown in Fig. 6. During the first proteolytic cleavage the signal sequence of the N-terminal (propeptide) region is removed by furin family enzymes (Lee, 2004). This small (24-amino acid) peptide is necessary to target the precursor protein to the secretory pathway. The second cleavage occurs at the site of amino acids 240–243 (Arg-Ser-Arg-Arg) and generates two fragments, the N-terminal (propeptide) and the C-terminal (mature myostatin) region, of ~28 Da and ~12 Da, respectively. These separate fragments are bound by non-covalent bonds in the one complex and remain in a complex which circulates in plasma as an inactive (latent) form of myostatin. The fragments (~12 Da) of the mature myostatin form a dimer. The propeptide plays several important functions (Lee, 2004). Firstly, it participates in the proper folding of the mature myostatin, and, secondly, it regulates the activity of the mature myostatin following the proteolytic processing. The protein becomes an active myostatin whenever the propeptide is cleaved from the dimer of the mature myostatin. The biologically active form of myostatin is the C-terminal dimer and all subsequent references to myostatin in this thesis should be taken to mean this form of the molecule. It is not quite clear which proteases are responsible for the cleavage of the propeptide. Those proteases most likely belong to the bone morphogenetic protein (BMP)-1/tolloid family of metalloproteinases. Members of this family cleave the propeptide at aspartate 76. Furthermore, the study in which the mutant propeptide

was generated by replacing the aspartate 76 to alanine demonstrated resistance to BMP-1/tolloid proteinases. Mice subjected to weekly injections of such propeptide displayed increase in muscle mass by 25 % (Wolfman et al., 2003).

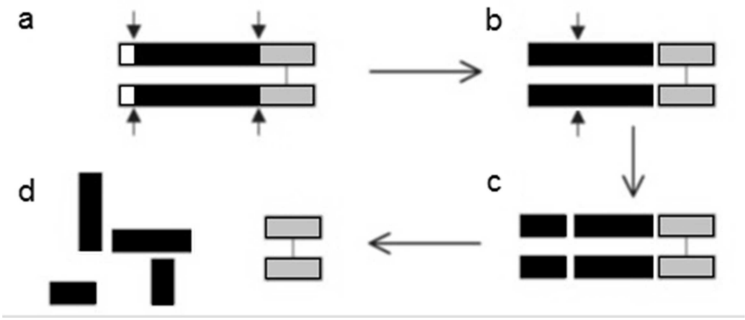

Fig. 6. *Processing of myostatin protein. Myostatin is synthesized as a precursor protein in muscle fibres. Following the initial cleavage events the signal sequence (white) of the protein is removed and generates two separate fragments (propeptide (black) and mature myostatin (grey)) which remain in the non-covalently binding (a, b). Members of BMP-1/tolloid family of metalloproteinases disrupt the non-active (latent) state of the protein by the cleavage of the propeptide (c) and release the mature myostatin in a form of C-terminal dimer which is an active form of myostatin (d). (Modified Lee, 2004)*

Binding to the target and signalling. The mature myostatin initiates signal transduction cascades within muscle fibre by binding to the specific receptors on the sarcolemma (Fig. 7). There are two types of activin II receptors, ActRIIA and ActRIIB, which possess slightly different characteristics of affinity to myostatin (Rebbapragada et al., 2003). The number of ActRIIB receptors which show high affinity to myostatin is greater in skeletal muscles which are composed of predominantly type II fibres (Mendias et al., 2006). Activated ActRIIB receptor complex induces a phosphorylation and thereby activation of the activin type I (ALK4 and ALK5) receptors which target downstream molecules, Smad2 and Smad3 (Small mothers against decapentaplegic homolog 2 and 3). In turn, activated and phosphorylated Smad2 and Smad3 form a complex with the adaptor protein, Smad4. These Smad complexes are key mediators of myostatin intracellular signalling. They translocate into the nucleus and regulate expression of targeted genes associated with myogenesis. For instance, myogenic transcription factors such as MyoD, myogenin are downregulated by overexpression of myostatin (Langley et al., 2002; Durieux et al., 2007). In addition to the Smad cascade, myostatin acts on several other signalling cascades participating in skeletal muscle size regulation. For example, signalling through IGF-1/PI3K/Akt/mTOR pathway, which is often

responsible for protein synthesis, can be downregulated by myostatin signalling which is able to inhibit activation of Akt (Trendelenburg et al., 2009). Furthermore, genetic loss of myostatin function leads to enhanced expression and activity of components of Akt, mTOR and p70S6K in mice (Morissete et al., 2009; Lipina et al., 2010). In muscle, protein synthesis and degradation pathways influence each other so that activation of anabolic signalling through Akt inactivates FoxO which controls genes of the proteolysis pathways and vice versa (Sandri et al., 2004; Schiaffino et al., 2013). For example, myostatin signalling cascade inhibits protein translation and anabolic activity of Akt and activates FoxOs (Forkhead box proteins O) which in turn upregulates critical components of ubiquitin-proteasome and autophagy systems to induce muscle protein catabolism (Lee et al., 2011; Han et al., 2013). Myostatin also serves to activate the MAPKs pathway (mitogen-activated protein kinases) such as extracellular signal-regulated kinases 1 and 2 (Erk 1/2), c-Jun N-terminal kinases (JNKs), p38 mitogen-activated protein kinases (p38MAPK). For instance, the activated Erk 1/2 MAPK pathway arrests cell proliferation and differentiation through the p21/Rb cascade in proliferating myoblasts (Thomas et al., 2000; McFarlane et al., 2011). *In vitro* studies of C2C12 muscle cell lines and *in vivo* embryonic muscles of chicken have shown that myostatin inhibits proliferation and differentiation of muscle cells (Taylor et al, 2001; Amthor et al., 2002a; Joulia et al., 2003). Meanwhile Erk 1/2 MAPK acts through p53 to promote anti-apoptotic pathways and stabilizes the acetylcholine receptors that underpin neuromuscular junctions in differentiated adult muscle (Bradley et al., 2008). Interestingly, myostatin is synthesized in increasing amounts during skeletal muscle development. Myostatin is produced in very low amounts during the prenatal phase of development when a rapid development of muscle tissue occurs with involvement of both fibre hyperplasia and hypertrophy. Myostatin production increases gradually during the postnatal development. This is associated with slowing in the rate of muscle growth and hypertrophy. Therefore, postnatal myostatin inhibition has a much smaller effect on skeletal muscles compared to the whole life span loss of myostatin function (Whittemore et al., 2003; Wolfman et al., 2003; Welle et al., 2007; Personius et al. 2010).

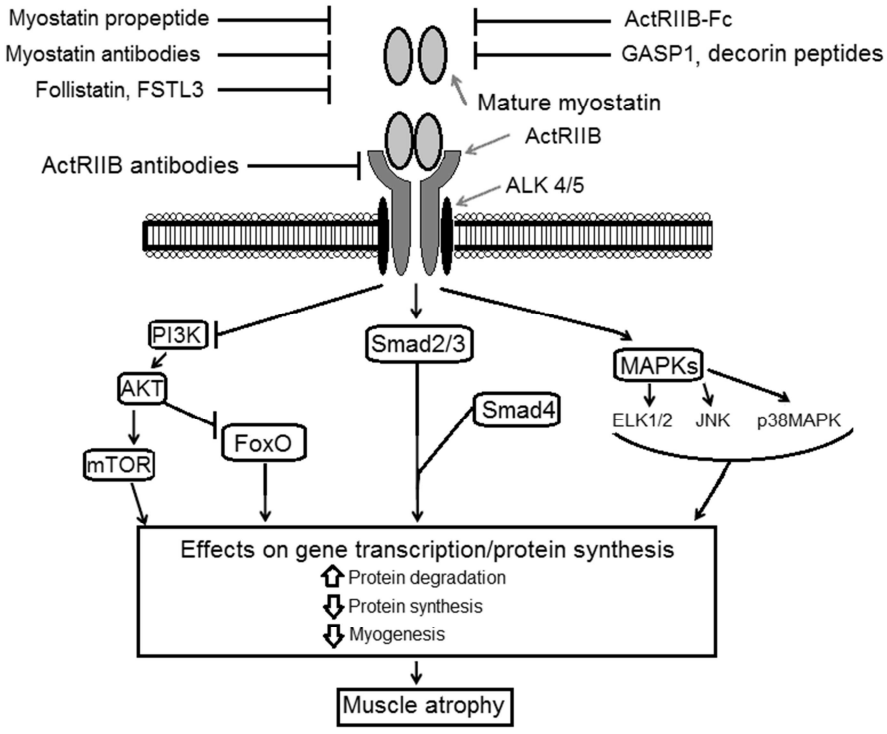

Fig. 7. *Regulation of extracellular myostatin activity and intracellular signalling cascade of events induced by active myostatin molecule (modified Smith, Lin, 2013)*

1.2.4. Myostatin blockade strategies

Previously described skeletal muscle phenotypes of animals with heritable mutations in the MSTN gene have inspired an interest in myostatin inhibition in otherwise-normal individuals and/or undergoing muscle wasting. There are multiple ways to inhibit myostatin function as both intereference with its activation and signalling can be applied. Almost all of these strategies are associated with extracellular myostatin inhibition (Fig. 7). Several naturally occurring proteins which bind to myostatin act outside the muscle cell. These proteins include myostatin propeptide, follistatin, follistatin-related proteins (e.g. FSTL3), growth and differentiation factor-associated serum proteins (GASPs) and decorin peptides (Smith, Lin, 2013). Thus, as long as myostatin is bound to these proteins it is not able to bind to ActRII receptors and activate the signal cascade within the muscle cell. There is a considerable interest in strategies to modify activities of these myostatin antagonists. There have been several successful attempts. An overexpression of myostatin propeptide in transgenic mice induced a significant enlargement of skeletal

muscle due to fibre hypertrophy (Yang et al., 2001; Lee, McPherron, 2001). Furthermore, the injections of a mutant myostatin propeptide, resistant to cleavage by BMP-1/TLD proteinases, into adult mice caused an increase of individual muscles by 18–27 % (Wolfman et al., 2003). In the latter study, the injections of WT myostatin propeptide did not provide such effects on skeletal muscle suggesting a superiority of mutant propeptide and/or a need of greater amounts of WT propeptide to attain comparable results. Interestingly, transgenic mice with skeletal muscle-specific overexpression of follistatin have larger skeletal muscle mass than mice with *Mstn* gene deletion (Lee, McPherron, 2001). Furthermore, when this follistatin overexpression was induced in supermuscular mice with myostatin deficiency the skeletal muscle mass of these mice doubled (Lee, 2007). In agreement with these findings, genetically engineered mice with a loss of function of follistatin have significantly reduced skeletal muscle mass (Matzuk et al., 1995a). Similarly, mice with a loss of GASP-1 and GASP-2 function exhibit a decrease in skeletal muscle mass, but to a lesser extent compared to the loss of follistatin function (Lee, Lee, 2013). On the other hand, GASP-1 overexpression promotes an increase of skeletal muscle mass but far less than follistatin overexpression (Monestier et al., 2012). It should be noted that GASP-1 is approximately 100 times more potent in inhibiting myostatin and shows higher levels of expression than GASP-2 when studied in adults (Walker et al., 2015). The differences in effectiveness between follistatin and GASPs are most likely associated with their effects on ligands other than myostatin. For instance, GASPs selectively inhibit only myostatin and growth and differentiation factor 11 (GDF-11) which is a 90 % analogue to myostatin whereas follistatin inhibits multiple ligands including myostatin, activin A, activin B, bone morphogenetic protein 7 (BMP-7) (Nakamura et al., 1990; Lee, McPherron, 2001; Amthor et al., 2002b; Walker et al., 2015). The multiple ligand binding is a disadvantage when considering a selective myostatin inhibition in order to avoid side effects. Indeed, although follistatin overexpression induces a skeletal muscle development, there might be negatives outcomes such as infertility (Guo et al., 1998).

There is also a considerable interest in artificial inhibitors which modify myostatin function postnatatally. The postnatal inhibition of myostatin has a mild effect on skeletal muscles as skeletal muscle mass increases by 13–40 % due to muscle fibre hypertrophy without hyperplasia (Whittemore et al., 2003; Wolfman et al., 2003; Welle et al., 2007; Personius et al. 2010). Among the artificial inhibitors,

there is a prominent group targeting ActRIIB receptor. As explained earlier, myostatin interacts with this receptor to trigger a signalling cascade in muscle cells after it disassociates from its propeptide and/or other binding proteins. It have been shown that a negative form of receptor (truncated ActRIIB) which do not activate a signal cascade and probably acts as a sink for extracellular myostatin enhances a growth of skeletal muscle in transgenic mice (Lee, McPherron, 2001). Thus, several pharmacological strategies have been developed in order to prevent an initiation of this signal cascade through the ActRIIB receptor. The first one is associated with a direct antibody against a receptor (Lach-Trifilieff et al., 2014). The second one is associated with using of a purified recombinant soluble form of the extracellular domain of the receptor fused to an immunoglobulin Fc domain (ActRIIB-Fc) which competes with natural receptors for myostatin binding (George Carlson et al., 2011; Pistilli et al., 2011; Attie et al., 2013). To date, ActRIIB-Fc is characterized as the most efficacious myostatin inhibitor reported and it affects all types of ActRII signalling (ActRIIB and ActRIIA) (Lee et al., 2005). Both of strategies have exhibited positive effects on skeletal muscle mass both in animals and in humans. It appears that better results can be achieved by a modification of the ActRIIB signalling compared to a direct regulation of myostatin activity with myostatin-specific antagonists (Lach-Trifilieff et al., 2014). This is probably due to interference with a variety of ligands that participate in regulation of skeletal muscle mass. In addition to myostatin, activin A, GDF-11, bone morphogenetic protein 9 (BMP-9) bind to the ActRIIB and initiate their signalling cascades (Smith, Lin, 2013). However, inhibition of those signalling cascades might have negative effects on other tissues. For instance, non-functional activin receptors lead to infertility and skeletal as well as facial abnormalities (Matzuk et al., 1995b). A blockade of ActRIIB signalling is associated with an extreme fatigability of skeletal muscle due to deficiency in oxidative capacity thus showing importance of this signalling pathway in energy production of muscle cells (Relizani et al., 2014). Nevertheless, inhibitors of ActRIIB signalling are being developed and tested in clinical trials due to their expected greater impact on skeletal muscles compared to myostatin-specific inhibition alone. One such agent is Bimagrumab (or BYM338), an antibody against ActRIIB. A single dose of this drug was well tolerated and increased thigh muscle volume in healthy volunteers (Rook et al., 2012). Phase 2 clinical trials with sporadic inclusion body myositis patients have shown a promising outcomes already after a single dose of BYM338 which increased thigh muscle

volume and lean body mass by 7 % and 6 %, respectively (Amato et al., 2014). 8 weeks of BYM338 administration were associated with improvement in quadriceps muscle strength and 6 min walking distance (Amato et al., 2014). This drug is being tested on volunteers showing symptoms of cachexia and/or sarcopenia in association with chronic obstructive pulmonary disease (COPD), lung and pancreas cancer patients or ageing (Smith, Lin, 2013). A single dose of the other drug Ramatercept (or ACE-031), which is ActRIIB-Fc agent, was well tolerated by healthy postmenopausal women and induced an increase in lean body mass (3.3 %) and thigh muscle volume (5.1 %) (Attie et al., 2013). However, later promotion of this drug was terminated probably due to the outcomes of subsequent trials with multiple doses in which a few patients exhibited more serious adverse effects. There is evidence from Phase 1 clinical trials that multiple doses of this drug can cause reversible nosebleeds and skin telangiectasias in some healthy volunteers (Borgstein et al., 2010). Similarly, subcutaneous injections of the drug were well tolerated, increased lean body mass (5.2 %) and tended to attenuate a decrease in thigh muscle volume as well as cause improvements in 6 min walking distance in boys with Duchenne Muscular Dystrophy (DMD), several adverse effects including reversible nosebleeds were registered (Campbell et al., 2012).

Specifically, myostatin-specific inhibitors seem to be the most promising strategy for intereference with myostatin signalling. It would be safer to monitor effects of the single protein inhibition. For this purpose myostatin neutralizing antibodies which have been developing and examining for a some time seem to be the best choice at the moment. A significant increase in mass of individual skeletal muscle (~20 %) and grip force (~10 %) was observed in healthy adult mice which were treated with such antibodies (Whittemore et al., 2003). Intraperitoneal injections of monoclonal antibodies against myostatin increased body and skeletal muscle mass, absolute muscle force and ameliorated degenerative processes in skeletal muscle of *mdx* mice which is a popular model of Duchenne Muscular Dystrophy (Bogdanovich et al., 2002). The treatment of skeletal muscle atrophy using these antibodies was accompanied by similar outcomes in Sgcg-/- mice (Limb girdle muscular dystrophy 2C model) (Bogdanovich et al., 2008). However, other studies demonstrated improvements only under certain conditions suggesting that the timing of the treatment as well as type and severity of the disease might be of importance (Holzbaur et al., 2006; Parsons et al., 2006). Several such myostatin antibodies have been tested in Phase 1 and 2 clinical trials. LY2495655 antibody

was well tolerated and increased thigh muscle volume in Phase 1 trials with healthy volunteers (Jameson et al., 2012). Meanwhile its effects on skeletal muscle mass and strength of patients with advanced cancer not receiving chemotherapy were dose-dependent (Jameson et al., 2012). Trials of the latter drug are continuing in Phase 2 with individuals prone to muscle wasting: elderly and patients undergoing skeletal muscle atrophy following hip replacement (Smith, Lin, 2013). Phase 1 and 2 clinical trials of another myostatin neutralizing antibody MYO-029 have been carried out with populations of various type of skeletal muscle atrophies such as Becker muscular dystrophy, facioscapulohumeral dystrophy, and limb-girdle muscular dystrophy showing a trend to increase skeletal muscle mass without any improvements of skeletal muscle function though (Wagner et al., 2008). Authors of this study acknownledged that a study was designed to examine a safety and adverse effects of the drug first of all, but not a direct effect of the antibody on muscle mass and function. The Phase 1 study of a new myostatin antibody called PF-06252616 financed by Pfizer company is being carrying out with healthy volunteers at the moment (Smith, Lin, 2013). The most recent antibody against myostatin which was studied in clinical trials is AMG 745 (Padhi et al., 2014). It is called a peptibody as it is composed of the peptide component and the Fc portion of an immunoglobulin in an overall structure that resembles an antibody. The warhead of the peptide interacts with myostatin and inactivates its signalling whereas the Fc portion stabilizes the whole complex in the body and extends its therapeutically useful range. Multiple dose administration (4 weeks) of this drug was well tolerated with small adverse effects in patients of prostate cancer receiving adrogen deprivation therapy. Higher doses of this drug were associated with a increase in lean body mass (2.2 %) and greater extremities muscle size (1.8 %), a decrease in body fat (2.5 %), but no effect on strength of lower extremities. More studies required to investigate a therapheutical potential of this antibody among patients with other conditions of muscle wasting.

The main strategies of myostatin blockade were reviewed in this chapter. It has to be mentioned that new strategies of myostatin function regulation are emerging. These strategies are based on RNA interference (Kawakami et al., 2013; Mori et al., 2014), exon skiiping (Lu-Nguyen et al., 2015) and other molecular methods.

1.3. Myostatin dysfunction and muscle function

The main function of skeletal muscle is a generation of the force within muscle fibres and its transmission through tendons to bones in order to perform movements required for everyday life. There are a positive relationship between skeletal muscle mass and force (Jones et al., 2008). Indeed, athletes involved in strength events are renowned for their muscularity (Ikegawa et al., 2008). Thus, possessing larger muscles might be more beneficial in terms of physical fitness. However, existence of such negative regulator of skeletal muscle as myostatin, which is highly conserved across the species, suggests that extremely large muscles are unnecessary from the standpoint of evolution. There might be an optimal range of muscle mass for specific force production. Beside force output, there are other requirements for muscle function which are linked to such intrinsic factors as fibre composition profile, mitochondrial enzyme activity and etc.

