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REVIEW

Androgens and skeletal muscle: cellular and molecular action mechanisms underlying the anabolic actions

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Abstract Androgens increase both the size and strength of skeletal muscle via diverse mechanisms. The aim of this review is to discuss the different cellular targets of androgens in skeletal muscle as well as the respective androgen actions in these cells leading to changes in proliferation, myogenic differentiation, and protein metabolism. Androgens bind and activate a specific nuclear receptor which will directly affect the transcription of target genes. These genes encode muscle-specific transcription factors, enzymes, structural proteins, as well as microRNAs. In addition, anabolic action of androgens is partly established through crosstalk with other signaling molecules such as Akt, myostatin, IGF-I, and Notch. Finally, androgens may also exert non-genomic effects in muscle by increasing Ca^{2+} uptake and modulating kinase activities. In conclusion, the anabolic effect of androgens on skeletal muscle is not only explained by activation of the myocyte androgen receptor but is also the combined result of many genomic and non-genomic actions.

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Abbreviations

AIS	Androgen insensitivity syndrome
ALP	Alkaline phosphatase
AMPK	Adenosine monophosphate-activated kinase
AR	Androgen receptor
ARE	Androgen response element
ARKO	Androgen receptor knockout
BC	Bulbocavernosus
BSA	Bovine serum albumin
c-Src	Cellular sarcoma
EDL	Extensor digitorum logus
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
FoxO	Forkhead box O
Fst	Follistatin
GH	Growth hormone
GPCR	G-protein coupled receptor
IGFBP	IGF binding protein
IGF-I	Insulin-like growth factor I
IGF-IR	IGF-I receptor
IP3	Inositol 1,4,5-triphosphate
JNK	c-Jun NH2-terminal kinase
LA	Levator ani
MADS	Mcm1 agamous deficiens serum response factor
MAFbx	Muscle Atrophy F-box
MAPK	Mitogen-activated protein kinase
mARKO	Myocyte-specific AR knockout
Mef	Myocyte enhancer factor
MGF	Mechanogrowth factor

miR	microRNA
mRNA	Messenger RNA
Mst	Myostatin
mTOR	Mammalian target of rapamycin
MuRF-1	Muscle Ring Finger 1
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
SARM	Selective AR modulator
SHBG	Sex hormone-binding globulin
SHBGR	SHBG receptor
SRC	Steroid receptor coactivator
SRE	Serum response element
SRF	Serum response factor
TCF	T cell factor
TGF-β	Transforming growth factor-β
WRE	Wnt response element
SRE	Serum response element
SARM	Selective AR modulator

Introduction

Androgens play crucial physiological roles in establishing and maintaining the male phenotype. Their actions are essential for the differentiation and growth of the male reproductive organs, initiation, and regulation of spermatogenesis, and the control of male sexual behavior. In addition, androgens also have anabolic actions on several extragenital structures including muscle and bone [1]. Indeed, testosterone, the main androgen in skeletal muscle [2], increases muscle size and strength both in young [3] and older men [4]. The testosterone-induced increase in muscle mass is partly due to muscle fiber hypertrophy, reflected by an increase in myonuclear number and cross-sectional area of both type I and type II muscle fibers [5]. The responsiveness of skeletal muscle to androgens could potentially be exploited clinically in the treatment of various chronic diseases that are accompanied by muscle wasting such as cancer cachexia, AIDS, chronic obstructive pulmonary disease, chronic renal disease, and burns [6]. Another important growing health issue associated with testosterone deficiency is the age-related increase in sarcopenia and frailty in elderly men and the accompanying risk for fractures due to increased falling [7]. Indeed, testosterone administration to frail elderly men may increase muscle strength [8]. These broad clinical potentials of androgens merit further review of the underlying cellular targets and mechanisms.

Although there is agreement that androgen administration increases muscle mass, data on the effects of testosterone supplementation on muscle performance and physical function are less clear. Meta-analyses indicate that testosterone therapy increases grip strength to a greater extent than placebo [9], but only few trials reported significant increases in maximal voluntary strength [10, 11]. While there is uncertainty about which measures of muscle performance are androgen-responsive [9], the tests of physical function used in most of the trials have serious limitations. Firstly, they have a low performance ceiling, so that at baseline the participants already perform above the test ceiling [12]. Secondly, sample size of most of the trials was relatively small. Therefore, it is likely that they did not have sufficient power to detect meaningful changes in physical function [9]. Finally, it has been suggested that the translation of muscle mass gain into improvements in physical function may require cognitive, behavioral, or functional training [12].

