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Perlecan deficiency causes muscle hypertrophy, a decrease in myostatin expression, and changes in muscle fiber composition

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Abstract

Perlecan is a component of the basement membrane that surrounds skeletal muscle. The aim of the present study is to identify the role of perlecan in skeletal muscle hypertrophy and myostatin signaling, with and without mechanical stress, using a mouse model (Hspg2^{-/-}-Tg) deficient in skeletal muscle perlecan. We found that myosin heavy chain (MHC) type IIb fibers in the tibialis anterior (TA) muscle of Hspg2^{-/-}-Tg mice had a significantly increased fiber cross-sectional area (CSA) compared to control (WT-Tg) mice. Hspg2^{-/-}-Tg mice also had an increased number of type IIx fibers in the TA muscle. Myostatin and its type I receptor (ALK4) expression was substantially decreased in the Hspg2^{-/-}-Tg TA muscle. Myostatin-induced Smad activation was also reduced in a culture of myotubes from the Hspg2^{-/-}-Tg muscle, suggesting that myostatin expression and its signaling were decreased in the Hspg2^{-/-}-Tg muscle. To examine the effects of mechanical overload or unload on fast and slow muscles in Hspg2^{-/-}-Tg mice, we performed tenotomy of the plantaris (fast) muscle and the soleus (slow) muscle. Mechanical overload on the plantaris muscle of Hspg2^{-/-}-Tg mice significantly increased wet weights compared to those of control mice, and unloaded plantaris muscles of Hspg2^{-/-}-Tg mice caused less decrease in wet weights compared to those of control mice. The decrease in myostatin expression was significantly profound in the overloaded plantaris muscle of Hspg2^{-/-}-Tg mice, compared with that of control mice. In contrast, overloading the soleus muscle caused no changes in either type of muscle. These results suggest that perlecan is critical for maintaining fast muscle mass and fiber composition, and for regulating myostatin signaling.

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Keywords

Perlecan; Muscle hypertrophy; Mechanical stress; Myostatin

1. Introduction

Skeletal muscle is the body's most abundant tissue. It functions not only in locomotion, but also acts as the body's main store of carbohydrates and protein. Therefore, its proper maintenance and repair are essential. Skeletal muscle is a dynamic tissue with a remarkable ability to maintain and regenerate in response to environmental stimuli that induce loading or injury. During these responses, for example after exercise, expression of local growth factors such as myostatin (also called GDF-8), transforming growth factor (TGF)- β , and insulin growth factor (IGF)-I are enhanced (Kjaer et al., 2006; Machida and Booth, 2004). Skeletal muscle can be classified into two broad fiber types, based on the type of myosin: slow twitch type I, and fast twitch type II fibers. Type I fiber is rich in mitochondria and myoglobin and can carry more oxygen and sustain aerobic activity over a long time period. Type II fibers are more efficient for short bursts of speed and power and use both oxidative metabolism and anaerobic metabolism, depending on the particular sub-type. Type II fibers have three major subtypes: Type IIa, IIx and IIb. Type IIa is aerobic, and rich in mitochondria, similar to a slow fiber. Type IIb is the major fast muscle in rodents. Type IIx is intermediate between Type IIa and Type IIb.

Myostatin is a highly conserved TGF- β superfamily member. It is a potent inhibitor of muscle growth and its deficiency dramatically increases muscle mass in diverse species (Anderson et al., 2008; Joulia-Ekaza and Cabello, 2006). Myostatin is produced primarily in skeletal muscle cells, circulates in the blood, and acts on muscle tissue. It is synthesized as a precursor protein that remains inactive until it is modified by several post-translational events (Hill et al., 2002; Lee and McPherron, 2001; McPherron et al., 1997; Thies et al., 2001). In serum, premature myostatin is found predominantly as a latent complex with its prodomain. In contrast, in skeletal muscle, uncleaved pro-myostatin predominates and is located in part in the extracellular space (Anderson et al., 2008) (supplemental Figure 2). Subsequent proteolytic cleavage generates an active dimer of myostatin (25 kDa) (supplemental Figure 2), which then binds to the activin type II receptor (ActRIIB), which in turn recruits and phosphorylates ALK4 or ALK5 (type I receptor) (Joulia-Ekaza and Cabello, 2006; Rebbapragada et al., 2003). Phosphorylated ALK4 or ALK5 induces signaling cascades through Smad-dependent and Smad-independent pathways (Massague and Chen, 2000; Tsuchida et al., 2008). In the Smad-dependent pathway, the ActRIIB/ALK4 or ActRIIB/ALK5-receptor complex activates Smad2/3, which binds Smad4 and induces expression of target genes. Myostatin is preferentially expressed in fast-type fibers; consequently, inactivating mutations are associated essentially with fast-twitch fiber hyperplasia (Carlson et al., 1999). Loss of myostatin causes a shift in fiber type from slow to fast. Two different types of muscular hypertrophy are recognized: size increase and number increase. Myostatin affects the fast muscle of both types of muscular hypertrophy induced by mechanical stress (e.g. exercise and loading).

Basement membranes consist of thin extracellular matrices and are present in most tissues (Martin and Timpl, 1987). They surround skeletal muscle fibers and provide scaffolding for the tissue. Genetic studies of muscular dystrophy in humans and mice have demonstrated that basement membranes, in addition to providing physical support, are critical for muscle function (Campbell and Stull, 2003; Sanes, 2003). Basement membranes are also essential for muscle development and regeneration (Arikawa-Hirasawa et al., 2002b; Campbell and Stull, 2003; Sanes, 2003).

Perlecan, a heparan sulfate proteoglycan (HSPG), is a component of basement membranes in many tissues, including skeletal muscle, but is also found in cartilage, which lacks basement membranes (Martin and Timpl, 1987; Noonan et al., 1991; Iozzo et al., 1994; SundarRaj, 1995; Iozzo et al., 1997; Handler et al., 1997). Perlecan interacts with extracellular molecules, growth factors, and cell surface receptors, and is implicated in many biological functions in tissue development, homeostasis, and diseases. Perlecan deficiency causes perinatal lethal chondrodysplasia in mice and in humans (Arikawa-Hirasawa et al., 1999; Arikawa-Hirasawa et al., 2001; Costell et al., 1999). Mutations in perlecan have been identified in patients with Schwartz-Jampel syndrome (SJS), a non-lethal condition characterized by myotonia and mild chondrodysplasia (Arikawa-Hirasawa et al., 2002a; Nicole et al., 2000; Stum et al., 2006). We found that perlecan is essential to the functioning of neuromuscular junctions (Arikawa-Hirasawa et al., 2002b). A hypomorphic mutation generated with one missense substitution, corresponding to a human familial SJS mutation, showed a similar myotonia phenotype in a knockin mouse (Echaniz-Laguna et al., 2009; Stum et al., 2008). These results indicated that perlecan is important in muscle function. However, the role of perlecan in homeostasis of adult skeletal muscle is unclear.