The hypothesis that myostatin-deficient individuals might have deteriorated function of skeletal muscles has arised from J. H. Holmes et al. (1973) study with cattle carrying a loss of myostatin function. According to this study, double muscle cattle subjected to forced exercise to exhaustion (≥8.8 km) displayed an acute rhabdomyolysis and elevation of serum creatine kinase level up to about 41.000 U/l in the most severe case. Remarkably, the one animal even died during the examination. However, this study was designated to evaluate the response of double muscle cattle to preslaughter stresses of fasting for 48-h but not to directly measure a functional capacity of double muscle cattle. It is difficult to draw conclusions from this study because in addition to the MSTN mutation the other mutations affecting skeletal muscles might have played a role as the animals had been affected by the extensive breeding programs in cattle for at least 200 years period. It has been determined that myostatin dysfunction increases muscle mass of these cattle by modest 20–25 % compared to 100–200 % increase in myostatin-deficient mice (Ansey, Hanset, 1979; McPherron, Lee, 1997). In fact, myostatin KO mice have not displayed a significant intolerance to physical exercise (Wagner et al., 2005). Muscle fibres of senescent myostatin KO mice were not prone to eccentric contraction-induced injury as an amount of prenecrotic fibres was neglible following prolonged exercise on the declined treadmill in those and WT animals. In other study, skeletal muscle of adult myostatin-deficient mice did not show any signs of muscle damage after exercise training (Savage, McPherron, 2010).

Muscle tendons are an integral part of musculotendinous unit and play a significant role in the modulation of forces transmitted between bones and skeletal muscles as well as in protection of muscle fibres from contraction-induced injuries (Griffiths, 1991; Monti et al., 1999). C. L. Mendias et al. (2008) showed that myostatin regulates directly the structure and function of tendons in skeletal muscles of mice. According to this study, there were substantial differences in tendons between normal and myostatin-deficient individuals. Myostatin-deficient mice had significantly lighter, more brittle and stiff tendons compared to WT mice. An expression of type I collagen, the major component of extracellular matrix (ECM) of tendons, is directly inducible by myostatin (Mendias et al., 2006; Mendias et al., 2008). The treatment of myostatin-deficient fibroblasts with myostatin increased cell proliferation, and increased the expression of type I collagen, scleraxis, and tenomodulin (Mendias et al., 2008). Taken together, this suggests that physical exercise especially involving eccentric contractions might have more negative consequences on individuals lacking of myostatin compared to individuals with the intact myostatin function. However, this might be limited only to mice because such negative consequences on structure and function of tendon have not been observed in rats (Mendias et al., 2015). Thus, to evaluate interspecies differences more animal and especially human models need to be examined.

Although animals lacking myostatin are characterized by large muscles it is not completely clear whether those muscles are functional enough. As myostatin is considered to be not only a promising therapeutic but also a performance enhancing target, it is obligatory to know whether athletes could benefit from the myostatin targeting (inhibition). So far, studies have revealed ambiguous data about it. Nevertheless, a few studies have shown a positive relationship between the loss of myostatin function and physical performance. One of such studies was examining racing dogs (Mosher et al., 2007). This study showed that a significant number of whippets harbor one allele mutation (*mh*/+) in the MSTN gene among the best performing runners (A and B grade) whereas the poorer runners (C and D grade) rarely had the mutated allele as only one dog with this mutation was identified. These dogs carrying the single copy of the mutation are more muscular than WT dogs. Individuals with two copies (*mh*/*mh*) of this mutation are characterized as extremely muscular known as the “bully” whippets. It is worth mentioning D. S. Mosher et al. (2007) examined a dog carrying two copies of the mutated allele. The dog was assigned to A grade runners. Authors concluded that the loss of

myostatin function might contribute to a better physical performance in short duration and high energy demand events such as sprinting. This positive effect of MSTN dysfunction might be dose dependent since a single copy of mutatant MSTN was predominant among the fastest runners. On the other hand loss of myostatin function might compromise physical events which rely on aerobic endurance. This contention is based on findings of a shift toward higher proportion of fast glycolytic fibers compared to slow oxidative fibers in myostatin-deficient animals compared to WT counterparts (Girgenrath et al., 2005; Gentry et al., 2011). In addition, some studies showed a decrease in the size and capacity of lungs in animals with the loss of myostatin function (Ansay, Hanset, 1979; Mosher et al., 2007).

M. Schuelke et al. (2004) have presented the case report where the loss of myostatin function in humans has been identified for the first time. Although the emphasis of this study was the double-muscled newborn with two copies of mutated alleles in the MSTN gene, the interesting insights into functioning MSTN might be offered by his mother possessing the single mutated allele of MSTN. The 24 year old mother of the double-muscled child had also a muscular appearance though not of the same degree. Interestingly, she was a former athlete participating in sprinting. Moreover, there were several other members of her family renowned for unusual muscularity and strength. For instance, her father a potential carrier of the mutated allele as well was a construction worker who was able to unload curbstones by hand. Thus, examples provided in this case report support hypothesis that loss of myostatin function might be beneficial for athletic performance requiring muscle power and strength, such as sprinting and etc.

1.3.1. Myostatin dysfunction and endurance

There is evidence to suggest that skeletal muscles with myostatin dysfunction have a reduced endurance capacity (Matsakas et al., 2010; Savage, McPherron, 2010; Ploquin et al., 2012). Myostatin-deficient mice subjected to treadmill running at an increasing speed until exhaustion ran 28 % less in time, covered 40 % shorter distance and had 38 % lower endurance capacity which was calculated by work performed compared to WT mice (Savage, McPherron, 2010). Similarly, the time to exhaustion during forced swimming activity was reduced approximately twofold in myostatin-deficient mice compared to WT counterparts (Matsakas et al., 2010). C. Ploquin et al. (2012) demonstrated that isolated EDL and SOL muscles from myostatin-deficient mice had ~50 % lower fatigue resistance compared to WT

muscles when tested using repetitive high-frequency tetani. An increased fatigability of myostatin-deficient muscles could partly be due to a shift in fibre type composition towards glycolytic fibre profile at the expense of oxidative fibres (Girgenrath et al., 2005; Gentry et al., 2011). In agreement with this interpretation, blood lactate levels were significantly higher in myostatin-deficient mice than WT mice following endurance swimming or exhaustion running (Matsakas et al., 2010; Mouisel et al., 2014). This is probably associated with the fact that mitochondria content and oxidative capacity is lower in skeletal muscles lacking myostatin (Amthor et al., 2007; Savage, McPherron, 2010; Ploquin et al., 2012). C. Ploquin et al. (2012) suggested that mitochondrial dysfunction but not altered redox status is the main cause of impaired resistance to endurance training. It is hypothesized that myostatin-deficient muscles are affected by reduction in ATP levels which might contribute to increased fatigue during repetitive exercise. In fact, impairment in ATP production was previously reported in myostatin KO muscles (Baligand et al., 2010). Furthermore, higher ATP cost of contraction has been demonstrated for the gastrocnemius muscle (GAS) of myostatin-deficient mice compared to WT muscles (Giannesini et al., 2013). The other reason for reduced endurance capacity could be associated with greater skeletal muscle fibre CSA in myostatin-deficient individuals (McPherron et al., 1997) which might limit a diffusion of oxygen and/or energy substrates into the contracting muscles (Degens, 2012).

E. Mouisel et al. (2014) have recently shown that myostatin might regulate oxidative metabolism independently of muscle fibre composition. A treatment with myostatin propeptide (inactivator of mature myostatin protein) caused a decreased endurance capacity in adult mice without change in fibre composition. A direct influence of myostatin on oxidative metabolism through a control of peroxisome proliferator-activated receptor (PPAR) activity was demonstrated in this study. Myostatin inactivation due to the overexpression of myostatin propeptide resulted in reduced expression of PPAR downstream targets, *Pparb* and *Pgc1α,* which are transcription factors involved in regulation of oxidative metabolism.

1.3.2. Myostatin dysfunction and strength

A significant amount of knowledge about a function of myostatin-deficient muscles has been obtained exlusively from studies with isolated mice muscles. These studies showed that the loss of myostatin function results in no change or an increase in production of peak tetanic force (P_0) (Mendias et al., 2006; Amthor et

al., 2007; Gentry et al., 2011; Qaisar et al., 2012). At the same time a decrease in specific P_0 (sP_0) was observed in mouse EDL (Mendias et al., 2006; Amthor et al., 2007; Qaisar et al., 2012) and SOL (Gentry et al., 2011; Ploquin et al., 2012). It is worth mentioning that slow-twitch SOL seems to be less affected as several studies have not identified a significant decrease in sP_0 of these muscles (Mendias et al., 2006; Qaisar et al. 2012). This might be associated with a lesser degree of hypertrophy in these muscles and muscle fibre composition. Mouse SOL is composed mainly of type I and type IIA fibres whereas EDL is composed of various type II isoforms (Qaisar et al., 2012). Quantity of ActRIIB receptors, to which myostatin binds, is approximately two-fold greater in type II muscle fibres. SOL appears to be less responsive to myostatin than EDL (Mendias et al., 2006). B. A. Gentry et al. (2011) in contradiction to C. L. Mendias et al. (2006) showed reduced sP_0 in SOL of myostatin-deficient mice. Authors suggested that differences between studies may be due to the method for evaluating contractile force generation (*in situ* vs. *in vitro*) and the age of the animals used (4 vs. 10–12 months). Thus alterations in type, number, and size of muscle fibres in myostatin-deficient mice due to the aging process could explain the differences in contractile function between these two studies. In general, however, a tendency toward lower sP_0 in skeletal muscle of myostatin-deficient mice is apparent in literature.

It is not fully understood why muscles of myostatin-deficient mice show low sP_0. Several hypotheses have been proposed to date. Firstly, C. L. Mendias et al. (2006), who first observed that EDL muscle of myostatin-deficient mice have a decrease in sP_0, hypothesized that these muscle due to extreme hypertrophy change in their architecture so that muscle fibre θ increases. Other studies confirm that large muscles have greater θ (Kawakami et al., 1993, Ikegawa et al., 2008). C. L. Mendias et al. (2006) measured θ in myostatin-deficient muscles undirectly using a model that estimates this parameter based upon the number of fibres in the muscle, L_f, L_0 and fibre CSA. They estimated that myostatin-deficient mice had a 38 % and 17 % greater θ for EDL and SOL muscles than WT equivalents, respectively. Thus, loss of myostatin function resulted in greater changes of muscle size and θ in EDL compared to SOL muscle. C. L. Mendias et al. (2006) suggested that there is a treshold of increase in muscle size for initiating a decrease in sP_0. This might explain why there was no decrease in sP_0 of SOL in most studies. Moreover, in support of this hypothesis EDL muscles of mice with a partial loss of myostatin function ($Mstn^{+/-}$) were not so large and had no decrease in sP_0 compared to

completely myostatin-deficient mice (Mendias et al., 2006). Although this hypothesis of increased θ is attractive, not all researchers agree with it since myostatin-deficient mice also show a decrease in sP_0 at a single fibre level (Qaisar et al., 2012).

Another explanation of lower sP_0 of myostatin-deficient mice came from H. Amthor et al. (2007) study where two distinct mouse strains lacking myostatin function were examined. Both strains demonstrated reduced sP_0 of EDL in myostatin-deficient mice compared to WT mice. Myostatin-deficient muscles were also depleted of mitochondria as the ratio of mitochondrial DNA to nuclear DNA and mitochondrial density were reduced in EDL muscle of myostatin-deficient mice compared to WT mice. As patients with impaired mitochondrial respiration also show muscle weakness (Moraes et al., 1991; Mancuso et al., 2002; Zhang et al., 2006), authors have proposed that low sP_0 in myostatin-deficient mice might also be due to reduced mitochondrial content. Moreover, these muscles accumulate tubular aggregates composed of sarcoplasmic reticulum components which are located exclusively in type IIB fibres (Amthor et al., 2007). Interestingly, this accumulation occurs with age as it has not been observed in young myostatin-deficient mice. Moreover, tubular aggregates have been produced far fewer in female mice. However, it is unclear if this morphological abberation is associated with the low sP_0 since sP_0 of myostatin-deficient mice is impaired in both genders at all ages (Amthor et al., 2007). Authors make a suggestion that these accumulations of tubular aggregates in sarcoplasmic reticulum might be a consequence of mitochondrial depletion in myostatin-deficient mice as within myofibre mitochondria can serve as fast calcium sinks as well as supplier of ATP for calcium uptake into sarcoplasmic reticulum (Andrade et al., 2005). It seems that some disturbances in calcium handling exist in myostatin-deficient muscles. For instance, myostatin-deficient muscles show a fast decay in tension during P_0 stimulation and increased the P_t/P_0 ratio (Amthor et al., 2007).

An elegant study has been carried out by R. Qaisar et al. (2012) who demonstrated that myostatin-deficient mice have reduced sP_0 at a single fibre level. Effects of muscle architecture could be avoided in this study. Thus, low sP_0 in myostatin-deficient muscles could not be simply due to changes in θ. The direct effects of ATP production by mitochondria on excitation-contraction coupling is also eliminated by this permeabilized single fibre technique. R. Qaisar et al. (2012) found that myostatin KO mice have lower sP_0 and larger myonuclear domain (MND) of single fibres in EDL compared to WT mice. It appears that myonuclei

generate gene transcripts for greater cytoplasmic volume in myostatin KO fibres compared to WT fibres. Indeed, WT and myostatin KO fibres do not differ in activation of satellite cells which serve as donors for new myonuclei (Zhu et al., 2000). As numbers of transcripts including those coding for contractile proteins is probably unchanged, there might be an inadequate supply of contractile proteins in myostatin KO fibres. Indeed, myosin concentration was lower in EDL fibres of myostatin KO compared to WT controls (Qaisar et al., 2012). Stiffness of those fibres was also lower suggesting a decreased number of strongly attached cross-bridges in muscle fibres with myostatin KO compared to WT fibres. Other studies also confirm fibre hypertrophy with low concentration of myonuclei in myostatin KO mice (Amthor et al., 2009; Matsakas et al., 2009). On the other hand, muscles of myostatin-deficient mice are capable of restoring their sP_0 whenever they are decreased in size (thus, in MND size either) in response to an atrophy stimulus induced by food restriction and strenuous endurance training (Matsakas et al., 2012; Matsakas et al., 2013; Collins-Hooper et al., 2015). This supports the hypothesis that myostatin deficiency leads to an increase in MND size beyond optimal level. In contrast to myostatin deficiency mice overexpressing mIgf1 did not demonstrate lower sP_0 despite a significant hypertrophy compared to WT mice (Qaisar et al., 2012). Remarkably, muscle fibre hypertrophy was accompanied by an increase in myonuclei number, thus maintaining MND size in these mice.

It has been recently suggested that low sP_0 in myostatin-deficient mice might be associated with accumulation of protein aggregates in the vicinity of contractile filaments of muscle fibres (Collins-Hooper et al., 2015). According to authors of this study, if there is an accumulation of intracellular proteins, continual autophagy processes should be activated. Judging by levels of lipidated form of LC3 (microtubule-associated protein 1A/1B-light chain 3) basal level of autophagy is higher in myostatin-deficient mice compared to WT counterparts. The other marker of autophagy, p62 showed a similar pattern. The formation of granules containing p62 which marks an accumulation of aberrant protein material was examined immunohistochemically. Analysis revealed different pattern of those formations between myostatin KO and WT mice. Microscopic images displayed a large number of punctate p62 bodies in the large predominantly IIB fibres of EDL muscle of myostatin-deficient mice compared to very few those bodies in WT counterparts. Interestingly, these patterns became opposite following catabolic stimulus induced by 24-h starvation. The presence of punctate p62 bodies was reduced in myostatin-

deficient mice whereas it increased in WT mice. The most important finding of this study was that sP_0 was restored in mice lacking myostatin following starvation to the level of WT counterparts. In addition, sP_0 did not change significantly but demonstrated a tendency toward a drop in WT mice after acute starvation. In addition, H. Collins-Hooper et al. (2015) showed that the levels of phosphocreatine and creatine are decreased in myostatin-deficient mice and closely resemble those of WT after acute starvation. As phosphocreatine is an energy store providing short-term replenishment of ATP to support cellular homeostasis, a low level of it may contribute to the reduced functionality of muscle. Whether the processes of authophagy and/or changes in phosphocreatine levels are really responsible for a low force generation in myostatin-deficient mice needs more research.

There are also differences in findings about the effect of myostatin deficiency on muscle function in species other than mice. Recently, the study has been conducted on isolated muscles of rats which demonstrated that these large rodents do not experience negative consequences on muscle force generation when myostatin is inactivated genetically (Mendias et al., 2015). The sP_0 was impaired neither at the whole muscle nor at single fibre level in this model. Moreover, a deleterious impact on the tendon morphology and mechanical properties seen in mice was not observed in rats. All this suggests that negative impact on muscle and tendon function might be limited to mice. Thus, it is necessary to examine the impact of myostatin deficiency on skeletal muscles in various species to understand better effects of loss myostatin function.

1.3.3. Myostatin dysfunction and exercise training

Plasticity is an important feature of skeletal muscle (Baldwin, Haddad, 2002; Flück, Hoppeler, 2003). Skeletal muscle can adapt to the various demands imposed on it. For example, concentration of mitochondria and oxidative enzymes increases after repeated bouts of endurance exercise while muscle CSA becomes larger in response to high resistance training. Although a significant effort has been spent on studying effects of myostatin inhibition in muscle cells and skeletal muscles, much less attention has been devoted to effects of myostatin inhibition on adaptation to exercise training. As previously mentioned, myostatin inhibition is considered to be a potent strategy to enhance athletic performance. Thus, it is important to examine how skeletal muscles with myostatin dysfunction adapt to exercise training and compare these responses to reponses of WT muscles.

Adaptation to endurance training. To date, only several studies have been carried out with aim of investigating adaptive responses of myostatin-deficient mice to exercise training and all of them focused on the endurance training exclusively. M. W. Hamrick et al. (2006) probably were the first who subjected myostatin-deficient mice to endurance training. Skeletal muscle tissue was not at the focus of that work and the effects on exercise performance or oxidative metabolism were not measured. Authors found that bone strength increased more in myostatin KO than WT mice after the training programme consisting of 30 min of treadmill exercises which were carried out 5 days per week for 4 weeks. In addition, triceps brachii muscle mass was reduced in myostatin-deficient but not WT mice after training. K. J. Savage and A. C. McPherron (2010) subjected mice to the same protocol of exercise training and found that glycolytic muscles of myostatin-deficient mice showed a trend towards an increased proportion of oxidative fibres. Furthermore, training increased significantly citrate synthase activity in one of the four muscles (triceps brachii) of myostatin-deficient mice. In contrast to the previous study, this protocol of endurance training induced a reduction in skeletal muscle mass of both WT and myostatin KO genotypes, although muscles were affected to a greater extent in myostatin KO group. Authors explain those discrepancies between studies by differences in genetic backgrounds of mice used in both studies. For instance, the CD-1 mice used in M. W. Hamrick et al. (2006) study have a greater critical running speed than the C57BL/6 strain in K. J. Savage and A. C. McPherron (2010) study. Thus the same protocol of running speed at 12 m/min may be relatively less intense for CD-1 mice. Two other protocols of endurance training, i.e. voluntary moderate intensity wheel running and forced high-impact swimming increased oxidative capacity and capillary density in skeletal muscles of myostatin KO mice more than WT mice (Matsakas et al., 2010; Matsakas et al., 2012). Irrespective of the training regime, there were significant changes in markers for oxidative metabolism such as increased concentration of succinate dehydrogenase (SDH) positive fibres, transition of muscle fibres from faster to slower type II fibres, increased levels of transcripts for uncoupling protein-3 (Ucp3), carnitine palmitoyltransferase 1α (Cpt1α), pyruvate dehydrogenase kinase isozyme 4 (Pdk4) and oestrogen-related receptor γ (Errγ). Analysis of muscle fibre CSA revealed that the muscles from myostatin KO mice responded differently compared to WT mice. For instance, in most cases fibres of myostatin-deficient mice underwent a greater decrease in fibre size after swimming and did not enlarge

after wheel running compared to fibres of WT mice (Matsakas et al., 2010). The running performance (active time spent in the wheel, average running distance and daily average wheel speed) was comparable and there was a tendency towards a greater average and daily maximum speed of running with training for both genotypes (Matsakas et al., 2010). Moreover, myostatin KO mice even demonstrated a particularly strong tendency ($P = 0.051$) towards an increase in maximum running speed compared to WT counterparts. Contrary to running exercise, myostatin-deficient mice were poorer swimmers than WT mice. Although myostatin KO mice improved their performance after training, time to exhaustion was still 50 % lower compared to WT values. Significantly higher blood lactate levels after the last swimming bout suggests that this type of exercise might be harder and more stressful for myostatin KO mice. In addition to reduced size of muscle fibres, both types of endurance training resulted in reduction of the MND in skeletal muscles of myostatin KO mice (Matsakas et al., 2012). These alterations correlated well with restored muscle function (P_0 and sP_0) in these mice. There were no changes of these force parameters in WT mice after endurance training.