The protein hypothesis states that testosterone administration induces an increase in skeletal muscle protein synthesis [13, 14] and an improved recycling of intracellular amino acids [14, 15]. The proposed effects of androgens on muscle protein degradation, however, are less clear: short-term treatment does not appear to change the breakdown rate [14, 15], whereas treatment for several months decreases muscle protein breakdown [10, 16]. Testosterone-induced muscle hypertrophy may thus be explained by changes in muscle protein metabolism. However, androgens also mediate changes in body composition characterized by an increase in lean body mass accompanied by a concomitant decrease in fat mass [17], which are difficult to explain only by muscle protein synthesis and/or breakdown. The question therefore arises how androgens may induce differential anabolic actions such as changes in body composition as well as muscle hypertrophy.

Androgens exert their effects largely by binding to the nuclear androgen receptor (AR). The AR is a ligand-inducible transcription factor that binds to specific DNA sequences called androgen response elements (AREs) and recruits coactivators, which will help affect the transcription of target genes [18]. Androgens also interfere with other signaling pathways [19], and several non-genomic androgen effects are described [20]. It should be noted that some effects of testosterone can be explained by the activation of estrogen receptors after conversion into estrogens [21]. Here we will summarize the current views on how androgens might act on skeletal muscle. Better knowledge of these mechanisms could lead to more targeted therapeutics acting downstream of androgens in a muscle-specific way. To what extent anabolic androgen action is mediated directly through the AR of the different muscular cells or indirectly through other cells or tissues that affect muscle physiology, also remains an important research question.

Cellular targets of androgen action in skeletal muscle

Skeletal muscles differ markedly in their responsiveness to androgens. For example, the perineal skeletal muscles

levator ani (LA) and bulbocavernosus (BC) are highly androgen responsive and depend on androgens for their normal maintenance and function, whereas the limb skeletal muscle extensor digitorum longus (EDL) is relatively unresponsive to androgens and does not depend on androgens to maintain fiber size [22]. Due to its high androgen responsiveness, the LA muscle is used widely as readout for androgen anabolic action in preclinical studies [6]. Immunohistochemical staining of muscle sections revealed that the BC/LA complex contains much more AR protein than do less responsive muscles like the EDL [23, 24]. Thus, differences in AR protein content of skeletal muscles seem to underlie differences in androgen responsiveness.

During growth and repair of the adult skeletal muscle, quiescent tissue-specific progenitor cells, also called satellite cells, are activated and start proliferating, at which stage they are often referred to as myoblasts [25]. Myoblasts further differentiate into myocytes that fuse to form multinucleated myotubes, which finally mature into contracting muscle fibers [26]. Satellite cells and myonuclei are reported to be the predominant sites of AR expression in muscle [27]. This observation supports the hypothesis that androgens might increase muscle mass mainly by stimulation of satellite cells [28]. However, other AR-expressing cell types may contribute to myogenic androgen action. Indeed, the AR is also expressed in CD34+ mesenchymal precursor cells within the human skeletal muscle that are capable of myogenic commitment [27], as well as in neurons that innervate skeletal muscle [29].

Satellite cells

Satellite cell biology

Satellite cells are located between the basal lamina and the plasma membrane of muscle fibers [30]. They can be identified as Pax7+ and CD34+ cells [31], but several other markers have been shown to be useful to isolate satellite cells such as SM/C-2.6, α7-integrin and caveolin-1 [32, 33]. During muscle development and regeneration, quiescent satellite cells become activated and start proliferating [25].

A progressive decline of skeletal muscle mass and strength is observed with ageing [34]. One potential underlying mechanism could be a decrease in the number of satellite cells [35, 36]. An alternative explanation may be a gradual age-related decline of the regenerative potential of skeletal muscle [37, 38], which may in large part be due to a decrease of Notch signaling [39]. Remarkably, the regenerative potential of satellite cells can be restored by exposure to a young systemic environment, suggesting that at least the intrinsic regenerative capacity of aged satellite cells remains intact [40]. Many factors, such as nitric oxide [41], interleukin-6 [42], and Notch signaling [43–45], may contribute to satellite cell activation but the exact underlying molecular mechanisms and interferences by androgens remain to be identified.

