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Androgens negatively regulate myostatin expression in an androgen-dependent skeletal muscle

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Abstract

Myostatin is an important negative regulator of skeletal muscle growth, while androgens are strong positive effectors. In order to investigate the possible interaction between myostatin and androgen pathways, we followed myostatin expression in the androgen-dependent levator ani (LA) muscle of the rat as a function of androgen status. By testosterone deprivation (castration), we induced LA growth arrest in young male rats, whilst atrophy in adult ones, however, both processes could be reversed by testosterone supplementation. After castration, a significant up-regulation of active myostatin protein (and its propeptide) was found, whereas the subsequent testosterone treatment reduced myostatin protein levels to normal values in both young and adult rats. Similarly, a testosterone-induced suppression of myostatin mRNA levels was observed in castrated adult but not in young animals. Altogether, androgens seem to have strong negative impact on myostatin expression, which might be a key factor in the weight regulation of LA muscle.

Keywords: Androgen; Myostatin; Rat; Levator ani; Castration; Testosterone supplementation

Myostatin, a member of the TGF-beta family, is a potent negative regulator of skeletal muscle growth and maintenance [1], exerting its final effect on cell proliferation and differentiation [2–4]. In contrast, androgens have long been regarded as positive regulators of muscle growth and strength [5], the mechanism of which, however, is still not completely clarified. A common target of androgens and myostatin seems to be the satellite cells, the main source of muscle growth and repair [5]. Whereas myostatin have been shown to inhibit satellite cell activation [6], androgens are considered to exert a positive effect on them through an androgen receptor (AR)-mediated pathway [7]. However, several data suggest that a direct or indirect link between myostatin and androgen pathways do exist. As an androgen-responsive element (ARE) has been described in the myostatin promoter [8], direct action of AR on myostatin transcription cannot be ruled out. In this regard, contradictory results have been reported in the literature showing no correlation between AR and myostatin mRNA levels [9,10]. Yet, the AR protein has not been measured in these studies, the level of which is more likely to correlate with the anabolic responsiveness of skeletal muscles [11]. In fact, lower levels of myostatin protein has been detected in the muscles of male versus female mice [12], suggesting a negative role of androgens in myostatin expression. However, additional effects of androgens on myostatin signalling should be also considered since the tissue-specific overexpression of myostatin resulted in muscle atrophy in males but not in females [13]. Thus, the potential (direct) regulation of myostatin pathway by androgens has to be further investigated.

An excellent model system to analyse androgen effects and the possible interaction between androgens and myostatin, is the rat levator ani (LA) muscle, the development, maintenance and function of which are regulated by sex hormones [14]. Involved in sexual behaviour, LA undergoes a pronounced hypertrophy due to the elevated serum testosterone levels during puberty in male rats, while endures atrophy in females [14]. Thus, prepubertal

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castration (testosterone deprivation) of young males prevents LA muscle from the postnatal hypertrophy resulting in growth arrest [15], whereas postpubertal castration of adult-size LA causes time-dependent atrophy [16]. Although both processes are reversible upon testosterone administration, less is known about their regulation.

Here, we addressed the issue whether myostatin (mRNA and protein) expression in LA muscle is regulated as a function of androgen status during muscle growth and maintenance. We revealed a highly significant down-regulation of the active myostatin protein by androgens in both young and adult rats, while a testosterone-induced mRNA suppression occurred in adult but not in young animals. Moreover, the active myostatin protein was identified in the rough nuclear fraction of LA muscles. In conclusion, we prove here that, although the mechanism might be altered during LA muscle growth (in young rats) and maintenance (in adult), androgens have strong negative impact on myostatin expression, which seems to be a key factor in the regulation of LA muscle weight.

Materials and methods

Animal treatment. Two different sets of male Wistar rats were used for the experiments. In the first set, four groups of young animals were randomly created. One group was castrated prior to puberty at 4 weeks of age, while another group, castrated at the same age, was treated with testosterone propionate (Sigma) daily, with a subcutanous dose of 100 μg/100 g body weight for 4 weeks [14,15]. LA muscles from castrated or castrated plus testosterone-treated groups were removed at 8 weeks of age (*8wC* and *8wC+T*, respectively). Four-week-old (prepubertal) or 8-week-old (postpubertal) untreated LA muscles were removed and served as time-matched controls (*4wN* and *8wN*, respectively).