Overall, endurance training might improve skeletal muscle function of myostatin-deficient mice by causing a reduction in muscle fibre CSA and remodeling metabolic profile towards WT individuals. Thus, although skeletal muscles lacking myostatin are not able to fully restore properties of WT muscles, they maintain capability to adapt to endurance training.

Adaptation to resistance training. Resistance exercise training is a well known method to increase skeletal muscle mass (Jones et al., 1989). Myostatin expression is significantly reduced in humans after acute and chronic resistance exercise (Roth et al., 2003; Raue et al., 2006). Thus, myostatin inhibition itself could be considered as resistance exercise mimetic because it induces a dramatic increase in skeletal muscle mass (Haidet et al., 2008). Therefore, athletes whose primary goal is to increase muscle mass and strength are highly interested in myostatin inhibition as a performance enhancing strategy even though such interventions might be unacceptable from the ethical standpoint. Although myostatin inhibition is beneficial for skeletal muscle of mice suffering from muscle dystrophies (Bogdanovich et al., 2002; Haidet et al., 2008) there is little data about effects of myostatin inhibition in healthy individuals. Studies suggest that such interventions might not produce significant functional benefits in otherwise-normal adult mice despite an increased skeletal muscle by 20–40 % (Personius et al., 2010).

Moreover, the question arises how these enlarged muscles would respond to resistance exercise training which is essential to strength and power athletes and create additional hypertrophy stimulus. So far, we failed to find studies examining adaptability of myostatin-deficient muscles to resistance training. Thus, one of our aims was to address this problem.

As it is quite difficult to engage mice and/or rats in voluntary resistance training, several other procedures are applied to induce functional overload (FO) of skeletal muscles (Lowe, Alway, 2002). One of such methods is synergist muscle ablation which results in a rapid and pronounced hypertrophy of other skeletal muscles with a similar function (Kandarian, White, 1989; Bodine, Baar, 2012). This method of muscle overloading induces a compensatory muscle hypertrophy. Similar to resistance exercise the synergist muscle ablation upregulates Akt/mTOR/p70S6K signalling pathway which is also responsible for an increased rate of protein synthesis after muscle exercise (Bodine et al., 2001a). The increased balance between protein synthesis and degradation is prerequisite for skeletal muscle hypertrophy (Schiaffino et al., 2013). According to C. Lipina et al. (2010) myostatin-deficient mice have significantly elevated expression and activity of components of Akt/mTOR/p70S6K signalling pathway. Thus protein synthesis in skeletal muscle might be closer to its maximum in these mice compared to WT counterparts. The excessive muscle hypertrophy in myostatin-deficient mice is consistent with this hypothesis and raises doubts whether further muscle hypertrophy is possible after FO of the skeletal muscles. Despite these concerns, myostatin-deficient mice can experience a further two fold increase in muscle mass if other regulators of muscle growth, such as follistatin, would be modified (Lee, 2007). The latter findings suggest that myostatin-deficient muscles do not reach the greatest possible muscle mass. However, it remains unclear if these muscles possess comparable adaptability to FO as WT muscles. As already mentioned, myostatin-deficiency leads to the shift from slower I and IIA toward faster contracting type IIX and IIB myosin heavy chain isoforms which might be advantageous for adaptations to high resistance training since type II fibres show greater enlargement in CSA compared to type I fibres (Verdijk et al., 2009). Thus, speculation about the potential for muscle hypertrophy of myostatin-deficient animals needs to be investaged using appropriate models of exercise training or FO.

As previously mentioned, myostatin KO mice generate low specific forces by their skeletal muscles (Mendias et al., 2006; Amthor et al., 2007; Gentry et al.,

2011; Ploquin et al., 2012; Qaisar et al., 2012). Irrespective of the mechanisms responsible for this impairment which are not completely elucidated, it seems that the size of skeletal muscles plays a critical role in this phenomenon. Whenever the size of skeletal muscle of myostatin-deficient mice is reduced and approach close to WT values after endurance training, caloric restriction and/or acute starvation, there is a restoration of sP_0 to the level similar to WT muscles (Matsakas et al., 2012, 2013; Collins-Hooper et al., 2015). Interestingly, that this occurs due to the decreased skeletal muscle mass and increased absolute force what is contrary to the widely held view about a positive relationship between skeletal muscle size and force (Widrick et al., 2002). Moreover, both the less muscular heterozyguous myostatin KO mice and mice with postnatal inhibition of myostatin function demonstrate sP_0 similar WT mice. This suggests the dose-dependent effect of myostatin inhibition on skeletal muscle force (Mendias et al., 2006; Morine et al., 2010; Gentry et al., 2011). Thus, synergist muscle ablation and/or resistance training leading to hypertrophy of skeletal muscle most likely would induce an additional impairment of force generation in skeletal muscles of myostatin-deficient mice if their mass would increased.

1.4. Myostatin dysfunction and muscle atrophy stimuli

1.4.1. Physiology of muscular atrophy

The term muscle atrophy is defined as the physiological process of a partial or complete breakdown of skeletal muscle tissue. It is often characteristic to various pathological conditions and diseases such as skeletal muscle dystrophies, neurodegenerative diseases or cachexia due to cancer, AIDS, congestive heart failure, chronic obstructive pulmonary disease (COPD), renal failure (Gonzalez-Cadavid et al., 1998; Costelli, Baccino, 2003; Debigaré et al., 2003; Holzbaur et al., 2006; Ohsawa et al., 2006; Argiles et al., 2007; Wang, Mitch, 2014). Muscle atrophy may lead to muscle weakness which is associated with increased risks of falls, fractures and even death. Muscle atrophy may also occur in otherwise-healthy individuals. Disuse, microgravity during a space flight, starvation and caloric restriction cause a rapid decrease in muscle mass (Melchior et al., 1998; Lalani et al., 2000; Reardon et al., 2001; Matsakas et al., 2013).

Muscle mass is dependent on a net protein turnover which is regulated by

muscle protein synthesis (MPS) and muscle protein degradation (MPD) (Schiaffino et al., 2013). There might be several scenarios. MPS may exceed MPD and thus cause muscle fibre hypertrophy. Equal rates of MPS and MPD mean no change in muscle mass. Muscle atrophy occurs when MPD exceed MPS. MPS and MPD pathways are intimately related with a possible cross-talk between each other. It is often assumed that MPD increases and MPS decreases during muscle atrophy. However, the actual changes in the pathways depend on the type of atrophy stimulus. Muscle denervation leads to a higher rate of MPD and increased rather than decreased MPS (Argadine et al., 2009; Quy et al., 2012). Starvation is accompanied by increased MPD and decreased MPS in both slow- and fast-twitch rat muscles (Li, Goldberg, 1976). All this suggests that a pathophysiology of atrophy may vary depending on the atrophic stimulus. In addition, skeletal muscles differ in fibre composition which might contribute to different responses of muscles to the same stimulus. Indeed, slow SOL is less sensitive to starvation-induced atrophy compared to fast EDL (Li, Goldberg, 1976). Slow-twitch type I fibres comprising a large number of the SOL fibres might be more resistant to atrophy due to differences in sensitivity to corticosteroids between slow and fast fibres (Goldberg, Goodman, 1969). Indeed, corticosteroid treatment causes atrophy of type IIB and IIX fibres but not type IIA and I fibres in the rat diaphragm (Verheul et al., 2004). Furthermore, oxidative status might also be of importance in protecting muscle fibres from autophagy (Mofarrahi et al., 2013; Collins-Hooper et al., 2015). It seems that high resistance to atrophy of SOL muscle is also related to high involvement of its fibres in locomotory activities. Interestingly, SOL of rodents is more prone to atrophy than EDL after subjection to disuse by hindlimb suspension (Fitts et al., 1986; Yamazaki, 2005). Similar findings were reported for mice after long-term microgravity during spaceflight (Sandonà et al., 2012). Both of these experimental models are depriving the muscles of weight-bearing and thus resemble a bed-ridden state. Thus, slow-twitch fibres of SOL tend to be quite resistant to atrophic stimuli when muscle activity and weight-bearing are not restricted. Interestingly, slow contracting muscles show higher rates of both MPS and MPD compared to faster contracting muscles (Li, Goldberg, 1976). There is also evidence for a complex interaction between various factors during muscle atrophy. For instance, denervation in the rat diaphragm muscle resulted in atrophy of fast-twitch type II fibres exclusively (Aravamudan et al., 2006). However, denervation of SOL caused a marked atrophy of type I fibres (Ciciliot et al., 2013).

Four major signalling pathways are controlling MPS and MPD (Schiaffino et al., 2013). MPS is promoted by IGF-1/Akt/mTOR pathway while myostatin-Smad2/3 pathway acts as as a negative regulator of MPS. These two pathways interact at the level of Akt and Smad3 (Sartori et al., 2009). Indeed, follistatin, which is a plasma protein, can act to inhibit Smad3 activity and activate Akt-mTOR signalling (Winbanks et al., 2012). On the other hand, expression of constitutively active Smad3 prevented follistatin-induced skeletal muscle hypertrophy and suppressed follistatin-induced Akt/mTOR/S6K signalling.

The signalling pathways for MPD are complex and not fully understood. During muscle atrophy two major proteolytic pathways, the ubiquitin-proteasomal and the autophagic-lysosomal, are activated at variable levels thus contributing to the loss of muscle mass (Schiaffino et al., 2013). Forkhead box (FoxO) proteins, which include three isoforms (FoxO1, FoxO3 and FoxO4), are a family of transcription factors that play important role in upregulating the expression of several genes involved in proteolytic pathways (Sandri, 2008). FoxO proteins might be considered as common triggers of atrophy signalling. Interestingly, Akt phosphorylates and inhibits activity of FoxO as transcrotion factors. Thus MPD and MPS processes are closely coordinated (Lee et al., 2004; Stitt et al., 2004). Atrophy-related genes also known as atrogenes are upregulated by atrophy transcription factors. Atrogenes belong to either the ubiquitin-proteosome or the autophagy-lysosome system.

A key regulator of proteolysis is the ubiquitin-proteosome system. During muscle atrophy sarcomeric proteins are undergoing ubiquitination process followed by their degradation with involvement of special structures called proteasomes. Proteosomes are large proteinase complexes with multiple catalytic sites (Coux et al., 1996). At least five peptidase activities in proteasome are required for cleaving peptide bonds after basic, acidic, and hydrophobic amino acids. Those processes normally start whenever a target protein (substrate) is ubiquitinated (i.e. marked by a covalent linkage with poly-ubiquitin chain) and then recognized by proteasomes. Some proteins are not ubiquitinated but are degraded directly by proteasomes. These reactions of substrate ubiquitination and degradation are catalyzed by various enzymes of which ligases E3 are especially important (Schiaffino et al., 2013). Although there are estimated 500–1000 E3 ligases only a few of them are both muscle-specific and upregulated during muscle atrophy in humans. Most prominent are the muscle atrophy F-box (atrogin-1/MAFbx) and the muscle RING finger 1

(MuRF1). Loss of function studies of these ligases revealed their critical role in skeletal muscle atrophy. Mice deficient in either atrogin-1/MAFbx or MuRF1 are resistant to denervation-induced atrophy whereas overexpression of atrogin-1/MAFbx in myotubes produced atrophy (Bodine et al., 2001b). Furthermore, inhibition of atrogin-1/MAFbx expression attenuates muscle atrophy in fasting mice (Cong et al., 2011). In those mice the level of a myogenic factor, MyoD, was upregulated whereas myostatin was suppressed. Treatment with the synthetic glucocorticoid dexamethasone in atrogin-1/MAFbx and MuRF1 KO mice showed skeletal muscle mass sparing properties of MuRF1 but not atrogin-1/MAFbx KO suggesting not similar function of these ligases under all atrophy models (Baehr et al., 2011). To date, only very small number of muscle proteins has been identified as substrates for atrogin-1/MAFbx and MuRF1. For instance, atrogin-1/MAFbx promotes degradation of MyoD and eukaryotic translation initiation factor 3 subunit F (eIF3-f) (Tintignac et al., 2005; Csibi et al., 2010; Cong et al., 2011). However, there might be many more muscle proteins that are interacting with these ligases and subsequently degraded. Although ubiquitination of muscle sarcomeric proteins (myosins, desmin, vimetin), transcription factors, translational components or mitochondrial proteins ha not been proven but interactions of atrogin-1/MAFbx with them have been found using immunoprecipitation experiments in C2C12 myoblast and myotubes (Schiaffino et al., 2013). In addition, similar interactions have been found between MuRF1 and muscle structural proteins (troponin I, myosin heavy chains, myosin light chains, myosin binding protein C, actin). Thus, both atrogin-1/MAFbx and MuRF1 could be potential molecular targets for treating muscle atrophy. It is likely that there are other E3s with a similar function in skeletal muscles. In fact, KO of muscle-specific TNF receptor-associated factor 6 (TRAF6) protects muscle mass during denervation or starvation in mice (Paul et al., 2010, 2012). It seems that this E3 ligase affects both proteasome and autophagic-dependent muscle degradation. Inhibition of TRAF6 reduces levels of atrogin-1/MAFbx and MuRF1 as well as p62, LC3B, Beclin1, Atg12, and Fn14 (Paul et al., 2012). There were almost no Lys63-polyubiquitinated proteins in fasted mice muscles. TRAF6 mediates the conjugation of Lys63-linked polyubiquitin chains to target proteins and those chains participate in regulation of autophagy-dependent cargo recognition by interacting with the p62 protein (Schiaffino et al., 2013). In turn, Lys48-linked but not Lys68-linked chains are a signal for proteasome-dependant protein degradation. Another E3, the mitochondrial ubiquitin protein

ligase 1 (Mul1), is upregulated by the FoxO proteins during atrophy stimuli and plays a significant role in the regulation of mitochondrial function (Lokireddy et al., 2012). KO of this ligase resulted in sparing of skeletal muscle mass during fasting (Lokireddy et al., 2012). Specific E3 ligases may be involved in different models of muscle atrophy as the Nedd4-1, which has been reported to be upregulated mainly during muscle disuse (Nagpal et al., 2012). Interestingly, muscle-specific Nedd4-1 KO positively affects denervated type II fibres protecting them from muscle atrophy.

The second major proeolytic pathway is the authophagy-lysosome system. This system is responsible for maintenance homeostasis of cells by removing and eliminating unfolded and toxic proteins as well as abnormal and dysfunctional organelles (Sandri, 2010). Autophagy system generates double membrane vesicles called autophagosomes that engulf portion of cytoplasm, organelles, glycogen and protein aggregates, which are then delivered to lysosomes for degradation of their contents. FoxO3 was identified as the critical factor for autophagy control in adult muscles (Sandri, 2010). Several autophagy genes including LC3, Gabarap, Bnip3, VPS34, and Atg12 are under its regulation. Basal autophagy acts as a quality control machinery for cytoplasmic components and is crucial for various cells including muscle fibres. However, a strong activation of autophagy contributes to excessive loss of muscle mass during catabolic states as starvation (Mizushima et al., 2004). Autophagy might be critical not only for protein degradation, but also for myofibre survival. Indeed, mice with conditional KO of Atg5 or Atg7, the critical gene of autophagy in skeletal muscle, underwent profound atrophy, weakness and several features of myopathy (Raben et al., 2008; Masiero et al., 2009). These mice showed accumulation of protein aggregates, abnormal mitochondria and concentric membranous structures in muscle fibres. Interestingly, conventional KOs for Atg genes leads to severe defects and ultimately to death of mouse neonates (Ichimura, Komatsu, 2011). Thus, autophagy might have a beneficial role and its absence and or excessive presence could be detrimental for muscle mass. However, there are still many unknowns in the role and regulation of the autophagic pathway in skeletal muscle. Inactivation of the proteins involved in the regulation of autophagy might be more risky than inactivation of the proteins of the proteasome system in terms of muscle health. However, the suppression of autophagy in muscles might be beneficial in preventing muscle atrophy. Runx1, the one of known suppresors of authopagy in skeletal muscles, preserve muscle mass during denervation. For

instance, KO of Runx1 results in excessive autophagy during denervation which leads to severe atrophy (Wang et al., 2005). Jumpy phosphatase is another negative regulator of autophagy in muscle cells (Vergne et al., 2009). The most potent autophagy inhibitor in skeletal muscles is Akt kinase (Sandri, 2010). Acute activation of Akt inhibits autophagosome formation and lysosomal-dependent protein degradation in adult mice during fasting or in muscle cell cultures (Sandri, 2010). Downstream Akt targets mTOR, S6K1 and S6K2 are less important in autophagy regulation as Akt.

1.4.2. Myostatin and muscle atrophy

The role of myostatin in muscle atrophy is not quite clear, but many studies support the idea that myostatin might be a major player here. Indeed, myostatin activity increases during muscle wasting diseases such as cancer, COPD, AIDS, renal failure, heart failure (Gonzalez-Cadavid et al., 1998; Ohsawa et al., 2006; Sun et al., 2006; Costelli et al., 2008; Plant et al., 2010; Breitbart et al., 2011; Gruson et al., 2011; Aversa et al., 2012). Myostatin levels correlate inversely with lean body mass index in HIV-infected men (Gonzalez-Cadavid et al., 1998). Higher levels of myostatin are characteristic for other conditions asscociated with muscle loss such as sarcopenia, disuse and microgravity (Lalani et al., 2000; Reardon et al., 2001; Yarasheski et al., 2002). Furthermore, myostatin overexpression or systemic administration causes skeletal muscle atrophy (Zimmers et al., 2002; Reisz-Porszasz et al., 2003; Amirouche et al., 2009). Therefore, it seems that myostatin is an important component of skeletal muscle atrophy, but it is unclear whether it is absolutely necessary or is just the one of mediators of muscle atrophy. In some cases, muscle atrophy is not associated with enhanced myostatin activity. For instance, myostatin levels are unchanged in denervation or hindlimb suspension induced skeletal muscle atrophy (Kawada et al., 2001; Baumann et al., 2003). It should be noted that in the hindlimb suspension study only myostatin mRNA levels were examined. This is an indirect measure of myostatin activity, since translational and post-translational events may be involved in regulation of myostatin activity. Moreover, B. M. Carlson et al. (1999) have found increased levels of myostatin mRNA only at early stages (post 24 h) but not at later stages following hindlimb suspension. Collectively, the evidences suggest that myostatin is not the primary cause of muscle atrophy but it plays an important role in mediating atrophy

signalling. In addition, the effect of myostatin on the every type of atrophy should be investigated separately as pathophysiology of different atrophy stimuli differ.

Molecular function of myostatin in skeletal muscle suggests that myostatin affects processes of both MPS and MPD and thus influencing a net protein balance. It seems that elevated levels of myostatin affect MPS to higher extent than MPD. For instance, components of the Akt/mTOR pathway but not ubiquitin-proteosome components are significantly downregulated in response to muscle-specific myostatin overexpression in rats by an electrotransfer of a myostatin expression vector into the tibialis anterior (TA) muscle (Amirouche et al., 2009). On the other hand, increased MPS have been observed in myostatin KO mice (Welle et al., 2007). C. Lipina et al. (2010) found an enhanced muscle expression of both Akt and mTOR/S6K signalling components of MPS in these mice. Moreover, M. R. Morissete et al. (2009) showed increased Akt protein expression and overall activity which was associated with an increase in Akt mRNA in myostatin KO mice. Akt is a potent inhibitor of MPD and especially autophagy signalling through suppression of FoxO3 (Sandri, 2010). Higher basal activity of Akt in myostatin KO mice suggests that myostatin inactivation might be beneficial in preserving muscle mass during catabolic states. Indeed myostatin blocking antibodies have a positive effect on muscle mass and function in *mdx* mice (Bogdanovich et al., 2002; Wagner et al., 2002). More detailed data on types of myostatin inhibition and its impact on various diseases were presented in chapter 1.2.4. Further we are going to focus on effects of myostatin inhibition in otherwise-healthy individuals during disuse or bed rest as well as various manipulations with diet such as caloric restriction or fasting. Strategies of preserving muscle mass would facilitate negative outcomes from muscle weakness accompanying these conditions.