Androgen effects on satellite cell proliferation

Studies performed by Sinha-Hikim et al. [46] showed that testosterone-induced muscle hypertrophy is associated with an increase in satellite cell number, both in young and older men [47]. Moreover, AR upregulation has been observed following testosterone treatment of cultured satellite cells from men [27] as well as pigs [48]. Satellite cells are therefore considered to be a direct androgen target in skeletal muscle. Testosterone has also been shown to stimulate satellite cell proliferation in rat [49, 50] and pig models [51]. This cell proliferation was followed by a subsequent increase in the myonuclei number [49]. Also, in in vitro cultured rat primary myoblasts as well as in the mouse myoblast cell line C2C12, testosterone induced proliferation [52, 53]. However, other groups found no direct effects of testosterone on C2C12 proliferation, nor on cultured porcine satellite cells [54, 55]. All together, these data indicate that androgens act on satellite cells by increasing AR expression as well as satellite cell number. This raises the question how and at which stages androgens may impact satellite cell differentiation.

Androgen effects on satellite cell differentiation

In the male complete androgen receptor knockout (ARKO) mouse model, levels of *Cdkn1c* and *Igf2*, both drivers of terminal myogenic differentiation [56, 57], are upregulated in ARKO versus wild-type muscle, whereas expression of *Itgb1bp3*, a negative regulator of muscle differentiation [58], is decreased [59]. From these results, the authors concluded that androgens promote muscle growth by maintaining myoblasts in the proliferative state and delaying differentiation. However, further evidence of testosterone action on satellite cell differentiation is contradictory [48, 53, 54, 60].

In addition, several other studies suggest that it is not proliferation nor differentiation, but other satellite cell functions that are targeted by androgens. Treatment of cultured bovine satellite cells with the synthetic androgen trenbolone, e.g., resulted in a dose-dependent increase in protein synthesis rate and a decrease in protein degradation rate, effects that could be counteracted by AR antagonists [61]. In conclusion, further well-controlled studies are required to elucidate the exact effects of androgens on proliferation and differentiation of satellite cells, myoblasts, and myocytes.

Mesenchymal precursor cells

Alternative muscle progenitor cells

Satellite cells are considered to be the main source of myonuclei in postnatal muscle [62]. However, vascular and bone marrow cells [63, 64] as well as other muscle-resident stem cells with myogenic potential [65, 66] have been reported. The precise anatomical location of these non-satellite cell progenitors is difficult to define due to the lack of suitable cellular markers, with the exception of mesangioblasts, which can be identified as blood vessel-associated alkaline phosphatase (ALP) positive cells [67]. A recent study describes a population of muscle resident stem cells located in the interstitium and expressing PW1 but being negative for Pax7, which can contribute to muscle regeneration [68]. At this moment, the effects of androgens on these cell types under normal physiological conditions remain unexplored.

Pluripotent muscle-adipose progenitor cells

The increase in muscle mass observed upon testosterone administration is accompanied by a reciprocal decrease in fat mass [17]. Conversely, lowering of testosterone concentration below baseline leads to an increase in total body adipose tissue [69]. In patients suffering from androgen insensitivity syndrome (AIS) secondary to disrupted AR signaling, an increase in body fat is observed as well as a higher prevalence of obesity [70], suggesting that these androgen effects on body composition are mediated via the AR.

Several animal studies support this hypothesis. Indeed, the ARKO mouse model developed by the Kato group shows a decrease in lean mass accompanied by a marked increase in visceral and subcutaneous fat [71]. Similarly, Chang et al. [72] report an obese phenotype with enlarged gonadal and perirenal fat pads and larger adipocytes in their AR-null model. However, a third ARKO model shows a decreased muscle cross-sectional area accompanied by reduced potential of voluntary running but without increased adiposity or obesity [73, 74]. Surprisingly, myocyte-specific AR knockout (mARKO) mice not only have a lower muscle mass but also a lower intra-abdominal fat mass [75]. Thus, although AR-related androgen effects on body composition are well established, the underlying AR pathways remain controversial.