In the present study, we examined skeletal muscle hypertrophy, and myostatin expression and its signaling pathway in perinatal-lethality rescued perlecan gene knockout mice ($Hspg2^{-/-}$; Col2a1- $Hspg2^{Tg/-}$, hereinafter, $Hspg2^{-/-}$ -Tg mice). These mice lack perlecan in skeletal muscle, but are rescued by expressing recombinant perlecan specifically in cartilage. We also examined the role of perlecan in skeletal muscle in response to mechanical stress created by tenotomy of co-operative muscle. Our results suggest that perlecan plays an important role in homeostasis of skeletal muscle and in the maintenance of muscle fiber types in response to unloading stress.

2. Results

2.1. Fiber cross-sectional area (CSA) and fiber type composition in tibialis anterior (TA) muscle of WT-Tg and $Hspg2^{-/-}$ -Tg mice

To study the role of perlecan in homeostasis of adult skeletal muscle, we rescued the perinatal-lethality of perlecan gene knockout ($Hspg2^{-/-}$) mice by expressing a full-length perlecan cDNA transgene specifically in cartilage under the control of the chondrocyte-specific Col2a1 promoter/enhancer (Col2a1- $Hspg2$) (Tsumaki et al., 1999) in $Hspg2^{-/-}$ mice. The rescued mice will hereafter be called $Hspg2^{-/-}$ -Tg mice. About half of the $Hspg2^{-/-}$ -Tg mice died around embryonic day (E) 10 of hemorrhage due to defective myocardium basement membranes, similar to what was previously observed in $Hspg2^{-/-}$ mice (Arikawa-Hirasawa et al., 1999; Costell et al., 1999). This was expected since the perlecan transgene was not expressed in basement membranes and did not rescue the myocardial defect (data not shown). Some of the perlecan null ($Hspg2^{-/-}$) mice developed exencephaly, wherein the brain is located outside of the skull, due to defects in the skull bones and embryonic calvarial cartilage (Arikawa-Hirasawa et al., 1999; Costell et al., 1999; Holmbeck et al., 2003). The perinatal-lethality rescued $Hspg2^{-/-}$ -Tg mice showed normal cephalic development.

We first examined perlecan expression in tissues of WT-Tg and $Hspg2^{-/-}$ -Tg mice. Immunostaining showed that perlecan was present in basement membranes surrounding the gastrocnemius muscle fibers of WT-Tg ($Hspg2^{+/+}$; Col2a1- $Hspg2^{Tg/-}$) mice (Fig. 1a). In $Hspg2^{-/-}$ -Tg mice, perlecan was absent in skeletal muscle, kidney, heart, and liver (Fig. 1a, supplemental Figure 1b), whereas it was expressed in those tissues in WT-Tg mice (supplemental Figure 1b). The recombinant perlecan was expressed in cartilage of $Hspg2^{-/-}$ -Tg mice (supplemental Figure 1a). Perlecan deficiency in muscle caused nonspecific myopathic changes, such as internal nuclei in muscle (Fig. 1a) similar to SJS muscle. Based on MHC isoform expression, muscle fibers have been classified as: i) slow type I, ii)

intermediate type IIa, iii) fast type IIb, and iv) fast type IIx. Myostatin is expressed preferentially in type IIb fibers (Carlson et al., 1999) and affects fast type fibers. We examined tibialis anterior (TA), a large fast type muscle, to identify both the morphological and molecular biochemical roles of perlecan in WT-Tg and Hspg2^{-/-}-Tg mice. Since the fiber cross-sectional area (CSA) of muscles of Hspg2^{-/-}-Tg mice appeared to be larger than those of WT-Tg mice, we analyzed the CSA and composition in the TA muscle for each MHC fiber type by immunohistochemical staining for MHC types. Figure 1b shows a representative immunostaining image for MHC types in cross-sections of gastrocnemius (composed of both fast and slow muscle) and TA (fast) muscle of WT-Tg and Hspg2^{-/-}-Tg mice: MHC I (red), IIa (blue), and IIb (green). Unlabeled fibers (black) are type IIx fibers. Figure 1c shows cross-sections of the TA muscle immunostained for MHC in WT-Tg and Hspg2^{-/-}-Tg mice. Fiber CSA analysis showed a 25% increase in type IIb MHC fibers in Hspg2^{-/-}-Tg TA muscle, compared to WT-Tg muscle, whereas there were no significant differences in fiber CSA for type IIa and type IIc MHC fibers ($P < 0.03$, Fig. 1d). Based on the tissue sections used for the immunohistochemical analyses, there was a 19% decrease in type IIb MHC fibers, and a 68% increase in type IIx MHC fibers, in the TA muscle of Hspg2^{-/-}-Tg mice, compared to WT-Tg mice (Fig. 1e). A change in type IIa MHC fibers in WT-Tg and Hspg2^{-/-}-Tg TA muscle was not statistically significant (Fig. 1e). These results indicate that perlecan deficiency resulted in hypertrophy of the TA muscle and changes in its fiber composition.

2.2. Expression of myostatin and its receptors decreases in perlecan-deficient muscle

Because myostatin is a negative regulator of muscle growth and differentiation, our next step was to examine myostatin protein in Hspg2^{-/-}-Tg muscle. Pro-myostatin was significantly reduced in the TA muscle of Hspg2^{-/-}-Tg mice compared to WT-Tg mice (Fig. 2a, b). Myostatin prodomain and mature myostatin were both significantly decreased in serum from Hspg2^{-/-}-Tg mice, compared to serum from WT-Tg mice (Fig. 2c, d, e, f). Next, we examined myostatin mRNA expression by quantitative RT-PCR. We found 61% less myostatin mRNA in the TA muscle of Hspg2^{-/-}-Tg mice than in that of WT-Tg mice (Fig. 3a). These results suggest that the decreased myostatin expression may cause hypertrophy of the TA muscle in Hspg2^{-/-}-Tg mice. The active myostatin dimer binds to the activin type II receptor (ActRIIB), which then recruits and activates the type I receptor (ALK4 or ALK5) by transphosphorylation. We analyzed the expression of myostatin receptors by qRT-PCR and found a 46% decrease in ALK4 in the TA muscle of Hspg2^{-/-}-Tg mice compared to WT-Tg mice. No statistically significant change was noted in the expression of ALK5 (major TGF- β type I receptor) (Franzen et al., 1993) (Fig. 3b, c). ActRIIB mRNA and Type II receptor for TGF- β (T β RII) mRNA levels in the TA muscle of Hspg2^{-/-}-Tg mice also did not change statistically compared with those in WT-Tg mice (Fig. 3d,e). Because myostatin regulates cell growth and cell death in concert with IGF-I (Yang et. Al., 2007), we examined IGF-I and its receptor (IGF-IR) mRNA expression by quantitative RT-PCR. We found 37% more IGF-I mRNA in the TA muscle of Hspg2^{-/-}-Tg mice than in those of WT-Tg mice (Fig. 3f). We also analyzed the expression of IGF-IR mRNA level by qRT-PCR and found no statistically significant change compared with that of WT-Tg mice (Fig. 3g).