Mice with conventional KO for myostatin are a popular model for examining role of myostatin in muscle atrophy. Few studies are carried out in this area but the results are ambiguous. During hindlimb suspension myostatin KO mice showed greater muscle atrophy than WT counterparts (McMahon et al., 2003). An expression of MyoD, Myf-5, and myogenin increased in WT mice and tended to decrease in muscles of myostatin KO mice. This suggests that hindlimb suspension induces myogenesis in WT mice to counter atrophy, but not induces myogenesis in myostatin KO mice, thereby resulting in a greater loss of muscle mass. H. Gilson et al. (2007) found that myostatin KO prevents from dexamethasone-induced muscle atrophy in mice. Muscle atrophy in WT mice was associated with increased mRNA of enzymes

involved in proteolytic pathways (FoxO3a, atrogin-1, MuRF1, and cathepsin L) and increased chymotrypsin-like proteasomal activity whereas all these enzymes were not significantly affected in myostatin KO mice. Dexamethasone reduced expression of IGF-1 in WT but not KO mice. Surprisingly, however, levels of muscle IGF-1 mRNA in KO mice were lower in both control and treated with dexamethasone groups compared to WT mice. It was attributed to a potentially compensatory increase of IGF-2 expression (2-fold) seen in KO mice of both groups.

Conflicting data on topic were collected in studies with food restriction studies. D. L. Allen et al. (2010) subjected mice to 48 h food deprivation (FD) and found that fast-twitch TA but not slow-twitch SOL muscles of myostatin KO were more resistant to atrophy compared to WT muscles. However, this resistance seems to occur after a period of FD as there was no difference in atrophy between genotypes after 24 h. The levels of myostatin mRNA were increased approximately 3-fold by 48 h, but not by 24 h in WT mice. Thus, authors suggested that myostatin contributes to muscle atrophy during prolonged FD. However, it is unclear by which mechanisms myostatin KO preserves skeletal muscle during prolonged FD. Authors examined only several atrogenes (Atrogin-1 and MuRF1) belonging to the ubiquitin-proteasomal system and did not find any differences in TA muscles between genotypes. As MPS and authopagy-lysosome pathways were not examined in this study, one might hypothesize that underlying mechanisms might be associated with them. H. Collins-Hooper et al. (2015) applied 24 h FD to myostatin KO and WT mice and found that skeletal muscles regardless of the contractile properties were more sensitive to FD-induced atrophy in myostatin KO mice. Reasons of differences between D. L. Allen et al. (2010) and H. Collins-Hooper et al. (2015) studies are unclear. In their study, H. Collins-Hooper et al. (2015) carried out an in-depth analysis of MPS and MPD pathways. After FD a combination of decreased MPS and increased MPD lead to a greater muscle atrophy in myostatin KO compared to the control mice. Interestingly, the basal levels of autophagy were elevated in the muscles of myostatin KO mice indicating the presence of unwanted material in the resting condition compared to WT mice. A. Matsakas et al. (2013) used a different model of muscle atrophy on myostatin KO and WT mice – food restriction by 40 % for a period of 5 weeks. They found that myostatin KO mice were more susceptible to catabolic processes than WT mice. The study also showed that myostatin KO mice maintained activity of molecules involved in MPS, did not

upregulate the expression of atrogenes, but increased expression of genes associated with autophagy during low calorie diet. Thus, autophagy might be upregulated to a great extent in absence of myostatin during muscle atrophy. This suggests that there are more important factors for inducing and regulating autophagy.

An overview of studies using myostatin KO mice shows a complex role of myostatin in muscle atrophy. However, mice with conventional myostatin KO exhibit an overt phenotype, which is rare in nature (Lee, 2004). In addition to muscle hypertrophy myostatin KO causes substantial changes in muscle fibre-type composition and metabolism (McPherron, Lee, 2002; Girgenrath et al., 2005; Guo et al., 2009). Therefore, muscles of myostatin KO mice and WT mice are at very different starting "set-points" before any experiments are carried out. Thus, effects of myostatin deficiency might be secondary to these additional changes. It seems that conditional myostatin KO would be a better model in studying effects of myostatin KO. Firstly, it does not provide such an overt phenotype (Whittemore et al., 2003; Personius et al., 2010). Secondly, it allows to have a homogenic animal groups before studies. So far, there are very few studies employing this model of myostatin deficiency because of the methodological difficulties. Nevertheless, E. M. MacDonald et al. (2014) applied a soluble ActRIIB receptor to mice during limb immobilization and denervation. This type of myostatin inhibition prevented immobilization-induced loss of muscle mass. Authors found that serum glucocorticoid-induced kinase (SGK) rather than Akt regulates a protection from disuse atrophy in ActRIIB-treated mice. Suprisingly, denervation atrophy was not protected by ActRIIB treatment. Of interest, upregulation of the pro-growth factors such as Akt, SGK and components of the mTOR pathway was induced during denervation. Treatment with the mTOR inhibitor rapamycin decreased activity of the Akt/mTOR pathway but did not alter denervation-induced muscle atrophy. Thus, effects of conditional myostatin KO might depend on the type of atrophy as a different underlying pathophysiological mechanisms are involved in denervation compared to disuse. It would also be useful to investigate effects of conditional KO of myostatin on catabolic states such as food restriction and FD before drawing firm conclusions about benefits of myostatin targeting.

2. METHODS AND MATERIALS

2.1. Animals

All the studies were performed on inbred laboratory mouse strains. The procedures involving mice were approved by the Lithuanian Republic Alimentary and Veterinary Public Office (no. 0223 in 2012 and no. 10 in 2014). Mice were bred and housed in the animal facility of Lithuanian Sports University. They were kept in standard cages, 1 to 3 individuals per cage at a temperature of 20–21 ºC and 40–60 % humidity with the reversed 12/12-h light/dark cycle. Animals were fed with standard chow diet (58 % kcal from carbohydrate, 28.5 % kcal from protein, 13.5 % kcal from fat; LabDiet 5001, USA) and received tap water *ad libitum*.

The following mice strains were studied: classical C57 black 6 (C57BL/6J), Berlin High (BEH), Berlin High with the both WT *Mstn* alleles (BEH+/+) and Dummerstorf High (DUH) (Fig. 8). The breeding pairs of C57BL/6J mice were obtained from the Jackson laboratory (USA) whereas the breeding pairs of the BEH, BEH+/+, DUH were a generous gift of prof. Lutz Bünger. BEH+/+ strain was generated from the BEH mice carrying MstnCmpt-dl1Abc (Compact; *Cmpt*) mutation in both *Mstn* alleles (Varga et al., 1997). Due to this mutation there is 12-bp deletion in the *Mstn* gene sequence coding a propeptide region of protein. As a result, the BEH mice are lacking the functional myostatin and have hypermuscular phenotype (Amthor et al., 2007; Lionikas et al., 2013a, b) (Fig. 9). The BEH+/+ strain with functioning myostatin or WT myostatin was generated by crossing BEH mice with the Berlin Low (BEL) strain and then repeatedly backcrossing the offspring to BEH using marker assisted selection for the WT myostatin (Amthor et al., 2007; Lionikas et al., 2013b).

In the *first study* force generating capacity of skeletal muscles was examined in the C57BL/6J, BEH+/+ and DUH which varied significantly in body and muscle mass. The BEH+/+ and DUH were examined in adulthood and at a young age to investigate the effect of natural growth on skeletal muscle function.

In the *second study* we investigated the effect of the myostatin dysfunction on skeletal muscle mass and function after functional overload (FO) which was induced by ablation of synergist muscles. Soleus (SOL) muscle of BEH+/+ and BEH mice with intact and dysfunctional myostatin were compared, respectively.

In the *third study* we investigated the effect of the myostatin dysfunction and genetic background on skeletal muscle mass and function in response to the atrophy

stimulus induced by the food deprivation (FD). The BEH+/+, BEH and C57BL/6J strains were examined.

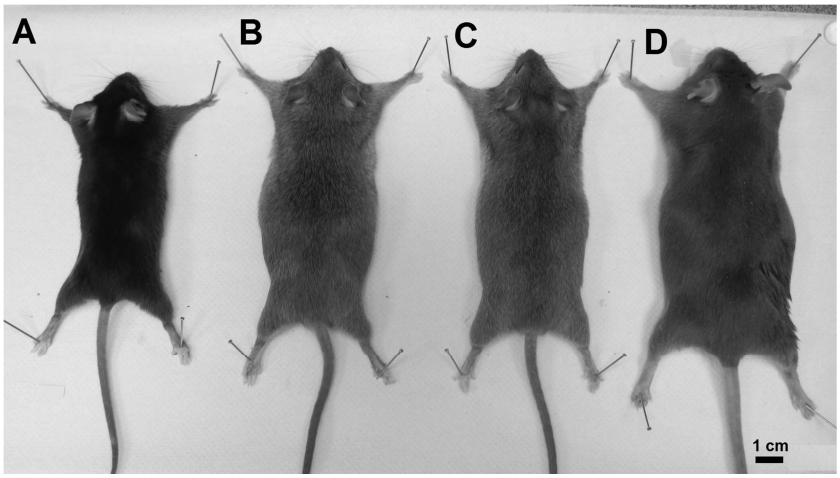

Fig. 8. *Mouse strains used in the experiments. They are the C57BL/6J (A), BEH+/+ (B), BEH (C) and DUH (D), respectively*

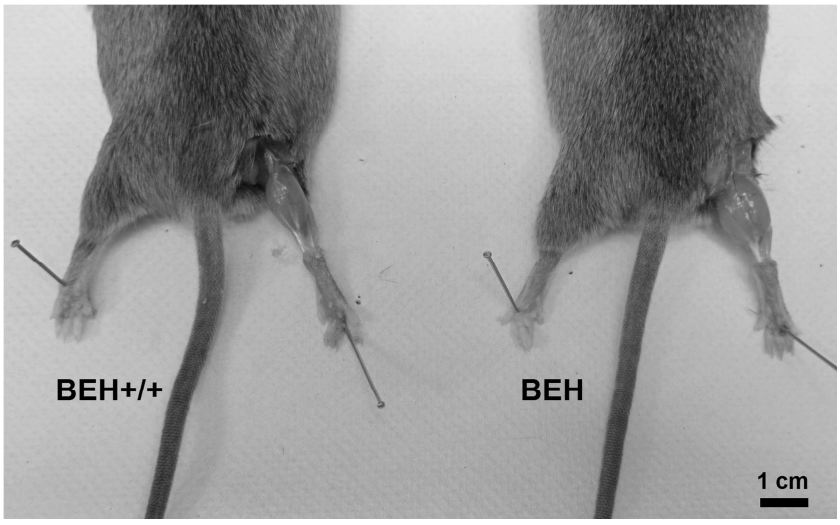

Fig. 9. *Musculature of hindlimbs in the BEH+/+ mice with wild-type allele and BEH mice with mutated MstnCmpt-dl1Abc (Compact) allele in the Mstn gene causing myostatin dysfunction*

More detailed data including the number, sex and age of the examined animals are shown in the Table 1.

Table 1. *Numbers and ages of mice used in all the studies*

Study	Strain	Groups	Age (weeks)	Sex	Mice number	Total number
1	C57BL/6J	SOL testing	14	Male	10	103
		EDL testing	15	Male	12	
	BEH+/+	SOL testing	14	Male	12	
			5	Male	8	
		EDL testing	15	Male	12	
			5	Male	8	
	DUH	SOL testing	15	Male	13	
			4	Male	8	
		EDL testing	16	Male	12	
			4	Male	8	
2	BEH+/+	CON	18	Male	12	45
		FO	18	Male	12	
	BEH	CON	18	Male	12	
		FO	18	Male	9	
3	BEH+/+	CON	18	Male	10	58
		FD	18	Male	10	
	BEH	CON	18	Male	10	
		FD	18	Male	10	
	C57BL/6J	CON	18	Male	9	
		FD	18	Male	9	

Note. SOL – soleus; EDL – extensor digitorum longus; CON – control, which were not subjected to any type of experimental intervention; FO – subjected to the functional overload; FD – subjected to the food deprivation.

2.2. Methods

2.2.1. Muscle dissection and *ex vivo* muscle functional testing

Isolated skeletal muscles were used in the all three studies. All measurements were carried out at room temperature (21–23 °C) except a slightly higher temperature in the first study (23–25 °C). Mice were euthanized either by cervical dislocation or by exposure to CO_2. Cervical dislocation was used in the first and second study while the exposure to CO_2 was applied in the third study.

Following the euthanasia the slow-twitch soleus (SOL) and/or the fast-twitch extensor digitorum longus (EDL) were dissected immediately in the Tyrode solution (121 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.4 mM NaH_2PO_4, 0.1 mM NaEDTA, 24 mM $NaHCO_3$, 5.5 mM glucose) in order to maintain their viability. The muscles were excised with 5–0 silk suture tied securely to the proximal and distal tendons. Following the dissection, muscles were attached in 100 ml Radnotti tissue bath with the Tyrode solution (pH adjusted to 7.4) that was bubbled with a gas mixture consisting of 95 % O_2 and 5 % CO_2. As shown in Fig. 10, the muscles were suspended vertically between two platinum plate electrodes with the proximal tendon attached securely to the lever arm of a dual-mode servomotor (model 300C with 2-cm length arm 300-2B, Aurora Scientific, Canada) and distal tendon to a stable hook. The muscle was then left to equilibrate in Tyrode solution for 10 min.

Muscle testing was performed using the muscle test system (1200A-LR Muscle Test System, Aurora Scientific Inc., Canada) which was controlled by Dynamic Muscle Control v5.3 software (Aurora Scientific Inc., Canada). Data on muscle force were analyzed using Dynamic Muscle Analysis v5.0 software (Aurora Scientific Inc., Canada). Muscles were stimulated by square pulses delivered by two platinum electrodes connected to a high-power custom-made current stimulator. The stimulus amplitude was 25 V which was supramaximal as determined using both twitch and tetanus contractions in a series of pilot experiments. The length of the muscle was increased in steps every 30 s just after delivery of electrical pulse to evoke a twitch contraction. This procedure was continued until twitch force did not increase with the increase in muscle length which took approximately 10–15 min. The muscle was then photographed with the length scale in the background to assess muscle length with a precision of 1 mm (Fig. 10). The muscle was kept at this optimal length (L_0) during the assessment of contractile properties. Firstly,

single twitch was repeated 3 times and peak twitch force (P_t) was measured. The twitch contraction time (CT) was assessed as the time from the beginning of the contraction to the peak of twitch force. Twitch half relaxation time (HRT) was measured as the time taken for force to decline from peak to 50 % of peak value. Afterwards, the muscle was subjected to 300 ms (EDL) or 900 ms (SOL) trains of stimuli (at 25, 50, 75, 100, 150, 200 Hz in the first study and at 20, 50, 80, 100, 150, 200 Hz in the second and third study, respectively) for assessment of peak tetanic force (P_0) from the force-frequency curve. This procedure took ~12 min. In the second study, we have also assessed muscle resistance to damage as described below. Following all measurements the muscle was released from tendons, blotted and weighed (Kern, ABS 80-4, Germany).

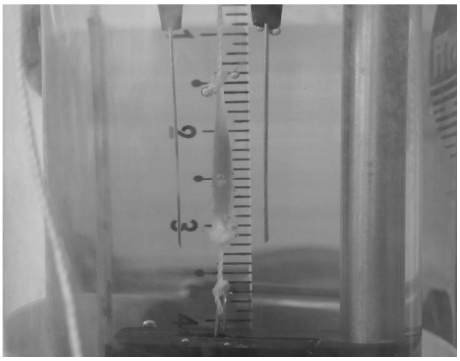

Fig. 10. *Representative image of soleus (SOL) muscle mounted between two platinum electrodes in the bath with Tyrode solution. SOL muscle of BEH+/+ mouse is shown in this particular case*

In the *first study*, SOL or EDL muscle was dissected from the random hindlimb. Specific P_0 (sP_0) was calculated as force (P_0) per physiological cross-sectional area (PCSA) of muscles fibres in mN/mm^2. PCSA was estimated by dividing muscle wet mass by the product of fibre length (L_f) and the density of mammalian skeletal muscle (1.06 g/cm^3), assuming that L_f/L_0 ratio is 0.45 and 0.70 for adult mice EDL and SOL muscles and 0.45 and 0.71 for EDL and SOL muscles of young mice, respectively (Brooks, Faulkner, 1988).

In the *second study* we studied SOL muscles of experimental mice with functionally overloaded SOL muscle and SOL of the control mice which were matched by age to the experimental mice, but were not subjected to any experimental treatment. The muscles were subjected to assessment of force generation capacity followed by the damage test consisting of 20 repeated eccentric contractions performed

every 10 s. Each eccentric contraction was induced by 1100 ms stimulation at a frequency needed to generate peak force (80 or 100 Hz). During the last 200 ms of this stimulation, a ramp stretch was imposed followed by a 200 ms gradual return of the muscle to the initial length without any stimulation. The amplitude of the stretch was 30 % of muscle fibre length which was assumed to be equal to 0.70 of muscle length (Brooks, Faulkner, 1988). The velocity of the stretch was ~2 L_f/s (Brooks et al., 1995). P_0 was measured during the initial 900 ms of contraction. Then these muscles were incubated in 1 mL of Tyrode solution for 1 h at room temperature. 250 μL of Tyrode solution was sampled for assessment of creatine kinase activity using biochemical analyser (Spotchem™ EZ SP-4430, Menarini Diagnostics, Winnersh-Wokingham, UK) with the reagent strips (Arkray Factory, Inc., Japan). Following all measurements muscles were freed from tendons, blotted and weighed.

As L_0 did not differ between the BEH+/+ and BEH mice, sP_0 was calculated simply as P_0 per muscle mass (N/g) as in the previous study with these mice (Amthor et al., 2007).

In the *third study* contractile properties of SOL and EDL muscles were examined in control and food deprivated mice. Both muscles were dissected from the right hindlimb simultaneously (SOL first followed by EDL) and subjected to the muscle force testing one after another. Contractile properties were measured in SOL first while EDL was left incubated in the Tyrode solution which was bubbled with 95 % O_2 and 5 % CO_2 gas mixture. Our pilot experiments showed that contractile properties of EDL were not affected by this 20 min incubation. sP_0 was calculated as in the first study. Muscles of the contralateral (left) hindlimb were also dissected without Tyrode solution to avoid effects of muscle swelling on estimates of muscle mass.

2.2.2. Synergist muscle ablation

Synergist muscle ablation is associated with functional overload (FO) of the remaining synergist muscles which as a result undergo compensatory muscle hypertrophy in mice and other animals (Lowe, Alway, 2002). It might be considered as a mimetic of the resistance training performed by humans.

In the *second study* BEH+/+ and BEH mice were subjected to FO of SOL and plantaris (PL) for 4 weeks starting at the age of 14 weeks. Prior to the synergist ablation procedure, mice were anesthetized using an intraperitoneal injection of

ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1}) cocktail. Then approximately two-thirds of the gastrocnemius muscle (GAS) was removed from a randomly selected leg (Fig. 11). After procedure, the fascia encasing the gastrocnemius and the incision place of the skin were stitched up accurately with 6.0 and 4.0 sutures, respectively. Mice were given buprenorphine postsurgery for pain relief and were monitored on a daily basis. Mice were weighed before the surgical intervention at 14 week and following 4 week period of FO (Kern 440-45N, Germany). Then mice were euthanized and their SOL muscles were subjected to the *ex vivo* muscle function analysis as described previously. The contralateral SOL of these mice served as internal control. Furthermore, other age matched BEH+/+ and BEH mice which were not subjected to any interventions served as independent controls.