Since satellite cells are already committed to myogenesis and do not spontaneously adopt an adipogenic fate [76, 77], androgen action on these cells cannot account for the observed effects on body composition. Therefore, the Bhasin group hypothesized that, in addition to direct effects on satellite cells, testosterone may promote the commitment of pluripotent precursor cells into the myogenic lineage and inhibit their differentiation into the adipogenic lineage [78].

Pluripotent progenitor cell differentiation

In adult skeletal muscle, a population of uncommitted pluripotent progenitor cells of mesenchymal origin serves as a reservoir for the generation of new satellite cells during muscle regeneration or hypertrophy [79] and of adipocytes [80]. Immunofluorescence experiments showing AR expression in CD34+ mesenchymal cells within the human skeletal muscle [27] support the hypothesis that these pluripotent progenitors may be a target of androgen action. In addition, male mice with targeted AR overexpression in mesenchymal stem cells have reduced visceral and subcutaneous fat accumulation with a reciprocal increase in lean mass [81].

Thus, there is increasing evidence that the myogenic action of androgens is partly mediated through the regulation of mesenchymal precursor cell commitment, a model that may explain both the increase in muscle mass as well as the decrease in fat mass following testosterone treatment. However, other studies provide alternative hypotheses to explain the reciprocal changes in body composition. Indeed, a transgenic rat model with selective overexpression of AR in myocytes shows that increased androgen signaling in muscle cells is sufficient to increase lean mass and decrease adiposity by virtue of increased muscular and systemic oxidative metabolism [82]. In addition, co-culture experiments reveal that adipogenesis of mesenchymal progenitors is strongly inhibited by the presence of satellite cell-derived myofibers [77].

In vitro experiments using the C3H 10T1/2 pluripotent mesenchymal cell line provided further evidence for androgen action on the commitment of precursor cells. Indeed, testosterone treatment of C3H 10T1/2 cells upregulated and downregulated myogenic differentiation markers and markers of adipogenic differentiation, respectively [83]. β-catenin signaling may play an important role in the androgenic regulation of precursor cell differentiation [84], as will be discussed further.

Motoneurons

Skeletal muscle is innervated by neurons whose nuclei originate within the spinal cord. Sarcopenia in men is considered to be primarily driven by motoneuron death, subsequently leading to a decrease in muscle mass [85]. In addition, immunohistochemical staining reveals that these motoneurons also express AR [29], hereby further suggesting that androgen anabolic action may be mediated via muscle innervation. Moreover, testosterone causes a

significant up-regulation of AR expression in these neurons [29] as well as an increase of the number and size of the motoneurons themselves [86, 87]. Androgen action on motoneurons may therefore contribute to their myogenic effects. However, whether androgen signaling in motoneurons is required for their anabolic action on muscle remains a matter of debate.

Muscle mass of mice selectively lacking AR expression in the nervous system does not differ significantly from that of their wild-type littermates [88]. In addition, complete denervation of the BC/LA complex in mice followed by testosterone administration did not prevent testosterone from sparing the muscle [89]. These data suggest that muscular maintenance is directly mediated by muscle and not by central androgen action. On the other hand, in another study, testosterone treatment failed to restore BC/LA weight following denervation [90], leaving open the possibility that androgens may also act upon motoneurons to affect muscle size. Thus, although AR expression has been demonstrated in motoneurons and both motoneuron number and size increase upon androgen administration, further studies are needed to fully elucidate the exact role of androgens in nerve cells and their relative contribution to the anabolic androgen action in skeletal muscle. A possible approach may consist of performing denervation studies also in limb muscles, as the validity of the perineal BC/LA complex as a general model of skeletal muscle is questionable.

In summary, the main cellular targets for androgens in skeletal muscle include satellite cells and myonuclei, but actions on pluripotent mesenchymal precursor cells and motoneurons may also contribute to the eventual outcome (Fig. 1).

Genomic actions of androgens in skeletal muscle

Androgens act predominantly through binding of the classical nuclear AR, inducing receptor dimerization, nuclear translocation, and coactivator recruitment to promote transcription of target genes [18]. Although the role of coactivators in androgenic action has been clearly demonstrated in secondary sexual and reproductive tissues [91], their role in AR action in muscle has not been comprehensively demonstrated. A variety of AR coregulator-deficient mice has been generated in the past decades, but none of them showed an obvious muscular phenotype [92, 93]. In an attempt to identify muscle-specific or abundant coregulators of the AR, Chang et al. [94] screened the skeletal muscle cDNA library and proposed that several actin-associated proteins, such as gelsolin and supervillin, function as AR coregulators and might modulate AR transcriptional activity in skeletal muscle. Thus, although efforts are being made to unravel the role of AR coactivators in myogenic androgen effects, their exact contribution to the genomic action of androgens in skeletal muscle remains unclear. The next section details this genomic action, including tethering of the AR by other transcription factors and androgenic regulation of polyamines and microRNAs in muscle.