2.3. Myostatin-induced Smad signaling is decreased in primary myotubes from perlecan-deficient muscle

Smad signaling downstream of myostatin was analyzed in a culture of myotubes from WT-Tg and Hspg2^{-/-}-Tg mice. Myostatin activated Smad2 signaling in myotubes from WT-Tg mice, but myostatin failed to activate Smad2 in myotubes from Hspg2^{-/-}-Tg mice (Fig. 4), consistent with the reduced expression of myostatin receptor ALK4 shown in Fig. 3b. TGF- β induced phosphorylation of Smad2 in myotubes from WT-Tg mice (Fig. 4). Smad activation by TGF- β in myotubes from Hspg2^{-/-}-Tg mice was only slightly reduced compared to that observed

in myotubes from WT-Tg mice (Fig. 4). This result was consistent with the expression levels of ALK4 and ALK5 in myotubes from Hspg2^{-/-}-Tg mice (Fig. 3).

2.4. Changes in CSA and fiber type composition after selective mechanical overloading and unloading of plantaris or soleus muscle by tenotomy

Mechanical stress is critical for muscle homeostasis. In rodents, the soleus muscle is composed of slow muscle and the plantaris muscle is composed of fast muscle. To analyze the role of perlecan in mechanical overload or unload on fast or slow type muscle, we performed two types of tenotomy in the distal tendons of left hindlimbs of WT-Tg and Hspg2^{-/-}-Tg mice (supplemental Fig. 3). First, we examined the effects of overloading on the plantaris muscle and of unloading the soleus muscle. For this, the gastrocnemius muscle (consisting of both slow and fast muscle) and the soleus muscle were tenotomized, but the plantaris muscle remained intact in WT-Tg and Hspg2^{-/-}-Tg mice (gastrocnemius and soleus muscles tenotomized, G/S-Ten). At 14 days post-surgery, wet weights of the plantaris and soleus muscles were compared with those of control sham operated, non-tenotomized muscles of the right hindlimbs of WT-Tg and Hspg2^{-/-}-Tg mice (Fig. 5a). The wet weight of the overloaded plantaris muscle in WT-Tg- and Hspg2^{-/-}-Tg-G/S-Ten mice was 43% and 68% larger, respectively, than in sham operated WT-Tg-Con and Hspg2^{-/-}-Tg-Con (without tenotomy) mice ($p < 0.02$, $p < 0.01$, respectively) (Fig. 5a). The wet weight of the unloaded soleus muscle in the G/S-Ten of WT-Tg and Hspg2^{-/-}-Tg mice was reduced equally compared to that of sham operated control WT-Tg-Con and Hspg2^{-/-}-Tg-Con mice ($p < 0.01$) (Fig. 5a). These results indicate that mechanical overloading leads to hypertrophy in perlecan-deficient plantaris muscle compared to control muscle. Under the unloading condition, the absence of perlecan does not affect the level of atrophy of the soleus muscle.

Next, we examined the effects of overloading on the soleus muscle and of unloading the plantaris muscle by tenotomizing gastrocnemius and plantaris muscles but not the soleus muscle (gastrocnemius and plantaris muscles tenotomized, G/P-Ten) (supplemental Fig. 3). The wet weight of the overloaded soleus muscle in WT-Tg- and Hspg2^{-/-}-Tg-G/P-Ten mice was larger, but not significantly (~1% increase), than each of the control hindlimbs (Fig. 5b). The wet weight of the unloaded plantaris muscle in WT-Tg- and Hspg2^{-/-}-Tg-G/P-Ten mice was 39% and 27% lower, respectively, than in WT-Tg-Con and Hspg2^{-/-}-Tg-Con mice ($p < 0.01$) (Fig. 5b). These results indicate that mechanical overloading did not lead to hypertrophy of the soleus muscle, regardless of the presence or absence of perlecan. Thus, perlecan deficiency might be advantageous for the plantaris muscle to sustain its volume under the unloading condition.

We further analyzed the CSA and fiber type composition of the plantaris muscle overloaded by the G/S-Ten operation (Fig. 6a, b). Compared to WT-Tg-Con and Hspg2^{-/-}-Tg-Con, overloading resulted in a 44% and 69% increase in the CSA of type IIa MHC fibers of the plantaris muscle in WT-Tg-G/S-Ten and Hspg2^{-/-}-Tg-G/S-Ten mice, respectively (Fig. 6a). Similarly, the CSA of type IIb fibers increased in both WT-Tg- and Hspg2^{-/-}-Tg-G/S-Ten mice by 9% and 19%, respectively, compared to WT-Tg-Con and Hspg2^{-/-}-Tg-Con (Fig. 6a). When compared to WT-Tg and Hspg2^{-/-}-Tg mice, loading increased the CSA of type IIa and IIb fibers in Hspg2^{-/-}-Tg plantaris muscle more effectively than it did in WT-Tg muscle (Fig. 6a). Overloading also increased the CSA of type IIx MHC fibers in the plantaris muscle of WT-Tg-G/S-Ten and Hspg2^{-/-}-Tg-G/S-Ten mice by 15% and 30%, respectively, compared to WT-Tg-Con and Hspg2^{-/-}-Tg-Con. However, no significant changes were seen in type I MHC fibers (slow muscle). Thus, the CSA of all fast fiber types of the plantaris muscle was significantly increased significantly in Hspg2^{-/-}-Tg mice compared to that of WT-Tg mice under the overloading condition.