In the second set, seven groups of sexually matured adult male Wistar rats (360–400 g) were analysed. Five groups were castrated, the LA muscles of four of them were removed after 4, 7, 11 or 14 days to reveal the dynamics of atrophy (*C4*, *C7*, *C11*, *C14*, respectively), while the fifth group, castrated for 7 days, was additionally injected *sc.* with a dose of 1 mg/day testosterone propionate for the next 7 days (*C7+T7*) [16]. Another group of intact normal rats was treated with the same dose of testosterone propionate for 7 days (*N+T7*). Intact normal rats served as controls (*N*). After removal of LA muscles, animals were sacrificed with an overdose of Na-pentobarbital. All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Szeged.

Measurement of the effect of castration/hormone treatment. The wet weights of LA muscles were measured. As the weights of adult castrated LA muscles did not decreased further after 11 days (weights of groups *C11*≈*C14*, see Fig. 1B) *C11* and *C14* groups were combined, referred to as *C11/14* group, and used for RNA and protein analysis. *C4* and *C7* groups were not further analysed since we concentrated here on the stage of most pronounced atrophy.

RT PCR analysis. From LA muscles of young (*4wN*, *8wN*, *8wC*, *8wC+T)* or adult rats (*N*, *C11/14*, *C7+T7*, *N+T7*) total RNA was isolated by the acid-guanidinium thiocyanate–phenol–chloroform (AGPC) method [17] and subjected to cDNA synthesis as described previously [18]. For amplifying rat muscle cDNA, PCR primers based on the rat myostatin cDNA sequence (GenBank Accession No. AF019624) were used and PCR reaction was carried out as described [18]. Myostatin mRNA levels were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [19]. After gelelectrophoresis and ethidium bromide staining PCR products were quantified by measuring the intensity of bands with Quantity One software (Bio-Rad).

Protein fractionation and Western blot analysis. A rough nuclear fraction from LA muscles in young (*4wN*, *8wN*, *8wC*, *8wC+T)* and adult rats (*N*, *C11/14*, *C7+T7*, *N+T7*) was prepared as we revealed in a previous study that the specific immuno-reactive signal of active (processed) myostatin accumulated in this fraction [18]. Using a much more sensitive Western blot detection method (ECL) in the present study, we additionally analyzed the supernatant fractions not to override any signal. For nuclear fractionation we applied a modified method of Zahradka et al. [20] as described previously [18]. Since we homogenized muscle tissue in a buffer of 31× volume followed by sedimentation and subsequent extraction of nuclear pellet in a buffer of 3× volume, the supernatant was about 10× less concentrated than the nuclear pellet. Thus, we loaded about 10× more volume from supernatant onto a 10% Laemmli-type acrylamide-gel corresponding to 65 μg and 25 μg protein from supernatant and nuclear pellet, respectively. Electrophoresis and wet-blotting to nitrocellulose membrane (Amersham) were carried out under standard conditions. The uniformity of protein loading and the efficiency of transfer were checked by staining the membrane with Ponceau-red. After blocking the membrane with TBS–Tween + 5% non-fat milk powder overnight, a polyclonal anti-myostatin antibody (1:1000, Chemicon AB3239) and a HRP-coupled secondary antibody (1:2000, Dako) was used for immunohybridization. The immunosignal for myostatin was developed by chemiluminescence reaction (ECL, Amersham) followed by densitometric quantification with Quantity One software (Bio-Rad).

Statistical analysis. Each group of rats consisted of 3–6 animals. Data are reported as mean ± SEM. Statistical differences between groups were analysed with one-way ANOVA followed by Newman-Keuls post-test (GraphPad Software, Inc.).

Results

LA muscle weights

Levator ani muscles were very small in prepubertal 4-week-old animals, however, an androgen-dependent pronounced hypertrophy was observed in 8-week-old postpubertal male rats (Fig. 1A, *4wN* versus *8wN*). This phenomenon is specific to the androgen-dependent skeletal muscle causing a significant increase in the proportion of muscle weight to body weight as well. In order to prevent young rats from the hormonal effects of puberty, prepubertal castration at 4-week of age was carried out, which indeed resulted in LA muscle growth arrest by the 8th week (*8wC*). However, 4-week-long testosterone supplementation of rats castrated at 4-week of age [15] successfully mimiced puberty by inducing a LA weight increase close to the normal level (*8wC+T*), consistent with morphological data as well (data not shown).