Actions involving direct and indirect DNA binding by the activated AR

Wyce et al. [95] recently identified over 30,000 AR-binding sites in the chromatin of myoblasts upon stimulation with dihydrotestosterone. The majority of these binding sites contain sequences resembling the ARE consensus indicating direct AR-mediated gene regulation events by coactivator recruitment [95, 96]. However, binding sites for the myocyte enhancer factor 2 (Mef2) family of MADS-box transcription factors are also enriched in these sites [95], indicating that at least for part of the target genes, AR could be recruited indirectly by tethering via Mef2 factors. Similar AR tethering has been described via other transcription factors such as serum response factor (SRF) [97] and T cell factor (TCF) [98] (Fig. 2).

AR-binding regions were found near genes encoding androgen-regulated microRNAs, as well as, e.g., the Mef2c gene, which controls muscle differentiation via regulating the expression of other muscle-specific genes [95]. AR binding was also observed near genes encoding factors involved in sarcomere integrity and muscle contraction, like myomesin, myotilin, and myozenin [95]. In conclusion, the definition of these binding sites results in a very valuable series of new putative androgen targets, but further in vivo validation experiments are needed.

Androgen-regulated polyamine biosynthesis

The polyamines putrescine, spermidine, and spermine play a role in cell proliferation and differentiation [99]. In skeletal muscle, too, several studies have demonstrated that hypertrophy is associated with increased polyamine levels [100, 101]. Conversely, decreased levels of putrescine, spermidine, and spermine have been observed in a rat model of muscle atrophy [102]. Androgens may directly regulate polyamine biosynthesis via an upregulation of the rate-limiting biosynthetic enzymes ornithine decarboxylase and *S*-adenosylmethionine decarboxylase, encoded by the genes *Odc1* and *Amd1*. Indeed, orchidectomized male mice show a decreased expression of *Odc1* and *Amd1*, which is restored by testosterone treatment [59]. Similarly, decreased expression of *Odc1* and *Amd1* is observed in male ARKO mice compared to their wild-type littermates [59]. In addition, expression of *Odc1*, which was recently shown to promote myoblast proliferation and delay