We found that the percentage of type IIa MHC fibers in the plantaris muscle in both WT-Tg- and Hspg2^{-/-}-Tg-G/S-Ten groups increased by 17%, compared to that of WT-Tg-Con and Hspg2^{-/-}-Tg-Con (Fig. 6b). Overloading decreased the percentage of type IIx MHC fibers of Hspg2^{-/-}-Tg mouse plantaris muscle but not WT-Tg mouse plantaris muscle (Fig. 6b). In contrast to type IIx MHC fibers, the percentage of type IIb MHC fibers decreased in Hspg2^{-/-}-Tg mice compared to WT-Tg mice. Overloading decreased the percentage of type IIb MHC fibers in the plantaris muscle of both WT-Tg- and Hspg2^{-/-}-Tg-G/S-Ten groups by 14% and 9%, respectively, compared to WT-Tg-Con and Hspg2^{-/-}-Tg-Con (Fig. 6b). These results indicate that overloading increases the CSA of fast muscle fibers and changes the fiber type composition in perlecan-deficient plantaris muscle.

2.5. Change in myostatin expression after tenotomy

Since myostatin regulates muscle hypertrophy under mechanical stress, we examined myostatin expression in skeletal muscle in overloading and unloading conditions created by tenotomy in WT-Tg and Hspg2^{-/-}-Tg mice. The myostatin mRNA level in the plantaris muscle of Hspg2^{-/-}-Tg mice decreased by 27%, compared to WT-Tg mice (Fig. 7a). G/S-Ten operation resulted in 41% and 75% decreases in the myostatin mRNA level in the overloaded plantaris muscle of WT-Tg- and Hspg2^{-/-}-Tg-G/S-Ten mice, compared to that in WT-Tg-Con and Hspg2^{-/-}-Tg-Con mice (Fig. 7b). The decrease in myostatin expression was significantly greater in the overloaded plantaris muscle of Hspg2^{-/-}-Tg mice than in that of WT-Tg mice ($P < 0.001$). G/P-Ten operation resulted in 27% and 22% increases in the myostatin mRNA level of the unloaded plantaris muscle in both WT-Tg- and Hspg2^{-/-}-Tg-G/P-Ten groups (Fig. 7c). Since the soleus muscle does not express myostatin, we did not examine myostatin expression in overloaded or unloaded soleus muscles.

3. Discussion

The perinatal-lethality rescued Hspg2^{-/-}-Tg mice lived to adulthood and showed no obvious defects in locomotor activity when we conducted tail suspension and rotarod tests (data not shown). Hspg2^{-/-}-Tg mice showed developed myotonia (unpublished data). However, unlike in perlecan morphants of zebrafish (Zoeller et al., 2008), we did not observe severe defects in the orientation of actin filaments or sarcomeres in the muscles of our mutant mice. Perlecan deficiency in skeletal muscle resulted in hypertrophy and decreased myostatin expression in fast muscles, such as the TA and plantaris muscles. These results suggest that perlecan serves to maintain fast type muscle mass and fiber type compositions. Mechanical overload on perlecan-deficient plantaris muscles induced by selective tenotomy further increased hypertrophy and promoted changes in fiber type compositions compared to the control plantaris muscle. Thus, perlecan deficiency appeared to increase muscle sensitivity to mechanical stress. However, no effect of mechanical overload was observed on the soleus muscle. Perlecan in basement membranes may buffer mechanical stress and regulate signaling pathways to maintain muscular function.

Myostatin is preferentially expressed in fast-type fibers, and inactivating mutations are associated essentially with fast-twitch fiber hyperplasia (Carlson et al., 1999). Loss of myostatin causes a shift in fiber type from slow to fast. We found that myostatin expression was decreased in the TA muscle and serum of Hspg2^{-/-}-Tg mice and that overloading significantly increased plantaris muscle wet weight. Myostatin expression decreased in overloaded plantaris muscles in both Hspg2^{-/-}-Tg and WT-Tg mice, and its expression increased in unloaded plantaris muscles. Thus, perlecan deficiency in muscles apparently causes a phenotype similar to that seen in myostatin gene knockout mice.

One explanation for the reduced myostatin expression in perlecan-deficient muscle may be that the myotonic phenotype of Hspg2^{-/-}-Tg mice affects muscle biosynthesis and reduces

myostatin production. However, myostatin expression in tenomized plantaris muscles was also decreased, suggesting that not only myotonia but also an additional mechanism is involved in the decrease in myostatin expression. Furthermore, Smad2 activation by myostatin treatment in perlecan-deficient myotubes was significantly lower compared to that in control myotubes from WT-Tg mice. These results suggest that the reduced myostatin signaling does not arise simply from the reduced myostatin expression. We found that the expression of myostatin receptor ALK4 was reduced in perlecan-deficient TA muscle. We also observed decreased levels of the active form of myostatin in Hspg2^{-/-}-Tg mice, suggesting that the maturation process of myostatin may also be affected by perlecan-deficiency. We also observed increased mRNA expression of IGF-I, another positive regulator of muscle growth, in Hspg2^{-/-}-Tg mice. Since myostatin inhibits IGF-I signaling in part through regulation of Akt (Yang et al., 2007; JBC 282:6 3799–3808, 2007), the decrease in myostatin may increase the expression of IGF-I and muscle hypertrophy in Hspg2^{-/-}-Tg mice. Therefore, multiple defects in cellular processes are likely to cause muscle hypertrophy in Hspg2^{-/-}-Tg mice.

In summary, our findings suggest that perlecan serves to maintain plantaris muscle mass and composition and to regulate myostatin expression. Perlecan might also act as part of the sensory complex in skeletal muscle that responds to mechanical stress.

4. Materials and Methods

4.1. Animals

Perlecan null (Hspg2^{-/-}) mice die perinatally due to premature cartilage development (Arikawa-Hirasawa et al., 1999; Costell et al., 1999). To restore cartilage abnormalities, we created a perlecan transgenic mouse line (WT-Tg, Hspg2^{+/+}; Col2a1-Hspg2^{Tg/-}) that expressed recombinant perlecan in cartilage, using a cartilage-specific Col2a1 promoter/enhancer (Tsumaki et al., 1999). We subsequently created lethality-rescued mice (Hspg2^{-/-}-Tg, Hspg2^{-/-}; Col2a1-Hspg2^{Tg/-}) by mating the transgenic mice with heterozygous Hspg2^{+/-} mice (paper in preparation). We maintained these mice in a mixed genetic background of C57BL/6 and 129SvJ. In the present study, we used 30 male adult mice (15 weeks old, 22–30 g), separated into six groups (five per group): three groups of WT-Tg mice and three groups of littermate Hspg2^{-/-}-Tg mice. All experimental procedures were performed following the guidelines for the care and use of animals at the Juntendo University Medical School, Japan.