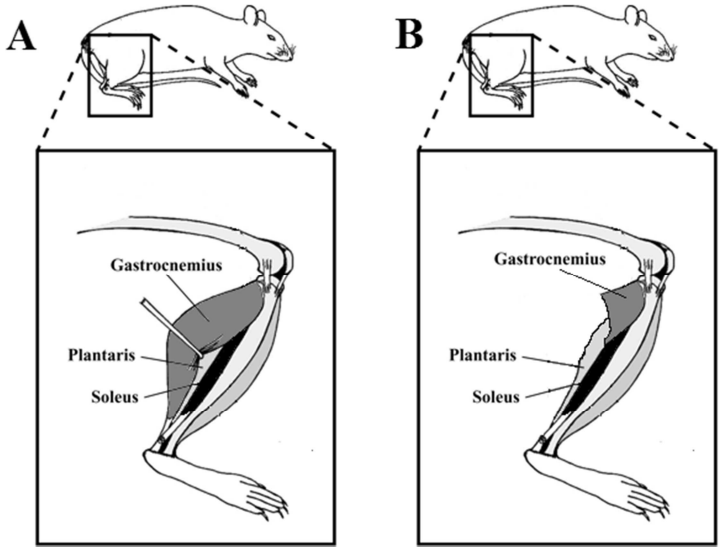

Fig. 11. *Illustration of the synergist muscle ablation procedure in mouse hindlimb. A) Hindlimb muscles before the procedure. B) Hindlimb muscle after the procedure which involved removal of 2/3 of the gastrocnemius (dark grey) muscle. Other synergist muscles, i.e. plantaris (light grey) and soleus (black) undergo the functional overload (FO) as a result of this procedure*

2.2.3. Food deprivation

In our *third study*, BEH+/+, BEH and C57BL/6J mice were subjected to food deprivation (FD) for 48 h using similar methodology as described previously (Allen et al., 2010). In brief, prior to the experiment mice have been fed *ad libitum* using a standard chow diet. On the day of experiment, two mice of the same strain, sex and similar age and body mass were assigned to either a control or FD

intervention. The control group mice were also kept one animal per cage provided with *ad libitum* food access. FD mice did not receive any food for 48 h. The water was provided to both groups *ad libitum*. Mice were weighed at 0 h, 24 h and 48 h following the initiation of the experiment with a precision of 0.1 g (Kern 440-45N, Germany).

At the end of fasting, mice were euthanized by the exposure to CO_2, tissue harvested and skeletal muscle function was assessed. All procedures were carried out and samples were collected in the early stage of the day to minimize diurnal fluctuations in glucocorticoid and/or glucose levels **(**Allen et al., 2010). Procedures were started with the FD mouse at 10:00 and then continued with the control mouse at ~13:00. Immediately following a sacrifice, the blood samples were taken and then heart and skeletal muscles such as GAS, PL, SOL, tibialis anterior (TA), EDL were dissected and weighed with a precision of 0.1 mg (Kern, ABS 80-4, Germany). Surgical dissection of the right hindlimb was done in Tyrode solution because the SOL and EDL muscles of this hindlimb were subjected to the *ex vivo* muscle function testing. The abovementioned muscles from contralateral (left) hindlimb were dissected without Tyrode solution in order to avoid a swelling from the fluid because these muscles were weighed for the examination of muscle atrophy.

2.3. Statistical analysis

All data are presented as means ± standard deviations (SD). All analyses were performed using IBM SPSS v21 and Prism 5.0 software packages for Windows. Before analyzing differences between experimental groups all data were tested for normality using the Shapiro-Wilk test which is more appropriate for small sample sizes (<50 samples) as in our case. For all statistical tests, the level of significance was set *a priori* at $p < 0.05$.

In the *first study,* all variables except CT and HRT presented normal distribution. Differences between strains were assessed by the one factor analysis of variance (one-way ANOVA) test for normally distributed variables. Bonferroni's *post hoc* test was applied. Data that failed normality test were compared with non-parametric Kruskal-Wallis H test. When significance of strain effect was detected, Mann-Whitney U *post hoc* comparison was used to assess the difference between individual strains.

In the *second study,* the two factor analysis of variance (two-way ANOVA) was used with strain (BEH or BEH+/+), treatment (FO or control) as main effects and strain-by-treatment. Bonferroni's *post hoc* test was applied. A repeated measures design was followed in the analyses of muscle mass (two levels; overloaded and contralateral leg) and muscle damage test (twenty levels). A Greenhouse-Geisser correction was applied in muscle damage test data analyses to compensate for the violation of sphericity.

In the *third study,* control and fasted mice were compared with unpaired Student's t-test. The one-way ANOVA with Bonferroni's *post hoc* test was used for comparing strains. HRT variables of SOL muscle in many groups failed normality test therefore they were compared with non-parametric Kruskal-Wallis H test. When significance strain effect was detected, Mann-Whitney U *post hoc* comparison was used to assess the difference between strains.

3. RESULTS AND DISCUSSION

3.1. Study 1. Muscle mass and force generating capacity in skeletal muscles of C57BL/6J, BEH+/+ and DUH mice

3.1.1. Results

Body mass and muscle properties of C57BL/6J, BEH+/+ and DUH mice are presented in Tables 2 and 3. These mouse strains differed ($p < 0.01–0.001$) in all measured parameters. The adult DUH mice had the greatest body and muscle mass ($p < 0.001$) while C57BL/6J mice were the lightest ($p < 0.001$). Young BEH+/+ and DUH mice were selected to match adult C57BL/6J mice by body masst, so effects of body mass on muscle properties could be studied in greater detail. In spite of similar body mass, the rest values such as muscle mass, ratio of muscle to body mass, L_0, L_f and PCSA of SOL and muscle masst, ratio of muscle to body mass, PCSA of EDL were lower in young BEH+/+ and DUH mice compared to the adult C57BL/6J mice ($p < 0.05–0.001$).

Table 2. *Body mass and properties of the soleus muscle (SOL) in C57BL/6J, BEH+/+ and DUH mice*

	Young		Adult		
	BEH+/+	DUH	C57BL/6J	BEH+/+	DUH
BM (g)	$25.0 \pm 1.8^{***}$	$26.9 \pm 2.2^{***}$	26.4 ± 0.6	$52.3 \pm 3.3^{\dagger\dagger\dagger}$	$77.6 \pm 8.1^{\dagger\dagger\dagger\ddagger\ddagger\ddagger}$
MM (mg)	$6.1 \pm 0.6^{***\dagger\dagger\dagger}$	$7.3 \pm 1.0^{***\dagger\dagger}$	10.5 ± 0.7	$13.7 \pm 1.0^{\dagger\dagger\dagger}$	$25.4 \pm 2.9^{\dagger\dagger\dagger\ddagger\ddagger\ddagger}$
MM/BM (%)	$0.024 \pm 0.002^{\dagger\dagger\dagger}$	$0.027 \pm 0.003^{***\dagger\dagger\dagger}$	0.040 ± 0.003	$0.026 \pm 0.002^{\dagger\dagger\dagger}$	$0.033 \pm 0.003^{\dagger\dagger\dagger\ddagger\ddagger\ddagger}$
L_0 (mm)	$11.4 \pm 0.7^{***\dagger\dagger\dagger}$	$10.7 \pm 0.9^{***\dagger\dagger\dagger}$	13.0 ± 0.6	$14.5 \pm 0.5^{\dagger\dagger\dagger}$	$15.1 \pm 0.4^{\dagger\dagger\dagger}$
L_f (mm)	$8.1 \pm 0.5^{***\dagger\dagger\dagger}$	$7.6 \pm 0.7^{***\dagger\dagger\dagger}$	9.1 ± 0.4	$10.1 \pm 0.4^{\dagger\dagger\dagger}$	$10.6 \pm 0.3^{\dagger\dagger\dagger}$
PCSA (mm^2)	$0.71 \pm 0.05^{***\dagger\dagger\dagger}$	$0.90 \pm 0.08^{***\dagger\#}$	1.08 ± 0.09	$1.29 \pm 0.10^{\dagger\dagger}$	$2.27 \pm 0.24^{\dagger\dagger\dagger\ddagger\ddagger\ddagger}$

Note. Values are means ± SD; BM – body mass; MM – muscle mass; L_0 – optimal muscle length; L_f – optimal fibre length; PCSA – physiological muscle cross-sectional area. † – $p < 0.05$, †† – $p < 0.01$, ††† – $p < 0.001$ vs. C57BL/6J; ‡‡‡ – $p < 0.001$ vs. BEH+/+; *** – $p < 0.001$ vs. adults of the same strain; $^{\#}$ – $p < 0.05$ vs. young BEH+/+.

Adult BEH+/+ and DUH mice had a lower ($p < 0.001$) muscle to body mass ratio compared to C57BL/6J mice (Tables 2 and 3). This ratio was also greater ($p < 0.01–0.001$) in adult DUH than BEH+/+ mice. There was no difference between young and adult mice of the same strain in this parameter except for SOL muscle of the DUH strain ($p < 0.001$).

Table 3. *Body mass and properties of the extensor digitorum longus muscle (EDL) in C57BL/6J, BEH+/+ and DUH mice*

	Young		Adult		
	BEH+/+	DUH	C57BL/6J	BEH+/+	DUH
BM (g)	$25.3 \pm 1.7^{***}$	$25.6 \pm 0.9^{***}$	26.2 ± 1.2	$50.1 \pm 3.6^{\dagger\dagger\dagger}$	$76.4 \pm 8.1^{\dagger\dagger\dagger\ddagger\ddagger\ddagger}$
MM (mg)	$8.8 \pm 0.5^{***\dagger\dagger}$	$9.3 \pm 0.7^{***\dagger\dagger}$	11.1 ± 0.7	$15.6 \pm 1.8^{\dagger\dagger\dagger}$	$27.7 \pm 4.7^{\dagger\dagger\dagger\ddagger\ddagger\ddagger}$
MM/BM (%)	$0.035 \pm 0.003^{\dagger\dagger\dagger}$	$0.036 \pm 0.003^{\dagger\dagger\dagger}$	0.042 ± 0.003	$0.031 \pm 0.003^{\dagger\dagger\dagger}$	$0.036 \pm 0.004^{\dagger\dagger\dagger\ddagger}$
L_0 (mm)	$13.6 \pm 0.8^{***}$	$13.4 \pm 0.7^{**}$	13.4 ± 0.5	$14.5 \pm 0.7^{\dagger\dagger}$	$17.3 \pm 0.8^{\dagger\dagger\dagger\ddagger\ddagger\ddagger}$
L_f (mm)	$6.1 \pm 0.3^{***}$	$6.0 \pm 0.3^{**}$	6.0 ± 0.2	$6.5 \pm 0.3^{\dagger\dagger}$	$7.8 \pm 0.4^{\dagger\dagger\dagger\ddagger\ddagger\ddagger}$
PCSA (mm^2)	$1.37 \pm 0.12^{***\dagger\dagger}$	$1.46 \pm 0.14^{***\dagger\dagger}$	1.74 ± 0.13	$2.25 \pm 0.27^{\dagger\dagger}$	$3.38 \pm 0.65^{\dagger\dagger\dagger\ddagger\ddagger\ddagger}$

Note. Values are means ± SD; BM – body mass; MM – muscle mass; L_0 – optimal muscle length; L_f – optimal fibre length; PCSA – physiological muscle cross-sectional area. †† – $p < 0.01$, ††† – $p < 0.001$ vs. C57BL/6J; ‡‡ – $p < 0.01$, ‡‡‡ – $p < 0.001$ vs. BEH+/+; ** – $p < 0.01$, *** – $p < 0.001$ vs. adults of the same strain.

Data on the force generating capacity of SOL and EDL are shown in Figures 12 and 13, respectively. Peak twitch force (P_t) was highly dependent on skeletal muscle mass and strains with a larger muscle mass had larger P_t ($p < 0.001$) (Figs. 12A and 13A). A similar trend was observed in peak tetanic force (P_0) as P_0 of SOL and EDL was greater in strains with heavier muscles ($p < 0.01$–0.001) (Figs. 12B and 13B). However, the difference in P_0 of EDL between adult C57BL/6J and BEH+/+ mice was not significant.

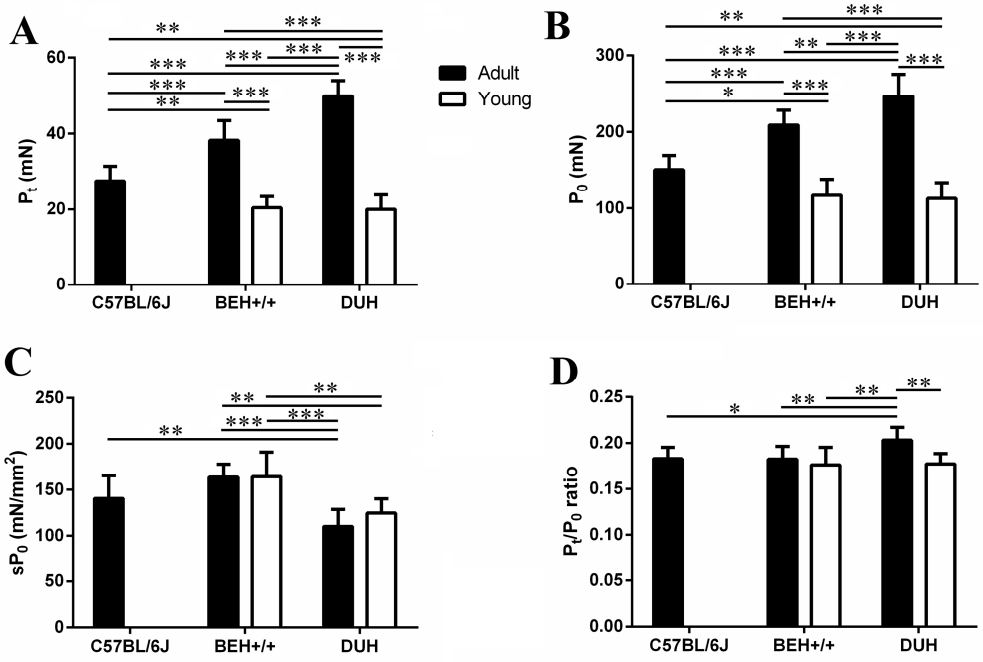

Fig. 12. *Isometric force generation capacity of soleus muscle (SOL) of C57BL/6J, BEH+/+ and DUH mice. Black and empty columns represent adult and young mice, respectively. Figs. A, B, C, D are depicting peak twitch force (Pt), peak tetanic force (P_0), specific tetanic force (sP_0) and ratio of peak twitch and tetanic force (P_t/P_0), respectively. Values are means ± SD, * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$ connected with the horizontal line*

Data on sP_0 are presented in Figs. 12C and 13C. DUH mice had lower sP_0 of both SOL ($p < 0.01$) and EDL ($p<0.05$) than C57BL/6J mice. DUH mice also had lower sP_0 of SOL ($p < 0.001$) than BEH+/+ mice while the difference between these strains in sP_0 of EDL was not significant. There were no differences between the C57BL/6J and BEH+/+ strains in sP_0.

Data on P_t to P_0 ratio (P_t/P_0 ratio) is shown in Figs. 12D and 13D. For SOL, P_t/P_0 ratio was greater in DUH compared to both C57BL/6J ($p < 0.05$) and BEH+/+ ($p < 0.01$) mice. For EDL, BEH+/+ and DUH showed similar P_t/P_0 ratios which were greater ($p < 0.05$) than for C57BL/6J mice.

We have also examined muscles of young BEH+/+ and DUH mice (Figs. 12 and 13, empty bars). P_t and P_0 values of SOL and EDL were highly dependent on the muscle masses of these mice. Young mice had lower ($p < 0.001$) force values than the adult counterparts or body mass-matched C57BL/6J mice ($p < 0.05$–0.001). When P_0 was normalized to PCSA young mice demonstrated the same

force ouput as adult counterparts (Figs. 12C and 13C). As in an adult case, young DUH mice had lower sP_0 compared to the two other strains. In young mice, sP_0 of SOL was lower ($p < 0.01$) in DUH strain compared to BEH+/+ strain. For EDL, young DUH mice had lower ($p < 0.05$) sP_0 than both BEH+/+ and adult C57BL/6J mice. Only DUH mice showed age-related difference ($p < 0.01$) in P_t/P_0 ratio of SOL (Fig. 12D). There were no differences between young mice and body mass-matched C57BL/6J (*F*igs. 12D and 13D).

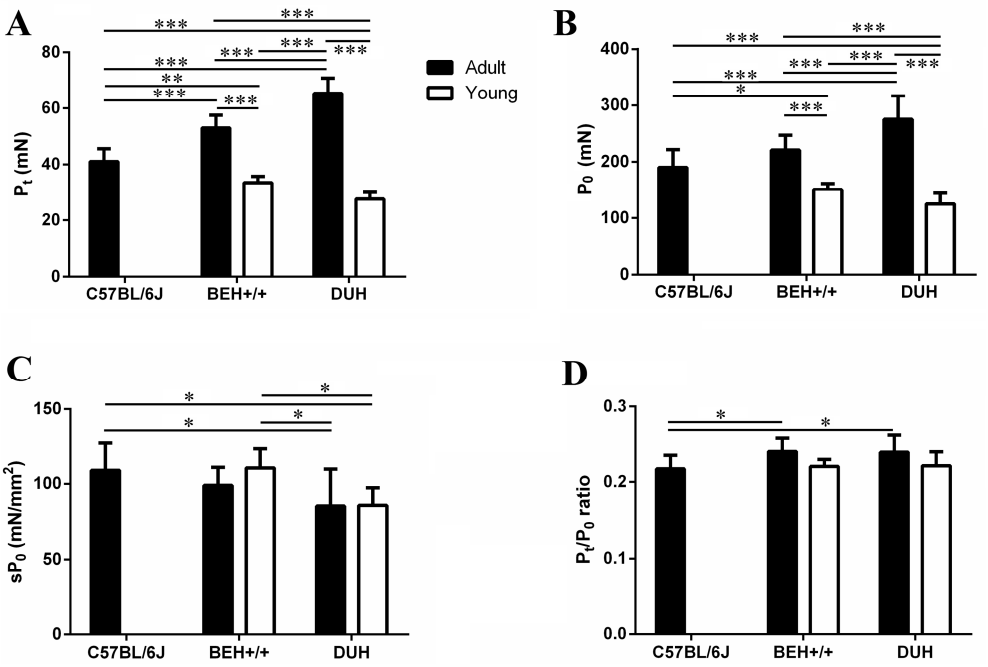

Fig. 13. *Isometric force generation capacity of extensor digitorum longus muscle (EDL) of C57BL/6J, BEH+/+ and DUH strains. Black and empty columns represent adult and young mice, respectively. Figs. A, B, C, D are depicting peak twitch force (P_t), peak tetanic force (P_0), specific tetanic force (sP_0) and ratio of peak twitch and tetanic force (P_t/P_0), respectively. Values are means ± SD, * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$ connected with the horizontal line*

We have also assessed twitch speed, i.e. contraction time (CT) and half relaxation time (HRT) (Fig. 14), which are often linked to muscle fibre composition. For SOL, adult C57BL/6J mice demonstrated a tendency towards shorter CT compared to the other two strains, but the differences were not significant. For EDL, there also were no differences between adult strains in CT. C57BL/6J mice showed a shorter ($p < 0.01$) HRT of SOL than both BEH+/+ and DUH mice

(Fig. 14B). For young mice, results were more complicated. CT of SOL was longer in young DUH compared to the adult DUH ($p < 0.05$), young BEH+/+ ($p < 0.05$) and adult C57BL/6J mice ($p < 0.01$) (Fig. 14A). Young BEH+/+ and DUH showed also a slightly prolonged HRT in both muscles compared to the adult counterparts and C57BL/6J mice. For SOL, HRT was longer in young DUH mice compared to adult BEH+/+ ($p < 0.05$) and C57BL6J ($p < 0.001$) (Fig. 14B). Young DUH mice showed the longest HRT of EDL compared to the adult counterparts ($p < 0.01$), BEH+/+ ($p < 0.01$), young BEH+/+ ($p < 0.05$) and adult C57BL/6J ($p < 0.001$) (Fig. 14D). Young BEH+/+ had longer HRT than C57BL/6J in both SOL ($p < 0.01$) and EDL ($p < 0.05$), respectively.