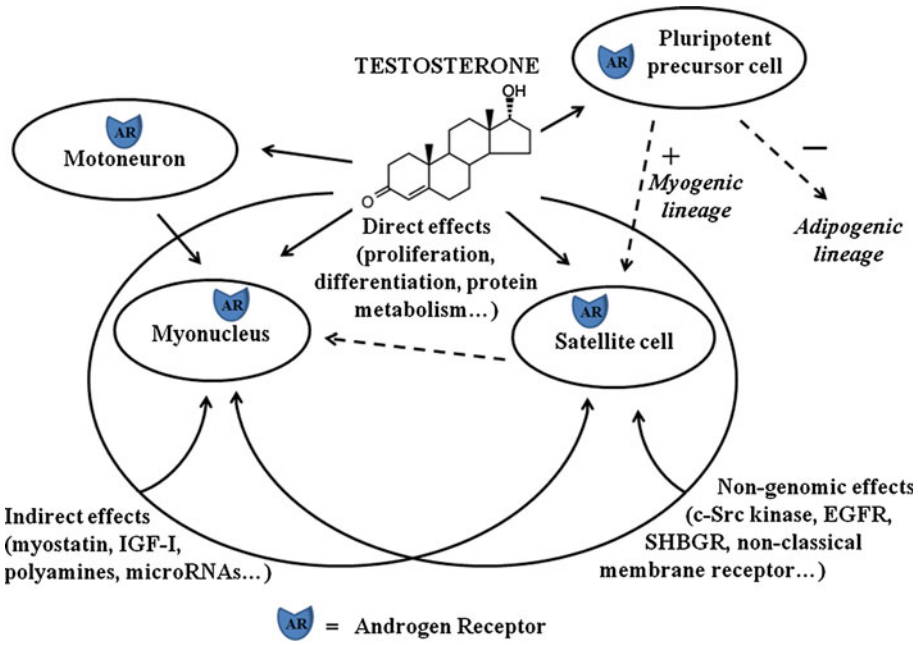

Fig. 1 Cellular targets of androgen action in skeletal muscle. Satellite cells and myonuclei are considered to be the main targets of androgen action in muscle. Other AR-expressing cell types such as pluripotent mesenchymal precursor cells and motoneurons may, however, contribute to myogenic androgen action as well. Apart from direct actions, including effects on genes regulating proliferation, myogenic differentiation, and muscle protein metabolism, indirect effects may explain at least part of the muscle hypertrophy observed following androgen administration. Non-genomic androgen pathways may be another mechanism by which androgens act on skeletal muscle. *Full arrows* indicate androgen action, *dotted arrows* depict cell differentiation. *IGF-I* insulin-like growth factor I, *EGFR* epidermal growth factor receptor, *SHBGR* sex hormone-binding globulin receptor

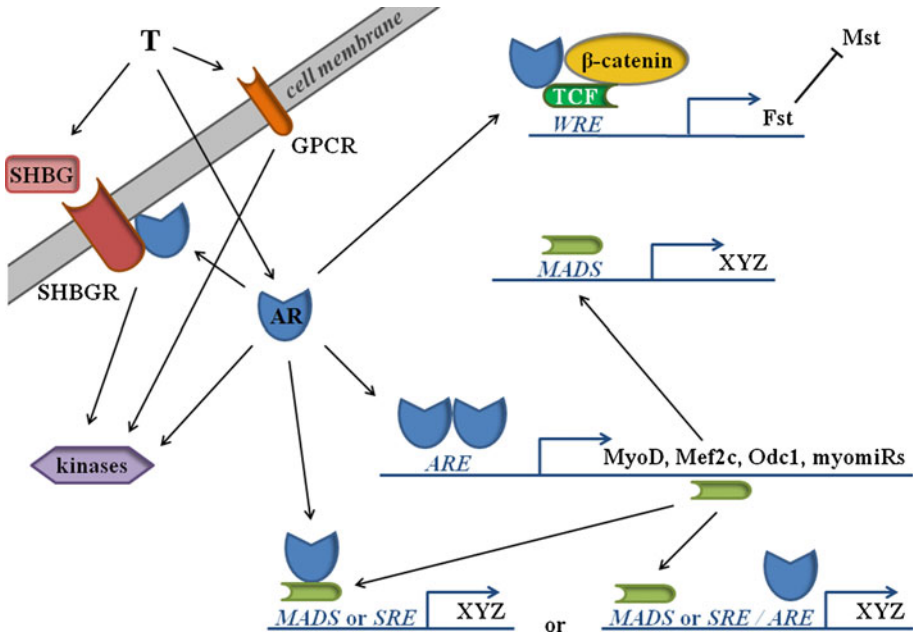

Fig. 2 Genomic and non-genomic effects of androgens in skeletal muscle. Testosterone (T) enters the cell and binds to the AR. This can act as a canonical transcription factor via binding to AREs, or be tethered (e.g., by TCF, Mef2c, or SRF) to muscle-specific enhancers. In this way, transcription of androgen target genes is activated. Some of the myomiRs are androgen targets, explaining part of the effect of androgens on translation. SHBG-bound T, T alone, or the T-activated AR can also activate membrane receptors that will act through activating specific kinases and increasing Ca^{2+} uptake. DNA response elements are depicted in *italics*. XYZ represent genes implicated in muscle development and maintenance. *SHBG(R)* sex hormone-binding globulin (receptor), *GPCR* G-protein coupled receptor, *Fst* follistatin, *Mst* myostatin

myogenic differentiation, is also decreased in a muscle-specific AR knockout mouse model [103]. Finally, a putative ARE has been described near the promoter of the *Odc1* gene [104], but this was not recovered as an AR-binding site by Wyce et al. [95].