4.2. Surgical procedures and tissue preparation

Two procedures were used: (1) In each group of WT-Tg and Hspg2^{-/-}-Tg mice, the right hindlimbs were used as controls (Con) and the left hindlimbs had the distal tendons of gastrocnemius and soleus muscles tenotomized (G/S-Ten); (2) In each group of WT-Tg and Hspg2^{-/-}-Tg mice, the right hindlimbs were used as controls (Con) and the left hindlimbs had the distal tendons of gastrocnemius and plantaris muscles tenotomized (G/P-Ten). Fourteen days after surgery, the hindlimb muscles were harvested, wet weighed, pinned on a cork, and immediately frozen in isopentane cooled with liquid nitrogen. The muscles were stored at -80°C for subsequent experiments. Surgery, casting, and tissue removal were performed under anesthesia (10% 2.5g/50mg nembutal).

4.3. Indirect immunofluorescence (Immunohistochemistry)

Immunofluorescent staining for type I, type IIa, type IIb, and type IIx MHC fibers was performed with antibodies against respective MHCs as described previously (Handschin et al., 2007) with some modifications. Briefly, frozen sections were cut in a cryostat on microscope slides. The sections were fixed in acetone for 10 min at 4°C and preblocked in 5% goat serum/PBS for 20 min at 37°C, followed by incubation with MHC-slow antibody (1:20; Novocastra, Newcastle upon Tyne, UK) for 1 h at 37°C. The sections then were washed in PBS, incubated

with Alexa Fluor 546 goat anti-mouse IgG (1:200; Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature, and washed again in PBS. The sections then were fixed in acetone for 5 min at 4°C, preblocked in 20% goat serum/PBS for 20 min at 37°C, and incubated with A4.74 antibody (1:10; Alexis Biochemicals, San Diego, CA, USA) overnight at 4°C. The sections were washed in PBS, incubated with Alexa Fluor 647 goat anti-mouse IgG (1:300; Invitrogen) for 1 h at room temperature, washed in PBS, and then incubated with BF-F3 antibody (1:5; ATCC, Rockville, MD, USA) overnight at 4°C. The sections then were washed in PBS before being incubated with biotinylated horse anti-mouse IgM (1:100; Vector, Burlingame, CA, USA) for 1 h at room temperature, washed in PBS, and finally incubated with streptavidin Alexa Fluor 488 (1:400; Molecular Probes, Carlsbad, CA, USA) conjugate for 1 h at room temperature. After washing, images were captured under a confocal laser microscope (Carl Zeiss LSM510 instrument). The mean of each fiber CSA and the composition of different MHC fiber types were quantified by counting five sections of each muscle bed (type I, red; type IIa, blue; type IIx, black; and type IIb, green) using Image-Pro Plus Version 6.0 software (MediaCybernetics, Bethesda, MD, USA). To immunostain perlecan in tissues, frozen sections were cut in a cryostat on microscope slides. The sections were fixed in acetone for 10 min at 4°C and preblocked in 5% goat serum/2% BSA/PBS for 20 min at 37°C, followed by incubation with perlecan antibody (1:200; Chemicon, Temecula, CA) for 1 h at 37°C. The sections were then washed in PBS, incubated with Alexa Fluor 488 goat anti-rat IgG (1:400; Molecular Probes, Carlsbad, CA, USA) for 45 min at room temperature, and mounted after being washed in PBS.

4.4. Detection of myostatin in skeletal muscle and serum of mice

Various forms of myostatin were detected as described previously (Anderson et al., 2008). Tibialis anterior muscle samples from WT-Tg or Hspg2^{-/-}-Tg mice were lysed in modified RIPA buffer (150 mM NaCl, 50 mM Tris, 25 mM β -glycerophosphate, 100 mM NaF, 2 mM Na₃VO₄, 10 mM sodium pyrophosphate, 2× Complete EDTA-free protease inhibitor mixture (Roche), 1 mM PMSF plus 2 mM EDTA, 1% nonidet P-40 (NP-40), 0.1% SDS, and 0.25% sodium deoxycholate). The samples were homogenized using a Polytron tissue homogenizer, lysates were centrifuged, and supernatants were collected. Concanavalin A (ConA) precipitation from the muscle samples and serum was performed using the Glycoprotein Isolation Kit, ConA (Pierce). The protein concentration was measured with a BCA kit (Pierce). Various forms of myostatin from the precipitants were analyzed by immunoblotting.

4.5. Cell culture and myostatin treatment

Primary myoblast cells were differentiated from satellite cells derived from the gastrocnemius muscles of 8–12-week-old WT-Tg and Hspg2^{-/-}-Tg mice, as described previously (Hashimoto et al., 2004). To prepare the myotubes, myoblast cells at 90% confluence were treated with DMEM (Invitrogen) supplemented with 2% horse serum (Invitrogen) and 0.05% chick embryo extract (MP Biomedicals) for five days. Myotubes were stimulated with 20 ng/ml TGF- β 1 (WAKO) or 10 ng/ml myostatin (R&D Systems) for 30 minutes, and were homogenized in lysis buffer (Cell Signaling) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM sodium orthovanadate (Na₃VO₄), and 2 mM sodium fluoride (NaF). After centrifugation, the supernatant was added to NuPAGE LDS sample buffer (Invitrogen) containing 50 mM dithiothreitol (DTT). The samples were analyzed by immunoblotting using antibodies against phospho-smad2 (Ser465/467) (1:1000; Cell Signaling) and smad2/3 antibodies (1:1000; Cell Signaling).

4.6. Quantitative RT-PCR

Total RNA was isolated from skeletal muscles using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was generated from 1.5 μ g of total RNA with MMLV

reverse transcriptase and oligo (dT) primer. Quantitative real-time PCR (qRT-PCR) was done with the ABI Prism® 7500 Fast Sequence Detection System (Applied Biosystems). Primers used are shown in Supplemental Table 1.