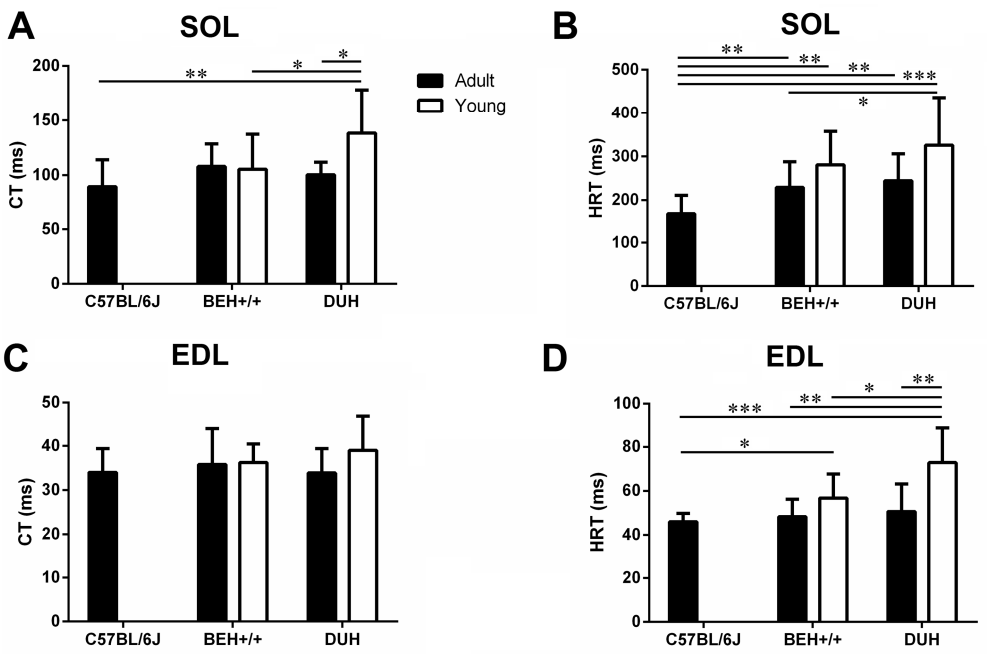

Fig. 14. *Twitch contractile properties of soleus (SOL) and extensor digitorum longus (EDL) muscles of C57BL/6J, BEH+/+ and DUH strains. Black and empty columns represent adult and young mice, respectively. Figs. A and B are depicting contraction time (CT) and half relaxation time (HRT) of soleus muscle obtained during twitch contraction. Figures C and D are depicting these variables of EDL muscle. Values are means ± SD,* * *– $p < 0.05$,* ** *– $p < 0.01$,* *** *– $p < 0.001$ connected with the horizontal line*

3.1.2. Discussion

The main aim of the study was to test the hypothesis that larger body and muscle mass and size are associated with reduction in specific force of skeletal muscles in mice. The results of the study do not agree with this hypothesis. C57BL/6J and BEH+/+ mice did not differ in specific force of soleus (SOL) or extensor digitorum longus (EDL) despite two-fold differences in body mass. Specific muscle force was also similar in young and adult mice which differed several fold in body mass. Interestingly, however, the heaviest DUH mice had lower specific force in both SOL and EDL.

We studied C57BL/6J mice which have similar body mass as many other inbred strains (Reed et al., 2007) and compared this strain with two high growth strains, i.e. BEH+/+ and DUH. The BEH+/+ and DUH strains were generated by selection for muscularity and/or body mass score over more than 30 generations (Bünger et al., 2004). These strains were referred to as strains with high protein amount and thus with large muscular mass (Varga et al., 1997; Bünger et al., 2004). While, extensive muscle hypertrophy of BEH strain is also associated with the *Compact* mutation in the *Mstn* gene which contributes to doubling mass of most muscles (Varga et al., 1997; Amthor et al., 2007), myostatin mutation was eliminated in BEH+/+ as WT allele was introduced instead of *Compact* allele in these mice. This was done to eliminate specific effects of myostatin gene which has been associated with low specific force in mice (Amthor et al., 2007). Interestingly, however, muscle to body mass ratio of BEH+/+ and DUH was lower than in C57BL/6J mice. Thus, enlargement of tissues other than skeletal muscles is probably of major importance in determining large body mass of BEH+/+ and DUH mice.

We did not find any differences in specific force (sP_0) between young and adult mice. We hypothesized that adult mice would show lower sP_0 than young mice. As discussed in the literature review, muscle hypertrophy leads to a decrease in sP_0 if it is associated with a large increase in muscle fibre diameter without increase in attachment area for muscle fibres at the myotendinous junction (Degens, 2012). It is well known that mice undergo a significant increase in muscle mass during maturation. It could be that this is matched by increase in attachment area for muscle fibres so that pennation angles (θ) of muscle fibres do not change significantly in spite of dramatic increase in muscle mass. These assumptions are supported by the data of *in vivo* studies of humans who do not show changes in muscle fibre architecture during maturation (Morse et al., 2008; O'Brien et al.,

2010). Interestingly, prepubertal children and adults have the same specific force of quadriceps (O'Brien et al., 2010). However, specific force of GAS was higher by 21 % in pubescent boys than in adult men (Morse et al., 2008). This was not due to changes in moment arm length, muscle architecture or antagonist coactivation and the authors attributed these findings to possible discrepancies in measuring PCSA between boys and men. In our case, however, there might be genetic differences in muscle architecture between the mouse stains. These genetic effects could explain low sP_0 in DUH mice as muscle fibre θ is an important determinant of muscle force output (Kawakami et al., 1995; Ikegawa et al., 2008).

In addition to body and skeletal muscle mass, muscle fibre-type composition is also determined by genetic factors. A. Lionikas et al. (2013a) showed that mouse strains differ in muscle fibre-type composition. DUH mice had twice as many type I fibres as C57BL/6J (64±11 vs. 31 ± 2 %) in SOL while BEH mice with the homozygous *Compact* allele had 35 ± 2% type I fibres (Lionikas et al., 2013a). A constitutive myostatin KO results in an approximately 20% increase in the relative content of type II fibres at the expense of type I fibres in SOL (Girgenrath et al., 2005). Thus it is likely that BEH+/+ mice with WT myostatin might be intermediate by proportion of type I fibres between C57BL/6J and DUH mice. Twitch speed measurements, especially HRT, suggest that SOL of C57BL/6J is faster than in the other two strains. This might suggest that lower sP_0 in DUH is somehow associated with greater relative content of slow type I fibres which tend to be weaker than type II fibres (Young et al., 1984; Bottinelli et al., 1991; Stienen et al., 1996; Krivickas et al., 2011). However, this explanation can hardly be valid for EDL which contains only negligible quantities of type I fibres.

It is also possible that contractile protein levels are lower in DUH mice compared to BEH+/+ and C57BL/6J mice. The average myonuclear domain (MND) is larger in myostatin-deficient mice which show excessive muscle hypertrophy compared to WT mice (Qaisar et al., 2012). Muscle fibres with large MNDs might not have sufficient levels of transcripts required for adequate expression of contractile proteins and force production (Qaisar et al., 2012). MND is not fixed and can increase in response to growth stimulus (Van der Meer et al., 2011). R. Qaisar et al. (2012) have also suggested that the size of MND has a threshold beyond which muscle fibres are not able to maintain adequate myofibrillar protein levels and number of functioning cross-bridges. According to A. Lionikas et al. (2013a), DUH mice has a larger CSA of both type I and IIA fibres than C57BL/6J

mice in SOL. Furthemore, DUH mice also has a larger CSA of type IIA but not type I fibres than myostatin-deficient BEH which demonstrates lower sP_0 in SOL (see Study 2). As isoforms of type II fibres are in both SOL (IIA) and EDL (IIB, IIX and very few IIA) muscles in considerable amounts they could be as a potential candidate for lower sP_0 due to enlarged MNDs in type II fibres. The drawback of this theory is that it cannot explain why young DUH mice with 3-fold smaller muscle mass (i.e. significantly smaller muscle fibres) and therefore supposedly smaller MNDs compared to adult counterparts have lower sP_0 as well.

One might argue that it is not reliable to compare skeletal muscle function between young mice showing rapid growth and adult mice due to unfinished development processes. We did not find any studies where force generation capacity was compared between young and adult mice of DUH and BEH+/+ strains. However, 2–3 month old C57BL/6J mice did not differ from 9–10 month mice in sP_0 of SOL and EDL (Brooks, Faulkner, 1988). Approximately 2-fold and 3-fold increase in skeletal muscle mass was observed in the BEH+/+ and DUH strains from the age of 4–5 weeks to 15–16 weeks in our study. Several studies show that skeletal muscles of ~1 month old mice are already displaying typical characteristics of mature muscle (Agbulut et al., 2003, Gokhin et al., 2008). During the first days after birth skeletal muscles of mice have lower density of contractile material and show a different fibre type composition compared to adults, but then undergo a rapid development during the several weeks. Following a period of 21 days after birth SOL and EDL muscles display a sequential transition from embryonic to neonatal and eventually to adult myosin heavy chain (MyHC) isoforms though few differences in a proportion of adult MyHC isoforms in muscles was still remaining after 21 days (Agbulut et al., 2003). D. S. Gokhin et al. (2008) demonstrated a robust increase in a myofibrillar packing from 48 to 93 % in mice tibialis anterior (TA) muscle fibres with an accompanying increase to a 6-fold in maximum isometric tension following a period of 28 days postnatal. Collectively, these evidences suggest that muscles of young mice of 28–35 days as in our study should demonstrate force generation capacity which is comparable to muscles of adult mice. Small differences in CT and HRT between muscles of young and adult mice herein might be associated with the remaining differences in composition of MyHC isoforms.

There are also methodological issues to consider when assessing sP_0. Some investigators calculate muscle force relative to a muscle mass while others normalize

force to muscle CSA. The calculations of sP_0 by dividing the absolute force by muscle mass are common in studies where muscle lengths are similar between the animal groups. In our case, however, there were significant differences in muscle length between the strains and age groups. Thus, differences in muscle length had to be accounted for and muscle CSA was used in assessment of sP_0. There are two different methods for assessment of muscle CSA. Some investigators calculate an anatomical CSA (ACSA) while others estimate the physiological CSA (PCSA). It appears that PCSA provides a better reflection of muscle force generating capacity since skeletal muscles differ in the length of muscle fibres as the ratio of fibre to muscle length (L_f/L_0) is 0.70 and 0.45 for SOL and EDL of C57BL/6J mice, respectively (Brooks, Faulkner, 1988). We used these ratios to evaluate a fibre length indirectly for a PCSA calculation in all three strains as we were not able to find any information about fibre to muscle length ratios for BEH+/+ and DUH mice. Thus, an applied ratio if it is inaccurate might result in an underestimation or overestimation of a real PCSA of these strains. However, it is unlikely that there are significant differences in a fibre to muscle length ratio between mouse strains. SOL of ICR mouse strain weighing 40–50 g, similar to BEH+/+ strain, showed L_f/L_0 ratio of 0.71 (Widrick, Barker, 2006; Choi, Widrick, 2009). EDL muscles of WI/HicksCar rats, which are significantly larger animals than mice, showed L_f/L_0 ratio of 0.4 which is also similar to C57BL/6J mice (Carlson, Faulkner, 1998).

Another methodological issue may concern viability of muscles differing in size during an *ex vivo* procedure. As muscles of the DUH strain are much thicker it might be argued that deeper fibres of these isolated muscles are affected by hypoxia. S. S. Segal and J. A. Faulkner (1985) demonstrated that *ex vivo* SOL and EDL (~70–90 mg) of rats show good contractile performance over significant periods of time when incubated as in our experiment using temperatures not exceeding 25 ^{o}C. Moreover, such temperature ensures an adequate O_2 diffusion and similar to fresh muscles glycogen content (Segal, Faulkner, 1985).

In summary, results of the study do not agree with the hypothesis that increase in body mass is associated with reduction in specific force of skeletal muscles in mice. However, the heaviest DUH mice had reduced specific force in both soleus and extensor digitorum longus muscles. It appears that this mouse strain could be an interesting model in studying factors limiting specific muscle force.

3.2. Study 2. The effect of myostatin dysfunction on muscle mass and force in response to functional overload

3.2.1. Results

Data on the body mass of mice is presented in Table 4. BEH mice were heavier ($p < 0.001$) than BEH+/+. Body mass did not change during the period of functional overload (FO) and did not differ between the experimental mice and the aged matched controls. This suggests that FO did not induce any adverse health effects which would be reflected in the mass loss of the animals.

Table 4. *Body mass of experimental BEH+/+ and BEH mice that were subjected to functional overload (FO) of soleus (SOL) muscle and body mass of the aged matched control mice which were not subjected to any interventions*

	Experimental mice		Control mice
	Before FO 14 weeks	After FO 18 weeks	18 weeks
BEH+/+ (g)	54.0 ± 3.3	54.1 ± 3.3	52.0 ± 3.6
BEH (g)	61.0 ± 4.0***	60.4 ± 4.4***	60.2 ± 3.1***

Note. Values are means ± SD. *** – $p < 0.001$ compared to BEH+/+ mice.

Morphometric and physiological SOL properties of control and FO mice of BEH+/+ and BEH mice are presented in Fig. 15. BEH mice had a significantly greater SOL mass compared to BEH+/+ ($p < 0.01$) (Fig. 15A). FO induced a significant ($p < 0.05$) compensatory hypertrophy of SOL in both strains only to a different extent. In BEH+/+ mice SOL mass increased by 46.7 % and 34.7 % compared to aged matched control mice and the contralateral control leg, respectively. Compensatory hypertrophy in BEH mice was inferior to that in BEH+/+. BEH mice showed an increase in SOL mass by 10.7 % and 17.9 % compared to the aged matched control mice and the contralateral control leg, respectively. For both strains, the SOL mass did not differ between the contralateral and the age matched controls. L_0 was the same in control BEH+/+ and BEH mice (Fig. 15B). However, FO induced a significant L_0 increase ($p < 0.05$) in BEH mice, but not in BEH+/+ mice. BEH mice produced a greater ($p < 0.01$) P_0 than BEH+/+ mice (Fig. 15C). This difference in P_0 between the strains disappeared after FO. This was associated with a dramatic reduction (~24.5 %, $p < 0.001$) in P_0 of BEH mice with no change for BEH+/+ mice. BEH+/+ mice had a greater ($p < 0.001$) sP_0 than BEH mice in the control condition (Fig. 15D). FO induced a decrease

($p < 0.001$) in sP_0 for both strains, but sP_0 still was consistantly lower ($p < 0.001$) in BEH than BEH+/+ mice.

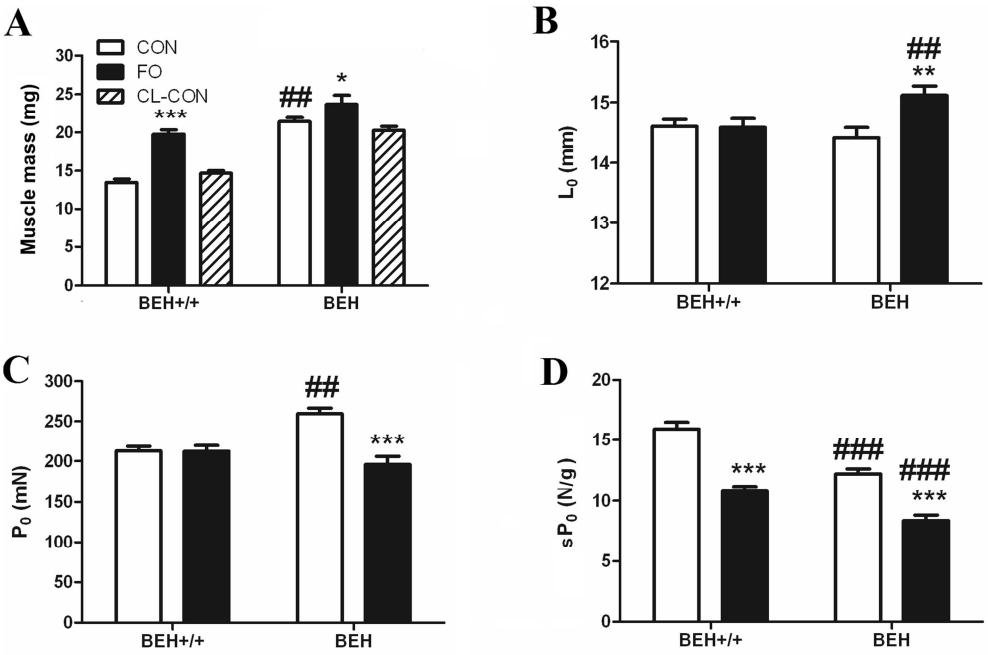

Fig. 15. *Muscle mass (A), optimal length (L_0) (B), peak tetanic force (P_0) (C) and specific tetanic force (sP_0) (D) of soleus muscles (SOL) in the control condition without any intervention (CON), after functional overload (FO) with the respective contralateral controls (CL-CON) where appropriate for BEH+/+ and BEH mice. Values are means ± SD. * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$ between CON and FO muscles; $^{\#\#}$ – $p < 0.01$, $^{\#\#\#}$ – $p < 0.001$ between BEH+/+ and BEH muscles*

Twitch contractile properties are shown in Fig. 16. BEH+/+ mice had longer ($p < 0.001$) CT and HRT than BEH mice. There were no changes in these parameters and differences ($p < 0.001$) in CT and HRT between strains still remained following FO. P_t/P_0 ratio was greater in BEH+/+ compared to BEH in the control condition ($p < 0.001$) and after FO ($p < 0.001$). No changes in P_t/P_0 ratio were observed following FO.

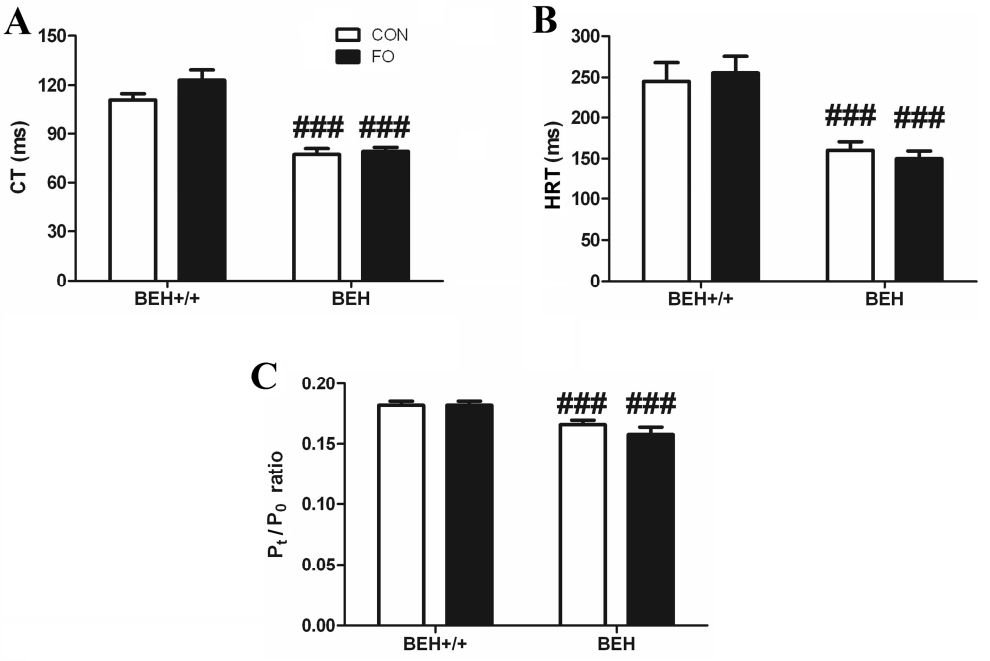

Fig. 16. *Twitch contractile properties of soleus muscle (SOL) in the control condition (CON) and after functional overload (FO) for BEH+/+ and BEH mice, respectively. Fig. A, B and C are depicting contraction time (CT), half relaxation time (HRT) and twitch/tetanic ratio (P_t/P_0), respectively. Values are means ± SD.* $^{###}$ *– $p < 0.001$ between BEH+/+ and BEH muscles*

SOL of both strains was subjected to a bout of 20 repeated eccentric contractions (Fig. 17). After the bout, P_0 drop was greater ($p < 0.001$) in BEH compared to BEH+/+ mice. FO increased ($p < 0.001$) resistance to force loss during the exercise in BEH muscles, but BEH+/+ muscles were less affected. Thus, after FO, there were no differences in P_0 drop between BEH+/+ and BEH during 20 eccentric contractions (24.4 ± 6.1 vs. 25.1 ± 3.6 %). Moreover, after FO a pattern of force decrements during the bout changed in both strains similarly. In control mice force loss was almost linear whereas after FO the greatest decrements occurred during first 5 eccentric contractions followed by stabilization and little change afterwards. There were no differences between BEH and BEH+/+ in muscle creatine kinase efflux after the exercise (Fig. 17B). Muscle creatine kinase efflux was not modulated by the adaptation to FO.