Androgen-regulated microRNAs

MicroRNAs (miRNAs) are small (~22 nucleotides) non-coding RNA transcripts able to inhibit translation or promote messenger RNA (mRNA) degradation by annealing to complementary sequences in the 3′ untranslated regions of specific target mRNAs [105, 106]. MiRNAs are synthesized by RNA Pol II as primary miRNAs, which are converted to mature miRNAs by the RNAse enzymes Drosha and Dicer [107]. Deletion of Dicer in embryonic skeletal muscle results in perinatal lethality and a decreased skeletal muscle mass accompanied by abnormal myofiber morphology [108], hereby illustrating the essential role of miRNAs in muscle development and function. Since expression of a large number of miRNAs in rat LA muscle is reduced by orchidectomy [109], muscle-specific miRNAs, also called myomiRs, are hypothesized to be mediators of myogenic androgen action. Wyce et al. [95] identified AR-binding sites near four miRNA-encoding genes, namely miR-206, miR-133, miR-221, and miR-222. They were selected for further analysis, as they are known to be involved in myoblast differentiation [110, 111]. All four myomiRs exhibited increased expression upon dihydrotestosterone treatment [95], hereby further suggesting that their genes are direct targets of the AR in muscle. Androgen regulation of miRNAs does not seem to be restricted to the genomic level. Indeed, miRNA maturation could also be regulated by androgens, as suggested by the ligand-induced interaction between AR and Dicer in a co-immunoprecipitation assay [109]. Collectively, these data illustrate that specific myomiRs may be androgen targets in skeletal muscle.

In conclusion (Fig. 2), androgens induce AR binding to DNA, either directly to AREs, or indirectly by tethering via other transcription factors that bind to muscle-specific enhancers. In this way, protein encoding genes are upregulated and muscle-specific functions become expressed. Alternatively, the transcription of myomiR-encoding genes is upregulated, and these miRNAs may in turn serve as a feedback loop to attenuate the expression of target genes like SRF.

Crosstalk between androgens and other signaling pathways in skeletal muscle

This section will describe the crosstalk between androgens and other signaling pathways in skeletal muscle, including those of PI3K/Akt, myostatin, insulin-like growth factor I and Notch.

Phosphatidylinositol 3-kinase/Akt

PI3K/Akt and muscle

Activation of the phosphatidylinositol 3-kinase(PI3K)/Akt pathway induces an increase in skeletal muscle mass. Indeed, transgenic mice in which a mutant, constitutively active form of Akt is conditionally expressed in skeletal muscle show a dramatic increase in muscle size [112]. Stimulation of skeletal muscle development by Akt relies on two distinct mechanisms, i.e., activation of protein synthesis pathways and blocking of the transcriptional upregulation of key mediators of muscle atrophy. Indeed, activation of Akt leads to phosphorylation and activation of downstream molecules including mTOR and $p70^{s6k}$, resulting in an increase in protein synthesis [113]. On the other hand, Akt activation also leads to phosphorylation and inhibition of forkhead box O (FoxO) transcription factors, which are required for the upregulation of the muscle-specific ubiquitin ligases MuRF-1 and MAFbx, resulting in a decrease in protein degradation [113]. These ubiquitin ligases induce the proteasome-mediated degradation of particular protein substrates, and have been shown to be induced in several models of skeletal muscle atrophy in both rodents and humans [113].

Crosstalk between PI3K/Akt and androgens

Several data sets indicate that androgens activate the PI3K/Akt pathway. Indeed, testosterone treatment of primary rat myotubes significantly increased Akt and mTOR phosphorylation [114], whereas decreased levels of phosphorylated Akt accompanied by an upregulation of MuRF-1 and MAFbx were observed following orchidectomy in both rats [115] and mice [116]. Both effects were reversed by testosterone replacement [115, 116]. Activation of Akt by androgens seems to be mediated by a direct interaction of the AR with the p85 regulatory subunit of PI3K, resulting in its activation and subsequent upregulation of Akt phosphorylation [117].

Thus, androgen-mediated increase in skeletal muscle mass is, at least partly, mediated through activation of PI3K/Akt signaling, resulting in both stimulation of protein synthesis and inhibition of protein degradation (Fig. 3).