4.7. Statistical analysis

All values are presented as means $\pm$ SE. Differences in mean values were evaluated with a two-sided Student's *t*-test, with significance accepted at $P < 0.05$. For all experiments, five mice/group were used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Con	control
CSA	cross-sectional area
G/P-Ten	gastrocnemius and plantaris muscles tenotomized
G/S-Ten	gastrocnemius and soleus muscles tenotomized
MHC	myosin heavy chain
Hspg2 ^{-/-} -Tg	knockout perlecan alleles, containing Col2a1-perlecan transgene (Hspg2 ^{-/-} ; Col2a1-Hspg2 ^{Tg/-})
SJS	Schwartz-Jampel syndrome
TA	tibialis anterior
WT-Tg	wild type of perlecan alleles, containing Col2a1-perlecan transgene (Hspg2 ^{+/+} ; Col2a1-Hspg2 ^{Tg/-})

References

- Anderson SB, Goldberg AL, Whitman M. Identification of a novel pool of extracellular pro-myostatin in skeletal muscle. *J Biol Chem* 2008;283:7027–35. [PubMed: 18175804]
- Arikawa-Hirasawa E, Le AH, Nishino I, Nonaka I, Ho NC, Francomano CA, Govindraj P, Hassell JR, Devaney JM, Spranger J, Stevenson RE, Iannaccone S, Dalakas MC, Yamada Y. Structural and functional mutations of the perlecan gene cause Schwartz-Jampel syndrome, with myotonic myopathy and chondrodysplasia. *Am J Hum Genet* 2002a;70:1368–75. [PubMed: 11941538]
- Arikawa-Hirasawa E, Rossi SG, Rotundo RL, Yamada Y. Absence of acetylcholinesterase at the neuromuscular junctions of perlecan-null mice. *Nat Neurosci* 2002b;5:119–23. [PubMed: 11802174]
- Arikawa-Hirasawa E, Watanabe H, Takami H, Hassell JR, Yamada Y. Perlecan is essential for cartilage and cephalic development. *Nat Genet* 1999;23:354–8. [PubMed: 10545953]
- Arikawa-Hirasawa E, Wilcox WR, Yamada Y. Dyssegmental dysplasia, Silverman-Handmaker type: unexpected role of perlecan in cartilage development. *Am J Med Genet* 2001;106:254–7. [PubMed: 11891676]

- Campbell KP, Stull JT. Skeletal muscle basement membrane-sarcolemma-cytoskeleton interaction minireview series. *J Biol Chem* 2003;278:12599–600. [PubMed: 12556456]
- Carlson CJ, Booth FW, Gordon SE. Skeletal muscle myostatin mRNA expression is fiber-type specific and increases during hindlimb unloading. *Am J Physiol* 1999;277:R601–6. [PubMed: 10444569]
- Costell M, Gustafsson E, Aszodi A, Morgelin M, Bloch W, Hunziker E, Addicks K, Timpl R, Fassler R. Perlecan maintains the integrity of cartilage and some basement membranes. *J Cell Biol* 1999;147:1109–22. [PubMed: 10579729]
- Echaniz-Laguna A, Rene F, Marcel C, Bangratz M, Fontaine B, Loeffler JP, Nicole S. Electrophysiological studies in a mouse model of Schwartz-Jampel syndrome demonstrate muscle fiber hyperactivity of peripheral nerve origin. *Muscle Nerve* 2009;40:55–61. [PubMed: 19367640]
- Franzen P, ten Dijke P, Ichijo H, Yamashita H, Schulz P, Heldin CH, Miyazono K. Cloning of a TGF beta type I receptor that forms a heteromeric complex with the TGF beta type II receptor. *Cell* 1993;75:681–92. [PubMed: 8242743]
- Handler M, Yurchenco PD, Iozzo RV. Developmental expression of perlecan during murine embryogenesis. *Dev Dyn* 1997;210:130–45. [PubMed: 9337134]
- Handschin C, Chin S, Li P, Liu F, Maratos-Flier E, Lebrasseur NK, Yan Z, Spiegelman BM. Skeletal muscle fiber-type switching, exercise intolerance, and myopathy in PGC-1alpha muscle-specific knock-out animals. *J Biol Chem* 2007;282:30014–21. [PubMed: 17702743]
- Hill JJ, Davies MV, Pearson AA, Wang JH, Hewick RM, Wolfman NM, Qiu Y. The myostatin propeptide and the follistatin-related gene are inhibitory binding proteins of myostatin in normal serum. *J Biol Chem* 2002;277:40735–41. [PubMed: 12194980]
- Holmbeck K, Bianco P, Chrysovergis K, Yamada S, Birkedal-Hansen H. MT1-MMP-dependent, apoptotic remodeling of unmineralized cartilage: a critical process in skeletal growth. *J Cell Biol* 2003;163:661–71. [PubMed: 14610065]
- Iozzo RV, Cohen IR, Grassel S, Murdoch AD. The biology of perlecan: the multifaceted heparan sulphate proteoglycan of basement membranes and pericellular matrices. *Biochem J* 1994;302(Pt 3):625–39. [PubMed: 7945186]
- Iozzo RV, Pillarisetti J, Sharma B, Murdoch AD, Danielson KG, Uitto J, Mauviel A. Structural and functional characterization of the human perlecan gene promoter. Transcriptional activation by transforming growth factor-beta via a nuclear factor 1-binding element. *J Biol Chem* 1997;272:5219–28. [PubMed: 9030592]
- Joulia-Ekaza D, Cabello G. Myostatin regulation of muscle development: molecular basis, natural mutations, physiopathological aspects. *Exp Cell Res* 2006;312:2401–14. [PubMed: 16793037]
- Kjaer M, Magnusson P, Krogsaard M, Moller J, Boysen, Olesen J, Heinemeier K, Hansen M, Haraldsson B, Koskinen S, Esmarck B, Langberg H. Extracellular matrix adaptation of tendon and skeletal muscle to exercise. *J Anat* 2006;208:445–50. [PubMed: 16637870]
- Lee SJ, McPherron AC. Regulation of myostatin activity and muscle growth. *Proc Natl Acad Sci U S A* 2001;98:9306–11. [PubMed: 11459935]
- Machida S, Booth FW. Insulin-like growth factor 1 and muscle growth: implication for satellite cell proliferation. *Proc Nutr Soc* 2004;63:337–40. [PubMed: 15294052]
- Martin GR, Timpl R. Laminin and other basement membrane components. *Annu Rev Cell Biol* 1987;3:57–85. [PubMed: 3318883]
- Massague J, Chen YG. Controlling TGF-beta signaling. *Genes Dev* 2000;14:627–44. [PubMed: 10733523]
- McPherron AC, Lawler AM, Lee SJ. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature* 1997;387:83–90. [PubMed: 9139826]
- Noonan DM, Fulle A, Valente P, Cai S, Horigan E, Sasaki M, Yamada Y, Hassell JR. The complete sequence of perlecan, a basement membrane heparan sulfate proteoglycan, reveals extensive similarity with laminin A chain, low density lipoprotein-receptor, and the neural cell adhesion molecule. *J Biol Chem* 1991;266(34):22939–47. [PubMed: 1744087]
- Nicole S, Davoine CS, Topaloglu H, Cattolico L, Barral D, Beighton P, Hamida CB, Hammouda H, Cruaud C, White PS, Samson D, Urtizberea JA, Lehmann-Horn F, Weissenbach J, Hentati F, Fontaine B. Perlecan, the major proteoglycan of basement membranes, is altered in patients with Schwartz-Jampel syndrome (chondrodystrophic myotonia). *Nat Genet* 2000;26:480–3. [PubMed: 11101850]