In sexually mature adult male animals (Fig. 1B), with LA muscle weights maintained normally due to physiological testosterone levels (*N*), castration caused a time-dependent atrophy (*C4*, *C7*, *C11*, *C14*) without any further decrease after *day 11* (*C11* versus *C14*). In contrast, the 7-day-long hormone treatment of 7-day-castrated rats [16] was enough to recover LA muscle from the pronounced atrophy to a close-to-normal weight (*C7+T7*) and morphology (data not shown). In case of normal animals, extra hormone supplementation (*N+T7*) did not cause significant hypertrophy likely due to its negative feedback on intrinsic testosterone production.

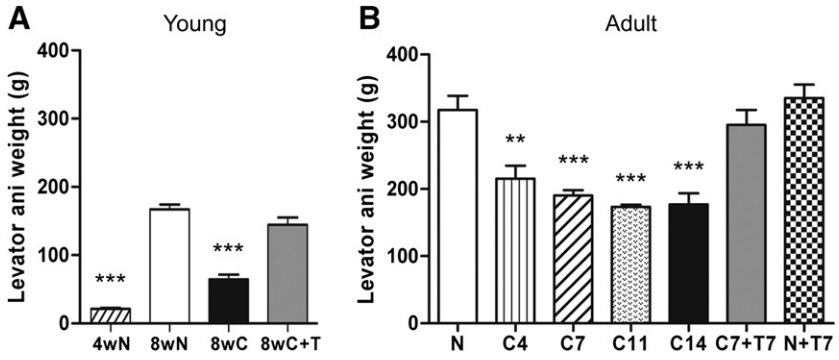

Fig. 1. LA muscle weights of young and adult rats after different treatments. An androgen-dependent hypertrophy occurs during puberty in young rats (A) (*4wN* versus *8wN*). LA muscles of castrated animals (*8wC*) show growth arrest, while subsequent testosterone-treatment (*8wC+T*) increases muscle weights close to normal. In adult animals (B), castration causes a time-dependent atrophy in LA muscle (*C4–C14*) but muscle weights do not further decrease after *day 11*. Testosterone treatment of castrated animals (*C7+T7*) turns LA muscle mass back to normal, while the hormone treatment of normal rats (*N+T7*) does not cause further hypertrophy. Bars represent mean values ± SEM., $^{**}p < 0.01$, $^{***}p < 0.001$ versus *8wN* (A) or versus *N* (B), $n = 3–6$.

Myostatin mRNA levels

As GAPDH mRNA levels were relatively stable in different animal groups, they proved to be appropriate internal controls for normalization of myostatin mRNA levels (Fig. 2). Despite the remarkable differences among experimental groups, no significant alterations in myostatin mRNA levels could be observed in young LA muscles (Fig. 2A). In adult rats, however, castration resulted in an increasing tendency of myostatin mRNA levels in the atrophied LA muscle (Fig. 2B, *C11/14*) compared to normal (*N*), whereas a significant decrease was clearly detectable after testosterone-treatment of castrated adult rats (*C7+T7*), when compared to both normal and castrated animals. A slight yet not significant decrease in myostatin mRNA levels were present in the testosterone-supplemented normal animals (*N+T7*) as well. These results suggest that testosterone treatment differentially influences myostatin transcript levels in young and adult animals.