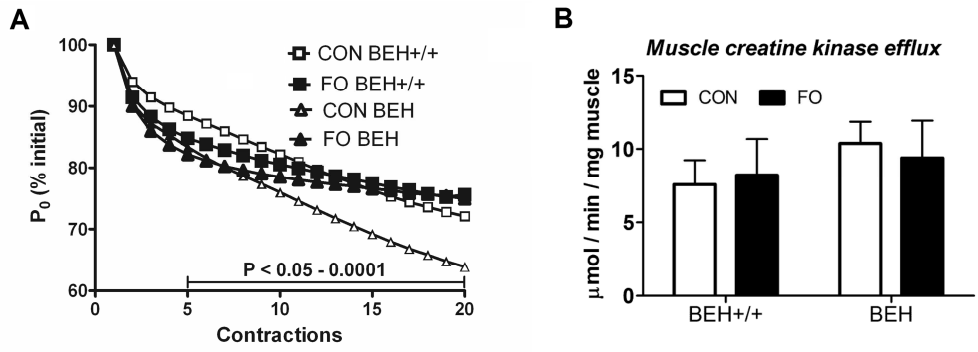

Fig. 17. *Peak tetanic force (P_0) in eccentric exercise (A) and the exercise-induced creatine kinase efflux (B) for soleus muscle in the control (CON) BEH+/+ and BEH mice as well as after 4 weeks of functional overload (FO) for BEH+/+ and BEH mice, respectively. Values are means ± SD. As indicated for the CON, the difference between BEH+/+ and BEH ranged from $p < 0.05$ after 5 contractions to $p < 0.001$ after 20 contractions*

3.2.2. Discussion

The main aim of the second study was to evaluate the adaptation of skeletal muscle to hypertrophic stimulus in mice with myostatin dysfunction. We compared the effects of ablation of the gastrocnemius (GAS) muscle on muscle properties of soleus (SOL) in BEH with myostatin dysfunction and BEH+/+ mice with the intact wild type (WT) myostatin. Our results show that SOL muscle of BEH mice exhibits reduced hypertrophy and significant deterioration of force generation capacity after functional overload (FO). These findings suggest that myostatin dysfunction might impair adaptation of some skeletal muscles to chronic high resistance exercises.

SOL muscle contains a mixture of muscle fibre types (Kilikevicius et al., 2013) and can be excised intact for assessment of contractile properties. Majority of studies on myostatin focused on the muscles dominated by type IIB fibres in mice (Amthor et al., 2007; Matsakas et al., 2010; Matsakas et al., 2012). However, skeletal muscles of humans are rather different as no type IIB fibres are present (Smerdu, Erzen, 2001). Thus, it is important to study effects of myostatin on SOL muscle which is comprised also of type I and IIA fibres and thus resembles more closer skeletal muscles in humans.

BEH mice were heavier than BEH+/+ mice in our study. This might be associated with enlargement of their skeletal muscles. Indeed SOL of BEH mice was ~1.6-fold heavier compared to SOL in BEH+/+ mice. Previous studies have also demonstrated that SOL (predominantly slow-twitch muscle) is ~1.3–1.6-fold

larger in myostatin KO compared to WT mice, whereas differences in other muscles (predominantly fast-twitch) between these mice are even greater (Mendias et al., 2006; Savage, McPherron, 2010; Gentry et al., 2011; Qaisar et al., 2012).

In addition to muscle hypertrophy myostatin-deficient mice show a shift in muscle fibre composition from type I to type II fibres (Amthor et al., 2007; Hennebry et al., 2009; Gentry et al., 2011; Qaisar et al., 2012). Consistently with these findings, BEH mice displayed significantly shorter CT and HRT than BEH+/+ in our study. Previously type II fibres have shown greater enlargement in the CSA compared to type I fibres after resistance training (Verdijk et al., 2009). Thus, our hypothesis was that SOL of BEH mice with myostatin dysfunction will be more responsive to FO and show a greater increase muscle mass after FO compared to BEH+/+ with the intact myostatin function. Contrary to the hypothesis, BEH mice gained significantly less muscle mass than BEH+/+ after FO lasting for 4 weeks.

It is well established that skeletal muscle hypertrophy occurs when a balance between protein synthesis and degradation is positive. Two major signalling pathways, the IGF1-Akt-mTOR and the myostatin-Smad2/3, are positive and negative regulator of muscle mass, respectively (Schiaffino et al., 2013). These pathways are not acting fully independently, but rather are connected intimately. Myostatin acts via ActRIIB receptor on Smad2 and Smad3, inhibitors of Akt, whereas mTOR acts in opposite manner and inhibits Smads (Sartori et al., 2009; Trendelenburg et al., 2009). Therefore, loss of myostatin function is associated with downregulation of Smads and upregulation of Akt and its downstream targets which promote protein synthesis. Indeed C. Lipina et al. (2010) has shown 1.8-fold increase of Akt expression and 3-fold elevation in expression of its downstream targets p70S6 kinase and ribosomal protein S6 in C57BL/6 mice with myostatin KO. BEH strain was generated by selection and crossbreeding of mice characterized by large accretion of carcass proteins (Bünger et al., 2004). Thus, presence of gene variants advantageous for protein synthesis might be greater in BEH compared to C57BL/6 mice. BEH mice represent high growth mice whose equivalent in human population could be individuals with a greater muscularity such as strength athletes. Activation of the Akt/mTOR pathway and its downstream targets is requisitely involved in muscle hypertrophy during FO triggered by synergist muscle ablation (Bodine et al., 2001b). Thus, FO might induce a lower activation of Akt/mTOR signalling in BEH mice with myostatin dysfunction compared to BEH+/+ mice with the intact myostatin due to initial redundant activation of these protein synthesis

pathways at basal level in former mice. This might cause a blunter SOL muscle hypertrophy after FO in BEH mice compared to BEH+/+ mice seen in our study.

Skeletal muscle mass is a major predictor of muscle strength (Jones et al., 2008). Thus, it is not surprising that ~60 % heavier SOL of BEH mice produced larger forces than SOL of BEH+/+ mice. However, force normalized to muscle mass (sP_0) was lower in BEH mice compared to BEH+/+ mice. There is now a significant amount of evidence suggesting that myostatin-deficient mice have a lower sP_0 of skeletal muscles than mice with the intact myostatin. However, such findings were based on the fast-twitch EDL (Mendias et al., 2006, 2011; Amthor et al., 2007; Qaisar et al., 2012), and only a few studies report that myostatin KO mice also show reduction in sP_0 for SOL when compared to WT controls (Gentry et al., 2011; Ploquin et al., 2012). Discrepancies between studies could arise due to difference in the mouse strains (C57BL/6J vs. FVB/N-C57BL), the age of animals and the method for evaluating contractile force generation (*in vivo* vs. *in situ*) used in these studies. In our experiments, we used high growth BEH mice. It appears that mice of different genetic background should be studied before the conclusions can be drawn about effects of myostatin dysfunction on sP_0 of SOL.

It is well known that high resistance exercise training leads to increase in skeletal muscle mass and force generating capacity (Tamaki et al., 1992). Synergist muscle ablation is often used in rodent studies to mimic effects of resistance exercise training (Lowe, Alway, 2002; Bodine, Baar, 2012). Despite muscle hypertrophy, however, P_0 of SOL did not change in BEH+/+ mice and even decreased in BEH mice. Thus sP_0 of SOL decreased in both BEH+/+ and BEH mice. Such findings have been previously reported for rodents after FO (Kandarian, White, 1989). One might argue that compensatory muscle hypertrophy could alter architecture of the muscle. It is possible that hypertrophy of the deep fibres in the muscle will push overlying fibres up and by necessity cause them to curve. This is expected to increase θ of these surface muscle fibres and reduce their forces that are transmited to the aponeurosis and tendon (Degens, 2012). However, this theory cannot fully explain the observed phenomenon because P_0 expressed per fibre CSA was also slightly depressed (3–8 %) in both PL and SOL chemically skinned fibres followed FO (Kandarian, Williams, 1993). In addition, these authors have found altered sensitivity of FO fibre to calcium ions (Ca^{2+}) where leftward shift in the force-pCa curve reflects greater Ca^{2+} sensitivity in hypertrophied fibres suggesting that during tetanic stimulation of whole muscle Ca^{2+} delivery to the contractile apparatus may

be impaired. A positive relationship has been previously pointed out between the rate of calcium ion release and the twitch to tetanus ratio (Celichowski, Grottel, 1993). However, we did not observe any differences in this ratio in SOL muscles following FO. It is worth to note that synergist muscle ablation induces rapid and robust skeletal muscle hypertrophy exceeding the hypertrophy levels observed in humans after resistance training (Lowe, Alway, 2002). Indeed, it is common that skeletal muscles mass of rodents increases by 50–100 % after FO what is not possible in humans following even much longer periods of high resistance exercise (Lowe, Alway, 2002). It can be argued that this model of hypertrophy is not physiological and a machinery of muscle contraction might not be able to accommodate with such degree of hypertrophy. Studies in rats demonstrate that muscle force deficit following FO is not temporarily but rather long-term persisting (Kandarian, White, 1990). These findings suggest that some of the gained muscle mass is not functional. Myonuclei play an important role in protein synthesis by providing the relevant gene transripts. The demand for gene transcripts increases during muscle hypertrophy. Fusion of satellite cells with existing muscle fibres help to increase the number of myonuclei in muscle fibres (Pallafacchina et al., 2013). However, satellite cells itself are not essential for muscle hypertrophy to occur. For instance, mice with ablated satellite cells underwent a significant muscle hypertrophy following FO (Rosenblatt, Perry, 1993; McCarthy et al., 2011). In such a case the cytoplasmic volume supported by individual myonuclei would expand thus increasing the MND. Large MND and low myosin content is probably the major cause of reduced sP_0 in muscle fibres of myostatin-deficient mice (Qaisar et al., 2012). However, studies of compensatory muscle hypertrophy in rodents are showing a robust activation of satellite cells and suggest that FO is a relevant model for studying satellite cell activation (Rosenblatt et al., 1994; Fry et al., 2014). However, activated satellite cells might not produce adequate amounts of contractile protein. Interestingly, after FO, SOL mass and sP_0 of BEH+/+ mice became very similar to BEH before exposure to FO. Thus, a critical threshold of muscle mass from which functional properties of muscle is deteriorating might be overstepped in BEH+/+ muscles after FO.

Particular features of adaptation to FO in the BEH strain was a decrease in P_0 and increase in L_0 which did not change in the BEH+/+ strain. Severe muscle exercise is associated with muscle damage, increased heterogeneity of sarcomere length and increased muscle L_0 (Proske, Morgan, 2001). FO induces muscle damage

especially during the first week of its application (Kandarian, White, 1989). It appears that myostatin dysfunction increases muscle susceptibility to damage after exercise (Mendias et al., 2006; Baltusnikas et al., 2015). Although there were no signs of excessive muscle damage in C57BL/6J mice with myostatin KO after exercise (Savage, McPherron, 2010), there were numerous reports of such effects in myostatin-deficient cattle (Holmes et al., 1973). We used BEH mice which were developed by selection using a muscularity score similar to breeding strategies of "double-muscled" cattle (Bünger et al., 2004). BEH mice have significantly greater body and muscle mass than C57BL/6J mice. Thus, in addition to the *Mstn* gene mutation they might accumulate other variants of genes for muscle hypertrophy. An extreme muscularity together with myostatin dysfunction might make BEH strain more susceptible to muscle damage compared to C57BL/6J strain. Indeed, a bout of eccentric contractions showed that BEH mice are losing more force than BEH+/+. As FO resembles chronic exercise training, it might be excessive for SOL muscle of BEH mice and thus cause severe muscle damage. Interestingly, improvement in resistance to eccentric exercise-induced muscle force loss was observed in BEH mice, but not BEH+/+ mice after FO. The underlying causes of this improvement are unclear. It is important to note that FO induced a decrease in overall muscle force generating capacity. Thus SOL of BEH generated less work after FO compared to the control muscles which were not subjected to FO. Morover, creatine kinase leakage, which is an indirect marker of muscle damage, did not differ between the muscles. Therefore it is unclear whether improved resistance to eccentric exercise-induced muscle force loss of BEH mice after FO is associated with muscle damage or not.

In summary, our results show that soleus of BEH mice experienced smaller rather than greater gain in mass as well as greater deterioration of specific force compared to BEH+/+ mice after functional overload. These findings suggest that myostatin inhibition might impair muscle adaptation to functional overload. Myostatin is impaired across the lifespan in BEH mice. Studies employing a conditional myostatin knockout limited to the period of functional overload are needed to confirm these findings. Moreover, effects of resistance training in conscious animals with myostatin knockout, which provide a more physiological model of hypertrophy, should also be studied for a better understanding of the functional implication of myostatin inhibition.

3.3. Study 3. Effects of myostatin dysfunction and genetic background on muscle mass and force after 48 h food deprivation

3.3.1. Results

Body masses as well as heart and muscle masses of mice are presented in Table 5. BEH mice consistently ($p < 0.001$) showed the greatest masses except for heart which was of similar mass as in BEH+/+ mice. C57BL/6J mice showed the smallest body mass and mass of the tissues ($p < 0.001$).

Table 5. *Body, heart and muscle mass in BEH+/+, BEH and C57BL/6J mice fed chow diet which served as control (CON) and the respective mice which were subjected to 48 h food deprivation (FD)*

Strain	Group	BM 0h (g)	BM 24h (g)	BM 48h (g)	Heart (mg)	GAS (mg)	PL (mg)	SOL (mg)	TA (mg)	EDL (mg)
BEH +/+	CON	49.1 ± 2.9	48.7 ± 3.7	48.5 ± 3.6	193.7 ± 15.5	157.4 ± 9.6	18.0 ± 1.7	10.3 ± 0.7	67.2 ± 3.3	13.0 ± 0.7
	FD	49.0 ± 2.9	42.3 ± 2.5 ***	39.8 ± 2.6 ***	175.4 ± 16.6 *	146.6 ± 9.0 *	17.2 ± 2.0	10.2 ± 0.9	68.2 ± 6.6	12.1 ± 1.1 *
BEH	CON	56.3 ± 1.8 †††	57.0 ± 1.6 †††	56.2 ± 1.6 †††	200.2 ± 14.8	325.0 ± 10.4 †††	44.7 ± 2.6 †††	17.3 ± 0.9 †††	124.8 ± 5.8 †††	27.4 ± 1.1 †††
	FD	56.4 ± 2.9 †††	49.9 ± 2.3 ***†††	46.9 ± 2.3 ***†††	182.5 ± 16.4 *	303.0 ± 10.8 ***†††	40.2 ± 2.8 **†††	16.6 ± 0.9 †††	119.4 ± 7.7 †††	24.8 ± 1.1***†††
C57BL/6J	CON	26.9 ± 1.5 ‡‡‡	26.5 ± 1.3 ‡‡‡	26.5 ± 1.4 ‡‡‡	115.1 ± 5.8 ‡‡‡	122.9 ± 5.2 ‡‡‡	16.3 ± 0.6 ‡‡	8.1 ± 0.6 ‡‡‡	49.5 ± 2.8 ‡‡‡	10.0 ± 0.5 ‡‡‡
	FD	26.9 ± 1.7 ‡‡‡	22.3 ± 1.6 ***‡‡‡	20.3 ± 1.4 ***‡‡‡	105.3 ± 11.0 *‡‡‡	110.6 ± 8.7 **‡‡‡	14.5 ± 1.1 ***‡‡	7.5 ± 0.6 ‡‡‡	44.6 ± 4.0 **‡‡‡	9.1 ± 0.5 **‡‡‡

Note. Values are means ± SD; BM 0h – initial body mass; BM 24h – body mass after 24h of the food deprivation (FD); BM 48h – body mass after 48h of the FD; GAS – gastrocnemius muscle; PL – plantaris muscle; SOL – soleus muscle; TA – tibialis anterior muscle; EDL – extensor digitorum longus muscle. * – $p < 0.05$, ** – $p < 0.01$ and *** – $p < 0.001$ between CON and FD mice, ††† – $p < 0.001$ vs. BEH+/+, ‡‡ – $p < 0.01$ and ‡‡‡ – $p < 0.001$ vs. BEH+/+ and BEH.

Effects of food deprivation (FD) varied between the strains. Body and heart mass decreased in all three strains ($p < 0.001$ and $p < 0.05$, respectively), but loss of muscle mass differed widely between the strains after 48 h of FD. C57BL/6J mice showed a marked ($p < 0.01$–0.001) loss of muscle mass for GAS, PL, TA and EDL. The same tendency was clear for SOL as well. For BEH strain, masses of 3 out of 5 muscles (GAS, PL, EDL) decreased ($p < 0.01$–0.001) while the decrease in masses for SOL and TA was not significant. BEH+/+ mice showed a mass loss only for 2 muscles (GAS and EDL) out of 5 ($p < 0.05$). Changes in masses for PL, TA and SOL were not significant.

The relative values of body mass loss are depicted in Fig. 18. All three

strains showed a largest loss of body mass during the first 24 hours ($p < 0.001$). Subsequent 24 hours induced a further loss but to a lesser extent compared to first 24 hours though still significant ($p < 0.001$). There was no difference in body mass loss between BEH+/+ and BEH mice. C57BL/6J strain showed the greatest body mass loss which was significantly different from BEH and BEH+/+ after 48 h of FD ($p < 0.01$).

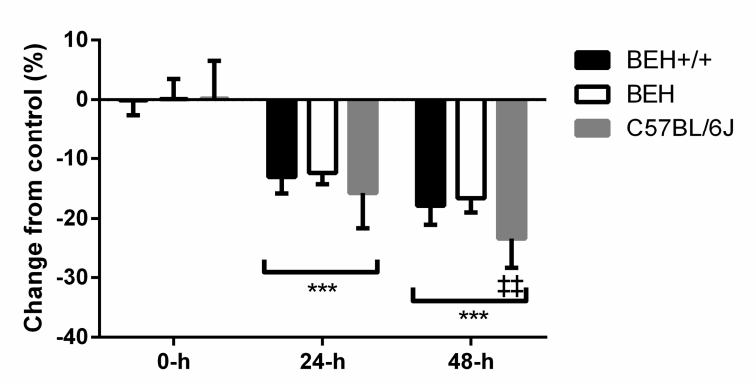

Fig. 18. *Body mass loss (% of control) in BEH+/+, BEH and C57BL/6J mice following food deprivation (FD) for 48 h. 0-h – initial; 24-h – after 24 h of FD; 48-h – after 48 h of FD. Values are means ± SD.* *** *– $p < 0.001$ vs. previous time point (0-h or 24-h),* ‡‡ *– $p < 0.01$ vs. BEH+/+ and BEH*

The relative values of heart and skeletal muscle mass losses are shown in Fig. 19. There was no difference between strains in a relative loss of heart, GAS, PL, SOL and EDL masses despite a trend for greater muscles losses in the C57BL/6J strain after 48 h of FD. However, there was a significant difference in TA mass loss between the C57BL/6J and BEH+/+ strain ($p < 0.05$).