- Rebbapragada A, Benchabane H, Wrana JL, Celeste AJ, Attisano L. Myostatin signals through a transforming growth factor beta-like signaling pathway to block adipogenesis. *Mol Cell Biol* 2003;23:7230–42. [PubMed: 14517293]
- Sanes JR. The basement membrane/basal lamina of skeletal muscle. *J Biol Chem* 2003;278:12601–4. [PubMed: 12556454]
- SundarRaj N, Fite D, Ledbetter S, Chakravarti S, Hassell J. Perlecan is a component of cartilage matrix and promotes chondrocyte attachment. *J Cell Sci* 1995;108:2663–72. [PubMed: 7593307]
- Stum M, Davoine CS, Vicart S, Guillot-Noel L, Topaloglu H, Carod-Artal FJ, Kayserili H, Hentati F, Merlini L, Urtizberea JA, Hammouda el H, Quan PC, Fontaine B, Nicole S. Spectrum of HSPG2 (Perlecan) mutations in patients with Schwartz-Jampel syndrome. *Hum Mutat* 2006;27:1082–91. [PubMed: 16927315]
- Stum M, Girard E, Bangratz M, Bernard V, Herbin M, Vignaud A, Ferry A, Davoine CS, Echaniz-Laguna A, Rene F, Marcel C, Molgo J, Fontaine B, Krejci E, Nicole S. Evidence of a dosage effect and a physiological endplate acetylcholinesterase deficiency in the first mouse models mimicking Schwartz-Jampel syndrome neuromyotonia. *Hum Mol Genet* 2008;17:3166–79. [PubMed: 18647752]
- Thies RS, Chen T, Davies MV, Tomkinson KN, Pearson AA, Shakey QA, Wolfman NM. GDF-8 propeptide binds to GDF-8 and antagonizes biological activity by inhibiting GDF-8 receptor binding. *Growth Factors* 2001;18:251–9. [PubMed: 11519824]
- Tsuchida K, Nakatani M, Uezumi A, Murakami T, Cui X. Signal transduction pathway through activin receptors as a therapeutic target of musculoskeletal diseases and cancer. *Endocr J* 2008;55:11–21. [PubMed: 17878607]
- Tsumaki N, Tanaka K, Arikawa-Hirasawa E, Nakase T, Kimura T, Thomas JT, Ochi T, Luyten FP, Yamada Y. Role of CDMP-1 in skeletal morphogenesis: promotion of mesenchymal cell recruitment and chondrocyte differentiation. *J Cell Biol* 1999;144:161–73. [PubMed: 9885252]
- Yang W, Zhang Y, Li Y, Wu Z, Zhu D. Myostatin induces cyclin D1 degradation to cause cell cycle arrest through a phosphatidylinositol 3-kinase/AKT/GSK-3 beta pathway and is antagonized by insulin-like growth factor 1. *J Biol Chem* 2007;282:3799–808. [PubMed: 17130121]
- Zoeller JJ, McQuillan A, Whitelock J, Ho SY, Iozzo RV. A central function for perlecan in skeletal muscle and cardiovascular development. *J Cell Biol* 2008;181:381–94. [PubMed: 18426981]

Fig. 1.

Fiber CSA and fiber type composition in TA muscle.

(a) Immunostaining of gastrocnemius muscle for perlecan. Perlecan is absent in Hspg2^{-/-}-Tg mice. Scale bar: 50 μm . (b) Immunostaining for MHC types. Cross-sections of gastrocnemius and TA muscles were immunostained for MHC I (red), IIa (blue), and IIb (green). Fibers with no label (black) are MHC IIx fibers. There were no MHC I (red) fibers in the TA section. Scale bar: 50 μm . (c) Cross-sections of the TA muscle were immunostained for MHC. IIa (blue) and IIb (green). There were no MHC I (red) fibers in this section. Unlabeled fibers (black) are MHC IIx fibers. Scale bar: 200 μm . (d) Mean ($\pm$ SE) fiber cross-sectional area (CSA) of each fiber type in TA muscles of WT-Tg and Hspg2^{-/-}-Tg mice. The CSA of type IIb MHC fibers in

Hspg2^{-/-}-Tg TA muscles increased, compared to WT-Tg mice. There were no significant differences in the CSA of type IIa and type IIc MHC fibers. * P>0.03 vs. WT-Tg. (e) The sum of all MHCs in the sample was taken as 100% and the percentage (means ± SE) of type IIa, IIx, and IIb MHC fibers in TA muscles of WT-Tg and Hspg2^{-/-}-Tg mice was based on immunohistochemical analyses. Percentages of Type IIa and type IIb MHC fibers in Hspg2^{-/-}-Tg TA muscles were decreased, compared to those in WT-Tg TA muscles, whereas type IIx MHC fibers increased. * P>0.01 vs. WT-Tg, † P>0.03 vs. WT-Tg.

Fig. 2. Relative quantitation of myostatin protein. TA muscle lysates and serum from WT-Tg and Hspg2^{-/-}-Tg mice were precipitated with concanavalin A. The same amount of sample protein was applied to reducing SDS-PAGE gels. Immunoblotting was performed using anti-myostatin prodomain antibody or anti-myostatin antibody. The relative abundance of all forms of myostatin was decreased significantly in Hspg2^{-/-}-Tg mice compared to in WT-Tg mice (a, c, & e). The intensity of signal was analyzed by Image J software and the individual bar graph shows the means and SDs for three experiments (b, d, & f). Protein ratios were quantified as the fold-increase relative to WT-Tg mice (defined as 1) * $P < 0.03$.

Fig. 3.

mRNA expression of myostatin and its receptors.

Gene expression levels were measured by qRT-PCR using mRNA extracted from TA muscles of WT-Tg and Hspg2^{-/-}-Tg mice and normalized to GAPDH levels. Myostatin and ALK4 mRNA was reduced in Hspg2^{-/-}-Tg TA muscles compared to those in WT-Tg TA muscles. (a) myostatin mRNA. * P > 0.004 vs. WT-Tg. (b) ActRIIB mRNA. (c) ALK4 mRNA. * P > 0.03 vs. WT-Tg. (d) ALK5 mRNA. (e) TβRII mRNA. (f) IGF-I mRNA. * P > 0.01 vs. WT-Tg. (g) IGF-IR mRNA.