Subcellular localization of myostatin protein

We isolated a rough nuclear fraction from the LA muscles based on our previous findings on the nuclear accumulation of the active form of myostatin protein in other skeletal muscles [18]. Here, with a polyclonal anti-myostatin antibody [21,22], we detected a single protein band (at about 30 kDa) in the rough nuclear fraction of both young and adult rats (Fig. 3A and B, RN) corresponding to active myostatin, however, this band was completely absent in supernatant (Fig. 3A and B, S) consistent with our previous data [18]. Surprisingly, a different protein at 40 kDa was found exclusively in the supernatant fraction of both young and adult animals (Fig. 3A and B, S) which prob-

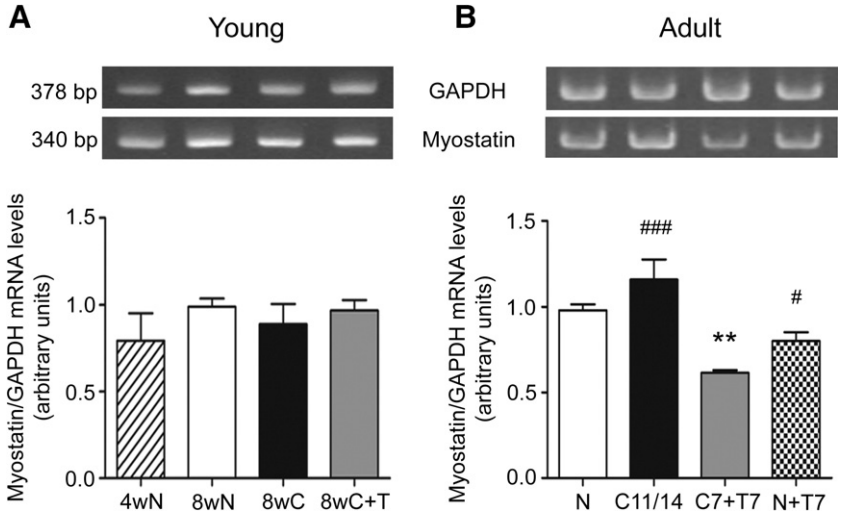

Fig. 2. Myostatin mRNA levels of LA muscles in young and adult rats. Myostatin mRNA levels are normalized to those of GAPDH. Upper panels show typical RT PCR amplifications of the 378 bp-long GAPDH and the 340 bp-long myostatin mRNA fragments. No difference in myostatin mRNA levels is detected in young rats (A), whereas a significant decrease in myostatin transcript levels is observed after testosterone-treatment of castrated adult rats (*C7+T7*) (B). Bars represent mean values ± SEM, $^{**}p < 0.01$ versus *N* (B), $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ versus *C7+T7* (B), $n = 3–6$.

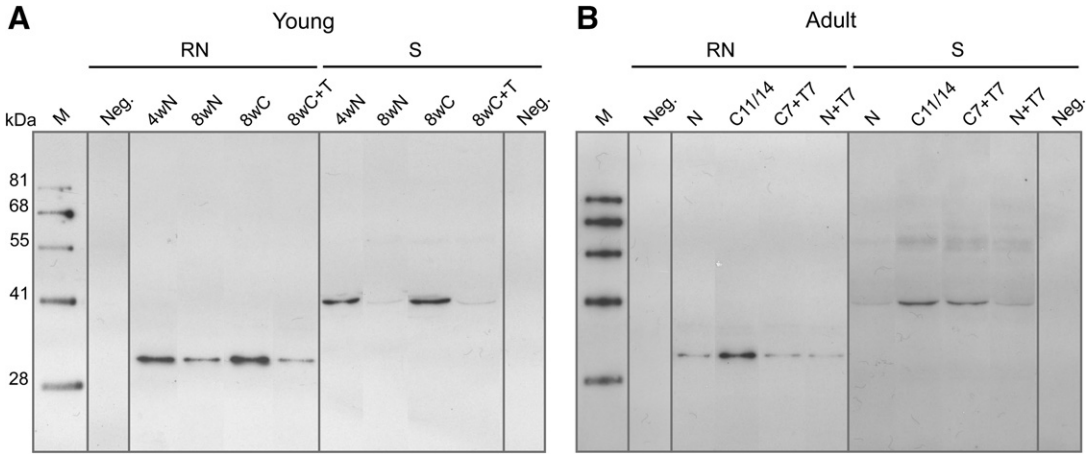

Fig. 3. Representative immunoblots of myostatin protein forms in different subcellular fractions of LA muscles in young and adult rats. The active form of myostatin protein (≈30 kDa) is exclusively present in the rough nuclear fraction of both young (A) and adult (B) rats, while the propeptide (≈40 kDa) is observed only in supernatant fraction. A very faint band at 55 kDa is occasionally observed in supernatant fraction as well. A quantity of 25 μg and 65 μg protein for rough nuclear and supernatant fractions, respectively, are loaded. M, molecular weight marker, RN, rough nuclear fraction; S, supernatant fraction; Neg, controls without primary (anti-myostatin) antibodies, group labels are the same as in Figs. 1 and 2.