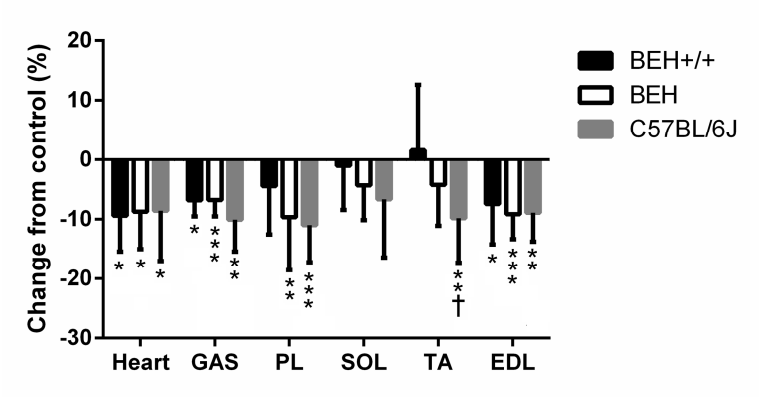

Fig. 19. *Mass loss (% of control) in the heart and skeletal muscles for BEH+/+, BEH and C57BL/6J strains after 48 h food deprivation. GAS– gastrocnemius; PL – plantaris; SOL – soleus; TA – tibialis anterior; EDL – extensor digitorum longus. Values are means ± SD.* * *– $p < 0.05$,* ** *– $p < 0.01$ and* *** *– $p < 0.001$ vs. control,* † *– $p < 0.05$ vs. BEH+/+*

Contractile properties of skeletal muscles with and without 48 h FD are presented in Table 6. P_0 of SOL and EDL were highly dependent on skeletal muscle mass and were greatest for BEH strain ($p < 0.001$) and lowest in C57BL/6J strain ($p < 0.001$). sP_0 was lower in the both muscles of BEH compared to BEH+/+ strain ($p < 0.05$–0.01), but did not differ between the muscles of BEH+/+ and C57BL/6J mice. BEH strain had a faster CT of SOL muscle than BEH+/+ and C57BL/6J mice ($p < 0.05$). There were no difference in CT and HRT of EDL between the BEH+/+ and BEH mice. For EDL muscle, CT was faster in C57BL/6J mice compared to BEH+/+ and BEH mice ($p < 0.001$). C57BL/6J mice also had a faster HRT of EDL compared to the two other strains, but the differences was significant only if compared to BEH+/+ strain ($p < 0.05$).

FD induced muscle-specific alterations in function of the skeletal muscles. Effect of FD on SOL was greater compared to EDL. After FD, P_0 and sP_0 of SOL declined in all three strains ($p < 0.001$). Although there was a fall in P_0 of EDL in all three strains ($p < 0.05$–0.01), sP_0 remained unchanged in BEH and C57BL/6J strains. CT and HRT of SOL became faster in BEH mice following FD ($p < 0.05$ and $p < 0.001$, respectively). CT and HRT showed a tendency to become prolonged in EDL muscle. CT became longer in the BEH ($p < 0.01$) and HRT became longer in the C57BL/6J strain ($p < 0.01$).

Table 6. *Contractile properties of soleus (SOL) and extensor digitorum longus (EDL) muscles in control (CON) mice and mice which were subjected to the 48 h food deprivation (FD). Data are shown for BEH+/+, BEH and C57BL/6J strains*

		SOL				EDL			
Strain	**Group**	**P_0 (mN)**	**sP_0 (mN/mm^2)**	**CT (ms)**	**HRT (ms)**	**P_0 (mN)**	**sP_0 (mN/mm^2)**	**CT (ms)**	**HRT (ms)**
BEH+/+	CON	193.8 ± 20.0	169.9 ± 17.0	90.7 ± 15.1	190.2 ± 72.2	213.7 ± 20.1	105.1 ± 14.1	29.4 ± 2.2	35.7 ± 10.0
	FD	129.8 ± 29.6 ***	116.8 ± 28.6 ***	102.2 ± 27.8	184.9 ± 121.7	185.3 ± 15.4 **	89.3 ± 8.5 *	31.4 ± 1.9 *	31.2 ± 4.0
BEH	CON	256.1 ± 24.4 †††	142.9 ± 15.5 ††	75.6 ± 5.6 †	196.9 ± 25.0	321.0 ± 26.0 †††	86.5 ± 10.8 †	28.4 ± 1.2	29.2 ± 3.4
	FD	155.9 ± 17.0 ***	89.0 ± 12.8 ***†	64.7 ± 10.2*†††	102.7 ± 30.7 ***†	283.5 ± 33.7 *†††	82.5 ± 10.3	30.1 ± 0.8 **	30.8 ± 3.9
C57BL/6J	CON	155.4 ± 17.1 ###	162.3 ± 27.5‡‡	81.5 ± 10.9	151.2 ± 28.9 ‡‡	172.2 ± 27.4 ###	100.6 ± 20.3	26.7 ± 1.7 ††	25.7 ± 5.0 †
	FD	86.2 ± 23.2***###	99.4 ± 27.9 ***	88.3 ± 15.8 ‡	187.4 ± 62.7 ‡	142.6 ± 10.8 *###	91.2 ± 6.1	28.0 ± 1.9 ††‡	33.0 ± 2.5 **

Note. Values are means ± SD; P_0 – peak tetanic force; sP_0 – specific tetanic force; CT – twitch contraction time; HRT – twitch half relaxation time. * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$ between CON and FD mice, † – $p < 0.05$, †† – $p < 0.01$, ††† – $p < 0.001$ vs. BEH+/+, ‡ – $p < 0.05$, ‡‡ – $p < 0.01$ vs. BEH, $^{\#\#\#}$ – $p < 0.001$ vs. BEH+/+ and BEH.

The relative changes in peak tetanic (P_0) and specific tetanic (sP_0) force of skeletal muscles following 48 h FD are shown in Fig. 20. Both P_0 and sP_0 of SOL declined in all three strains ($p < 0.05$–0.001) and there were no differences in relative declines between the strains. P_0 but not sP_0 of EDL declined in all three strains ($p < 0.001$). There were no differences in relative changes of EDL between the strains.

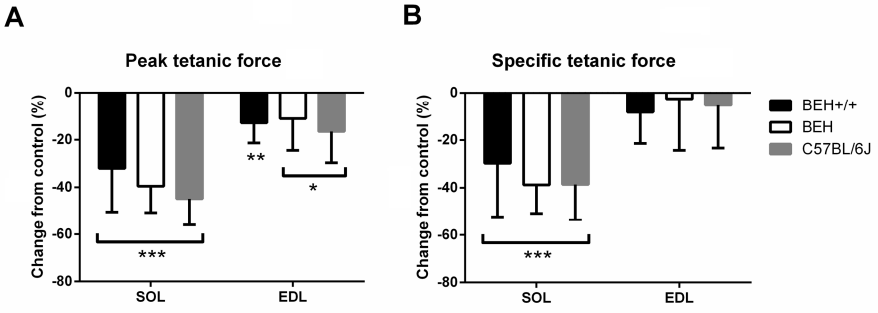

Fig. 20. *Changes (% of control) in peak tetanic (A) and specific tetanic force (B) for soleus (SOL) and extensor digitorum longus (EDL) muscles in BEH+/+, BEH and C57BL/6J strains after 48 h of food deprivation. Values are means ± SD.* * *– $p < 0.05$,* ** *– $p < 0.01$,* *** *– $p < 0.001$ vs. control*

3.3.2. Disscusion

The main aim of the third study was to evaluate effects of myostatin dysfunction and genetic background on muscle wasting after food deprivation (FD). We studied BEH and BEH+/+ mice with and without myostatin dysfunction, respectively, as well as C57BL/6J mice as independent control which was used to evaluate effects of genetic background. The main findings of the study were as follows. Firstly, loss of muscle mass tended to be greater in BEH compared to BEH+/+. Thus myostatin dysfunction does not protect against muscle wasting during FD. Secondly, body mass loss as well as muscle wasting tended to be greater in C57BL/6J mice than BEH+/+ suggesting that genetic background is of importance in muscle wasting under these conditions. Thirdly, muscle wasting varied between skeletal muscles. This suggests that specific functional requirements posed on skeletal muscles might be of importance for muscle wasting during FD. Fourthly, impact on force generation capacity of FD differed between slow-twitch and fast-twitch muscles irrespective of mouse strain. Thus, fibre type specific alterations might affect muscle function during FD.

We studied the effects of 48 h FD as previously on myostatin dysfunction in C57BL/6J mice (Allen et al., 2010). In general, myostatin KO is associated with improved functioning of PI3-kinase/Akt/mTOR signalling pathway which promotes protein synthesis in skeletal muscles after exercise, as well as in response to IGF1 and leucine elevations (Lipina et al., 2010; Schiaffino et al., 2013). It appears that the same pathway is involved in control of proteasome and autophagic lysosome pathways which are responsible for protein breakdown (Glass, 2003; Schiaffino et al., 2013). Thus it is logical to assume that myostatin dysfunction should protect muscles from wasting under various catabolic conditions, including FD. Our findings, however, reject this hypothesis. If anything, BEH mice tended to be more susceptible to FD-induced muscle wasting than BEH+/+ mice. Despite similar body masst loss, muscles of BEH mice were affected more than those of BEH+/+ mice. This was reflected in the fact that 3 out of 5 muscles lost their mass in BEH mice compared to 2 out of 5 muscles showing mass loss in BEH+/+ mice. The rest 2 muscles of BEH also tended to show larger decrements although not significantly. In contrast, D. L. Allen et al. (2010) showed that TA but not SOL of myostatin KO mice is more resistant to muscle wasting after 48 h FD compared to WT mice. In agreement with our study, however, H. Collins-Hooper et al. (2015) found that GAS, SOL, TA and EDL muscles of myostatin KO mice were more prone to

wasting after 24 h FD compared to WT mice. It is unclear why studies of D. L. Allen et al. (2010) and H. Collins-Hooper et al. (2015) generated contradicting results. The experimental procedures were similar and both studies used C57BL/6 mice. It might be that duration of FD (48 vs. 24 h) could have been of importance. Our results are consistant with H. Collins-Hooper et al. (2015) though we used BEH mice and different duration of FD (48 vs. 24 h). Discrepancies in several skeletal muscles such as SOL and TA between their and our study might be influenced by distinct genetic backgrounds of mice. Interestingly, a study employing 40 % food restriction for 5 weeks also showed that myostatin KO mice were more prone to skeletal muscle wasting compared the WT controls (Matsakas et al., 2013).

It is important to identify physiological mechanisms which are responsible for this increased rate of muscle wasting in mice with myostatin dysfunction compared to WT mice. There is a need to maintain blood glucose levels for normal brain and other tissue function during starvation. This is probably reflected in a decrease of physical activity and body temperature during FD (Swoap, 2008). During FD, energy substrates are mobilized from adipose tissue and other tissues, including skeletal muscles, to provide a continued source of nutrients for gluconeogenesis in liver (Allen et al., 2010). As myostatin-deficient mice have reduced adipose tissue mass (McPherron, Lee, 2002), amino acids from their skeletal muscles might be used for energy to a greater extent than in WT during FD. Indeed, myostatin KO mice have reduced rate of lipid oxidation in skeletal muscles, higher insulin sensitivity and higher glucose uptake compared with WT mice in the fed state (Guo et al., 2009). Moreover, the respiratory exchange ratio was elevated in myostatin-deficient mice suggesting an increased carbohydrate utilization, reduced lipid utilization, or both (Guo et al., 2009). A systemic administration of Follistatin288, which acts as myostatin inhibitor, increases muscle mass, reduces fat accumulation and pushes metabolism towards glycolysis (Gangopadhyay, 2013). Skeletal muscle is known to be a tissue of high energy demand (Baker et al., 2010). This implies that FD might induce a greater impact on individuals with larger skeletal muscle mass whose resting metabolic rate (RMR) might be greater. However, myostatin KO mice do not show greater RMR than WT counterparts when RMR is normalized to lean mass or to total body mass (McPherron, Lee, 2002; Guo et al., 2009). Thus, the evidence suggests that increased lean body mass rather than increased metabolic rate might be of major importance for greater FD-induced muscle wasting in myostatin-deficient mice

compared to WT controls.

Our results suggest that genetic background might also play a significant role in muscle wasting as C57BL/6J mice showed greater muscle wasting compared to BEH+/+ and BEH strains. The C57BL/6J mice are approximately 2-fold lighter than BEH+/+ mice. We hypothesize that C57BL/6J mice might have higher RMR than BEH+/+ mice which could explain a greater loss of body mass and muscle atrophy in C57BL/6J mice compared to BEH+/+ mice. RMR does show a high variability among inbred mice strains even with similar body mass (Konarzewski, Diamond, 1995). RMR correlates well with the size of metabolically active organs such as heart, kidney, liver and small intestine, and strains with exceptionally high (or low) RMR tended to have disproportionately large (or small) organs. Liver size was the most significant morphological trait linked to differences in RMR (Selman et al., 2001). We only measured the mass of heart which was relatively heavier in C57BL/6J strain compared to the BEH+/+ or BEH strain. Interestingly, C57BL/6J mice had also a greater relative muscle mass compared to BEH+/+. Thus, relatively greater metabolically active body mass could be mainly responsible for greater susceptibility of C57BL/6J mice to FD-induced muscle atrophy compared to BEH+/+ mice.

There was a muscle-specific response to FD. SOL with a greater percentage of type I fibres (Burkhodler et al., 1994) showed greater resistance to atrophy compared to other muscles in all three mouse strains. There is evidence that 48 h FD increases mRNA levels of ubiquitin ligases MuRF-1 and atrogin-1 in the faster contracting TA, but not in the slower contracting SOL (Allen et al., 2010). This may be due to the lower sensitivity of SOL compared to TA to glucocorticoid signalling which plays a major role in FD-induced muscle atrophy (Livingstone et al., 1981; Wing, Goldberg, 1993). Myostatin is involved in various types of muscle wasting conditions as its gene expression or protein levels increase (Schiaffino et al., 2013). SOL muscle might be less affected by myostatin alteration due to the lower amount of ActRIIB receptors which mediate myostatin signalling (Mendias et al., 2006). However, greater muscle wasting in BEH mice with myostatin dysfunction suggests that myostatin is not a key player to induce muscle loss during FD.

Our results show that effects of FD on muscle function also differ between SOL and EDL muscles. Despite no change in mass, SOL exhibited 32–45 % drop in P_0 after FD in all three strains. This translated into 30–40 % reduction in sP_0. In contrast to SOL, EDL exhibited only 11–16 % drop in P_0 and no significant change

in sP_0 since EDL mass was also reduced by 7–9 % after FD. We were not able to find any other study confirming our results. In rats, SOL has a higher protein turnover rate than EDL with a protein half-life of 7–8 days, almost two times faster that of EDL (Lewis et al., 1984). Thus, a renewal of muscle proteins, especially those critical to a generation of muscle contractions in slow-twitch type I fibres, might be impaired more in SOL compared to EDL during FD. It should be noted that following muscle length adjustments and single twitches in early stage of L_0 assessment, basal tension of SOL was decreasing more after FD compared to the control condition (unpublished observation). Inability to maintain a basal tension suggests that muscle stiffness might be altered after FD. Muscle stiffness depends also on the number of strongly attached cross-bridges (Seow et al., 1997). Decreased stiffness with a concomitant loss of myosin was measured in fibres of myostatin-deficient mice experiencing reduced sP_0 (Qaisar et al., 2012). In rats 48 h FD did not affect myosin heavy-chain (MyHC) composition in either the SOL or EDL (Mizunoya et al., 2013). As possible interspecies differences exist, similar studies with mice would provide us with more comprehensive understanding about changes of skeletal muscle contractile proteins in response to FD.

There is evidence showing that FD and/or food restriction restores sP_0 of EDL in myostatin KO mice of C57BL/6J background (Matsakas et al., 2013; Collins-Hooper et al., 2015). Endurance exercise training also increased sP_0 of EDL in these mice (Matsakas et al., 2012). Both FD and endurance training induced a loss of muscle mass in the myostatin KO mice and this was associated with the improved contractile function. This reduction in size of muscle fibres caused an increase in the nuclear-to-cytoplasmic volume ratio such that myonuclear domain (MND) approached values of WT mice (Matsakas et al., 2012). It might be that this change helped to improve contractile function of EDL in myostatin KO mice. It is believed that increased MND is associated with decreased sP_0 in muscle fibres of myostatin KO mice (Qaisar et al., 2012). Interestingly, sP_0 of EDL did not improve in BEH mice with myostatin dysfunction after FD. It is likely that restored EDL force generation in myostatin KO mice of C57BL/6J background in abovementioned studies is due to a greater muscle mass decrease toward WT phenotype after FD and caloric restriction. Muscle mass of myostatin KO mice of this background was only ~30 % greater or even similar to WT counterparts after FD or caloric restriction (Matsakas et al., 2013; Collins-Hooper et al., 2015). We did not observe such dramatic muscle mass decrements in the BEH background.

Following FD the mass of EDL was still near 2-fold greater in the BEH than BEH+/+ strain with WT myostatin.

In summary, our results show that myostatin dysfunction does not provide protection from food deprivation-induced skeletal muscle atrophy. In BEH mice myostatin is dysfunctional across the lifespan and animals display an overt phenotype prior to food deprivation. Therefore, studies employing a model of the conditional myostatin knockout limited to the period of food deprivation are needed to confirm these findings. It is also important to investigate how myostatin function is modulated by genetic background, especially in view of genetic heterogeneity of human population.

CONCLUSIONS

Study 1:

1. Skeletal muscles of mouse strains vary significantly in muscle mass and force generation capacity. Specific muscle force, however, does not change with increase in body size and as a result of muscle growth during maturation.
2. DUH background shows lower specific muscle force compared to both BEH+/+ and C57BL/6J backgrounds.

Study 2:

1. Myostatin dysfunction is associated with increased muscle mass, but reduced specific muscle force.
2. Myostatin dysfunction is also associated with reduction in hypertrophy and impairment in force generation capacity of soleus muscle after functional overload.

Study 3:

1. Skeletal muscle atrophy after food deprivation is dependant on myostatin dysfunction, genetic background and muscle properties. Myostatin dysfunction is associated with a greater loss of muscle mass. C57BL/6J background is more susceptible to muscle atrophy than BEH+/+ background under these conditions.
2. After food deprivation, the slower contracting soleus shows less severe muscle atrophy, but greater loss of specific muscle force compared to a faster contracting extensor digitorum longus.

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PUBLICATIONS

The thesis is based on the following articles:

1. Minderis, Petras; Kilikevičius, Audrius; Baltušnikas, Juozas; Alhindi, Yosra; Venckūnas, Tomas; Bünger, Lutz; Lionikas, Arimantas; Ratkevičius, Aivaras. Myostatin dysfunction is associated with reduction in overload induced hypertrophy of soleus muscle in mice. *Scandinavian Journal of Medicine & Science in Sports*, 2016, 26 (8), 894–901. doi: 10.1111/sms.12532. [Impact Factor: 3.025; ISI Journal Citation Reports © Ranking: 2015: 11/82 (Sport Sciences)].
2. Minderis, Petras; Fokin, Andrej; Ratkevičius, Aivaras. High growth dummerstorf mice have reduced specific force of slow and fast twitch skeletal muscle. *Baltic Journal of Sport & Health Sciences*, 2016, 2 (101), p. 44–52.
3. Minderis, Petras; Libnickienė, Indrė; Ratkevičius, Aivaras. Muscle wasting after 48 hours of food deprivation differs between mouse strains and is promoted by myostatin dysfunction. *Baltic Journal of Sport & Health Sciences,* 2016, 2 (101), p. 53–60.

Other publications:

1. Minderis, Petras; Baltušnikas, Juozas; Venckūnas, Tomas; Kilikevičius, Audrius; Lionikas, Arimantas; Ratkevičius, Aivaras. Repeated eccentric contractions induce a progressive increase in creatine kinase leak from the isolated soleus muscle. *Current Issues and New Ideas in Sport Science: 5th Baltic Sport Science Conference: Abstracts,* Kaunas, 18–19 April 2012. ISBN 9786098040708, p. 145.
2. Minderis, Petras; Venckūnas, Tomas; Lionikas, Arimantas; Ratkevičius, Aivaras. Reduced activity of citrate synthase has a limited effect on muscle function in mice. *Physiology 2014: Proceedings of the Physiological Society*, London, UK, 30 June – 2 July 2014 / The Physiological Society. 2014, vol. 31, PCB136, p. 277P–278P.
3. Baltušnikas, Juozas; Venckūnas, Tomas; Kilikevičius, Audrius; Minderis, Petras; Fokin, Andrej; Lionikas, Arimantas; Ratkevičius, Aivaras. Creatine

kinase efflux from isolated mouse soleus after contractile activity depends on age, gender and contraction type. *Acta Kinesiologiae Universitatis Tartuensis= 7th Conference of Baltic Society of Sport Sciences: Abstracts,* May 7–9, 2014, Tartu, Estonia. ISSN 1406-9822 2014, vol. 20 (Supplement), p. 65.

4. Kvedaras, Mindaugas; Minderis, Petras; Venckūnas, Tomas; Ratkevičius, Aivaras; Lionikas, Arimantas. A mouse model for revealing endurance. *Current issues and new ideas in sport science: 9th conference of the Baltic sport science society: Abstracts*, Kaunas, 27–29 April 2016. ISBN 9786098040968, p. 155.