Fig. 4.

Smad signaling in myotubes by myostatin and TGF-β.

Myotubes from WT-Tg or Hspg2^{-/-}-Tg mice were treated with 20 ng/ml TGF-β1 or 10 ng/ml myostatin for 30 minutes. Activated Smad2 was analyzed by immunoblotting with anti-phospho-smad2 (Ser465/467) antibody. The same blots were reprobed with anti-smad2/3 antibodies. Both TGF-β and myostatin activated Smad signaling in myotubes from WT-Tg mice, whereas myostatin did not activate Smad2 in myotubes from Hspg2^{-/-}-Tg mice. The intensity of signal was analyzed by Image J software. The bar graph shows the means and SDs for three experiments. Relative activity, expressed as the ratio of activated phospho-smad2 to total smad2/3, was quantified as the fold-increase relative to non-stimulation conditions (defined as 1) * $P < 0.05$.

Fig. 5.

Wet weights of plantaris and soleus muscles under overloading and unloading conditions. (a) Wet weights of overloaded plantaris and unloaded soleus muscles. To examine the effect of overloaded plantaris muscles and of unloaded soleus muscles, gastrocnemius and soleus muscles, but not plantaris muscles, in the distal tendons of left hindlimbs were tenotomized (G/S-Ten) and measured wet weights of plantaris and soleus muscles 14 days after surgery, as shown in supplemental Fig. 3b. The right hindlimbs were used as controls. Overloading increased the wet weight of the plantaris muscle in WT-Tg- and Hspg2^{-/-}-Tg-G/S-Ten mice. Unloading reduced the wet weights of the soleus muscle in WT-Tg- and Hspg2^{-/-}-Tg-G/S-Ten mice. WT-Tg-Con, sham operated WT-Tg control mice; Hspg2^{-/-}-Tg-Con, sham operated

Hspg2^{-/-}-Tg mice; WT-Tg- and Hspg2^{-/-}-Tg-G/S-Ten, gastrocnemius and soleus muscles, but not plantaris muscles, tenotomized, in WT-Tg and Hspg2^{-/-}-Tg mice, respectively. * P>0.02 vs. WT-Tg-Con, † p>0.01 vs. Hspg2^{-/-}-Tg-Con. ** P>0.01 vs. WT-Tg-Con, †† p>0.01 vs. Hspg2^{-/-}-Tg-Con. (b) Wet weights of unloaded plantaris and overloaded soleus muscles. Gastrocnemius and plantaris muscles, but not soleus muscles, in the distal tendons of left hindlimbs were tenotomized (G/P-Ten) and measured wet weights of plantaris and soleus muscles 14 days after surgery as shown in supplemental Fig. 3c. Overloading caused no significant changes in the wet weight of soleus muscles in WT-Tg- and Hspg2^{-/-}-Tg-G/P-Ten mice. Unloading reduced the wet weight of plantaris muscles in WT-Tg- and Hspg2^{-/-}-Tg-G/P-Ten mice. WT-Tg-Con, sham operated WT-Tg control mice; Hspg2^{-/-}-Tg-Con, sham operated Hspg2^{-/-}-Tg mice; WT-Tg- and Hspg2^{-/-}-Tg-G/P-Ten, gastrocnemius and soleus muscles, but not plantaris muscles, tenotomized, in WT-Tg and Hspg2^{-/-}-Tg mice, respectively. * P>0.01 vs. WT-Tg-Con, † P>0.01 vs. Hspg2^{-/-}-Tg-Con.

Fig. 6.

Changes in the CSA and fiber type compositions of overloaded plantaris muscles.

(a) Mean ($\pm$ SE) fiber CSA of each fiber type of overloaded plantaris muscles in both WT-Tg and Hspg2^{-/-}-Tg mouse plantaris muscles 14 days after G/S-Ten surgery. All MHC fiber types except MHC type I in WT-Tg and Hspg2^{-/-}-Tg mice increased under the overloading condition. * $P > 0.02$ vs. WT-Tg-Con, † $P > 0.03$ vs. Hspg2^{-/-}-Tg-Con. (b) The sum of all MHCs in the sample was taken as 100% and the percentages (means $\pm$ SE) of different MHC fiber types were calculated. Overloading increased the percentage of MHC type IIa fibers in plantaris muscles of WT-Tg and Hspg2^{-/-}-Tg mice. MHC IIx and IIb types reduced. * $P > 0.03$ vs. WT-Tg-Con, † $P > 0.05$ vs. Hspg2^{-/-}-Tg-Con.

Fig. 7.

Myostatin mRNA expression in plantaris muscles in WT-Tg and Hspg2^{-/-}-Tg mice. Myostatin mRNA levels were measured by qRT-PCR using mRNA extracted from plantaris muscles and normalized to GAPDH levels. (a) Myostatin mRNA expression in plantaris muscles of non-tenotomized WT-Tg and Hspg2^{-/-}-Tg mice. Myostatin expression was decreased in Hspg2^{-/-}-Tg mice. * P>0.003 vs. WT-Tg. (b) Myostatin mRNA expression in overloaded plantaris muscles of WT-Tg and Hspg2^{-/-}-Tg mice 14 days after G/S-Ten surgery. Overloading substantially reduced myostatin expression in Hspg2^{-/-}-Tg plantaris muscles. WT-Tg-Con and Hspg2^{-/-}-Tg-Con, plantaris muscles from sham operated mice; WT-Tg-G/S-Ten and Hspg2^{-/-}-Tg-G/S-Ten, plantaris muscles from tenotomized mice. * P>0.002 vs. WT-Tg-Con, † P>0.001 vs. Hspg2^{-/-}-Tg-Con. (c) Myostatin mRNA expression in unloaded plantaris muscles of WT-Tg and Hspg2^{-/-}-Tg mice 14 days after G/P-Ten surgery. No significant change in myostatin expression levels was noted. WT-Tg-Con and Hspg2^{-/-}-Tg-Con, plantaris muscles from sham operated mice; WT-Tg-G/P-Ten and Hspg2^{-/-}-Tg-G/P-Ten, plantaris muscles from tenotomized mice. * P>0.02 vs. WT-Tg-Con, † P>0.01 vs. Hspg2^{-/-}-Tg-Con.