ably corresponds to myostatin propeptide arising from the proteolytic cleavage of promyostatin into active myostatin. Additionally, a very faint band of 55 kDa—possibly representing promyostatin—was occasionally observed in supernatant fraction (Fig. 3A and B, S) but we could not quantify it. In accordance with our Western blot results, Shyu et al. [22] detected the same protein bands of similar relative intensity in normal skeletal muscles, using the same antibody. However, they analysed total muscle homogenates, whereas we identified the active myostatin and its propeptide in two different fractions of LA muscle. We also addressed the question whether there is a redistribution of the active myostatin protein as a function of androgen status but we did not find any alteration in this respect (RN versus S).

Myostatin protein levels

In young rats, both the active and propeptide forms of myostatin (Fig. 4A and B, respectively) were detected at high levels in prepubertal LA (*4wN*), whereas a significant down-regulation occurred by the end of puberty (*8wN*). Myostatin proteins were up-regulated in growth-arrested LA muscles of castrated animals (*8wC*), while testosterone-treatment of castrated rats (*8wC+T*) significantly reduced both myostatin forms close to normal levels (*8wN*). In adult rats, active myostatin and its propeptide (Fig. 4C and D, respectively) were expressed at low level in normal LA muscles (*N*). A significant up-regulation was observed in the atrophied LA muscle (*C11/14*), whereas myostatin levels were again depressed after testosterone treatment of castrated rats (*C7+T7*). Testosterone-treatment of normal animals (*N+T7*) did not change myostatin protein levels consistent with their unaltered muscle weight. In summary, we detected similar alterations in myostatin protein levels in both young and adult rats, *i.e.* the active myostatin (and its propeptide) were up-regulated when serum testosterone levels and the respective LA weight were low (*4wN*, *8wC*, *C11/14*), whereas elevated testosterone levels induced the down-regulation of myostatin proteins accompanied by LA weight increase (*8wN*, *8wC+T*, *N*, *C7+T7*, *N+T7*).

Discussion

Taking advantage of the fact that in the androgen-dependent skeletal muscles significant muscle mass alterations take place as a function of androgen status, we successfully used the rat LA muscle as a model system for analyzing the possible regulation of myostatin by androgens. Here we provide evidence that androgens exert a negative effect on myostatin expression which seems to be a key element in the weight regulation of LA muscle.

Our results, *i.e.* the testosterone-induced suppression of myostatin mRNA levels in adult LA (*C7+T7*), suggest an AR-dependent transcriptional depression of myostatin, in accordance with the documented presence of ARE in the myostatin promoter [8]. Indeed, in adult castrated rats, it has been described that testosterone supplementation induced an increase in AR protein level [23,24], which would also fit the remarkable (AR-related) down-regulation of myostatin transcript in our case. If so, it is puzzling why no mRNA alteration was observed in young animals upon increasing testosterone levels during puberty. A possible, yet not full explanation could be that the AR level and/or its reaction to testosterone is different in young and adult animals. In fact, intact prepubertal rats (*4wN*) and the ones castrated at 4-week of age (*8wC*) have never experienced, in contrast to adults, high testosterone levels, which certainly determines their basic AR (mRNA and protein) status and the potential

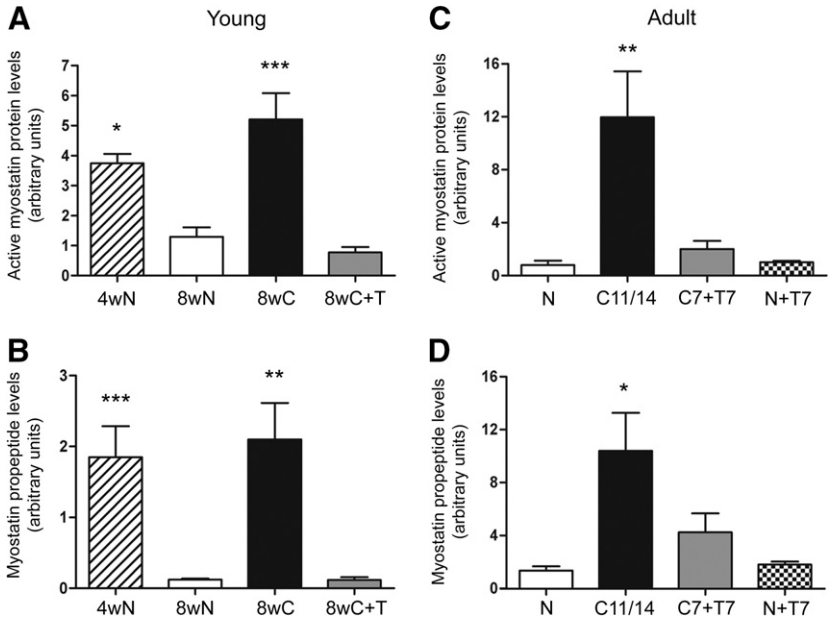

Fig. 4. Myostatin protein levels in different subcellular fractions of LA muscles in young (A,B) and adult (C,D) rats. In young rats both the active myostatin (A) and its propeptide (B) are detected at high levels in prepubertal LA muscle (*4wN*), while a significant down-regulation occurs by the end of puberty (*8wN*). Myostatin proteins are expressed at high levels in castrated animals (*8wC*), but testosterone-treatment of castrated rats (*8wC+T*) significantly reduces both myostatin forms close to normal levels (*8wN*). In adult rats, active myostatin (C) and its propeptide (D) are expressed at low level in normal LA muscles (*N*). A significant up-regulation is observed 11–14 days after castration (*C11/14*), whereas myostatin levels are again reduced after testosterone-treatment of castrated rats (*C7+T7*). Testosterone-treatment of normal animals (*N+T7*) does not alter myostatin levels. Bars represent mean values ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus *8wN* (A,B) or versus *N* (C,D), $n = 3$–6.

myostatin reactivity upon testosterone increase. Another possibility is that the activity of AR is altered during LA growth as it is influenced by numerous kinases affecting AR stability [25]. However, the role of other non-transcriptional or AR-independent factors should not be underestimated in this process either.

Indeed, we reported here that, despite the different levels of myostatin mRNA in young and adult rats, the final effect of androgen action seems to be the significant down-regulation of the active myostatin protein (and it propeptide) in both cases. Different mRNA and protein levels of myostatin have been already described in other skeletal muscle models [18]. Even in adults, where testosterone-treatment seemed to suppress both mRNA and protein levels in the present experiments, the order of magnitude of protein alterations was higher than that of mRNA. Thus, several other steps, such as the mRNA stability, the translation or posttranslational modification of promyostatin including the cleavage of promyostatin into active myostatin [2], or even the extracellular recruitment of myostatin protein might be considered as target points of further regulatory mechanisms. Due to its low level [21,22], we could however not quantify promyostatin so it is still unclear whether the alterations in the level of active myostatin are preceded by the same alterations in its precursor. This makes it difficult to deduce at which point androgen signalling might be effective.

Another exciting finding is the nuclear localization of the active myostatin protein in the LA muscle, which corroborated our previous results on this issue [18]. Although, our crude nuclear pellet probably entraps other cellular (or extracellular matrix) elements, another group has also proven the presence of active myostatin in the nucleus, using different nuclear isolation methods and immunohistochemistry in C2C12 cells [26]. In contrast to their findings on up-regulation and a subsequent cytoplasmic redistribution of myostatin induced by glucocorticoid treatment, we could not observe similar redistribution upon castration or androgen administration. The possible role of myostatin in the nucleus (as a potential transcription factor) is still an open issue.

In conclusion, myostatin expression in LA muscle is fine tuned in a way that not the mRNA, but the active myostatin protein correlates negatively with the respective LA weight. Thus, myostatin seems to function as an androgen-responsive growth regulator. Moreover, as myostatin protein itself has been shown to down-regulate ARA70, the co-activator of AR [27], it seems reasonable to speculate that a negative feedback-loop exists between myostatin and androgen pathways. However, further analysis is needed to elucidate the exact molecular mechanisms.

Acknowledgments

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