Myostatin

Myostatin and muscle

Myostatin (Mst) is a member of the transforming growth factor-β (TGF-β) superfamily that is expressed specifically in skeletal muscle [118]. Mst is a strong negative regulator

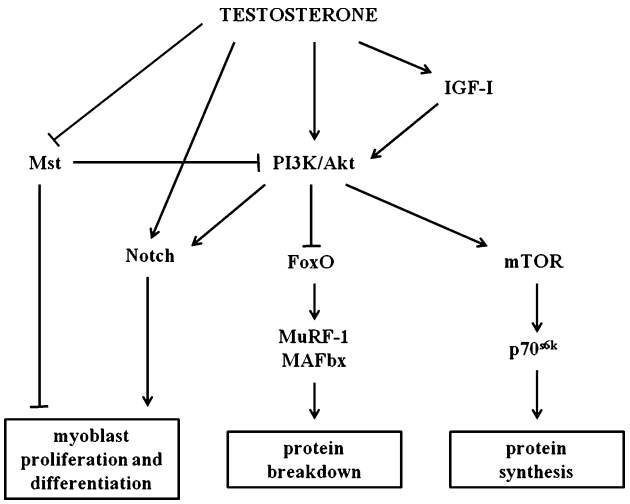

Fig. 3 Crosstalk between androgens and other signaling pathways in skeletal muscle. Testosterone activates PI3K/Akt signaling, either directly or through IGF-I stimulation. Activation of Akt leads to phosphorylation and activation of downstream molecules including mTOR and $p70^{s6k}$, resulting in an increase in protein synthesis. Furthermore, Akt activation leads to phosphorylation and inhibition of FoxO transcription factors, which are required for upregulation of the ubiquitin ligases MuRF-1 and MAFbx, resulting in a decrease in protein degradation. Testosterone also inhibits expression and activity of Mst, which represses protein synthesis and stimulates muscle atrophy though inhibition of PI3K/Akt signaling and also negatively regulates myoblast proliferation and differentiation. Finally, testosterone increases Notch signaling, which is also a downstream effector of Akt and is essential for satellite cell proliferation and myogenic progression

of muscle growth, since disruption of the Mst gene in mice, cattle and dogs induces a dramatic increase in muscle mass due to both muscle hypertrophy and hyperplasia [118–120]. Similarly, muscle-specific overexpression of Mst in mice is associated with lower muscle mass an decreased fiber size [121].

Mst seems to be involved in several processes that control muscle development and maintenance (Fig. 3). It inhibits both proliferation [122, 123] and differentiation of C2C12 myoblast [124, 125]. These inhibitory actions correlate with the upregulation of the cell cycle proteins p21 and p53 [126] and the downregulation of the myogenic factors MyoD and myogenin [125, 126]. In the same cell line, Mst was shown to dose-dependently inhibit DNA and protein synthesis [123]. Mst could also cause muscle cell atrophy by reversing the PI3K/Akt pathway, resulting in an increased FoxO transcriptional activity, which induces the expression of atrogenes [127].

The effect of Mst on satellite cells is still unresolved. As Mst knockout mice show increased satellite cell numbers [128], it has been proposed that Mst blocks the activation of satellite cells and also negatively regulates their self-renewal, thereby maintaining them in quiescence [129, 130]. However, another study showed that muscle hypertrophy in the absence of Mst involves no input from satellite cells [131].

Mst was also proposed to act on the commitment of pluripotent mesenchymal precursor cells, since the increased muscle mass in Mst knockout mice is associated with a significant reduction in adipogenesis and body fat [132, 133]. Moreover, Mst induces the expression of adipogenic markers in the pluripotent mesenchymal cell line C3H 10T1/2, whereas markers of myogenic differentiation are downregulated [134].

At the molecular level, Mst exerts its activity through the activin receptors type I and type II. Upon tetramerization of the receptor complex, the signal is relayed to the cytoplasm via SMAD proteins. Phosphorylated SMAD4 will translocate to the nucleus and regulate the expression of a specific set of target genes [135]. Moreover, the Mst-mediated effects are antagonized by follistatin (Fst) [136–138], a protein of which the expression is regulated through β-catenin signaling [84]. Fst antagonizes Mst by direct protein interaction, which prevents Mst from binding to its receptor [137].

Crosstalk between myostatin and androgens

The muscle hypertrophy observed in Mst knockout mice is more pronounced in males compared to females [118] and, conversely, muscle-specific Mst overexpression lowers muscle mass more in male than in female mice [121]. This gender specificity suggests a crosstalk between androgens and Mst, consistent with the finding of elevated Mst expression in LA muscle after orchidectomy [139]. Moreover, androgen regulation of Mst does not seem to be restricted to the repression of Mst expression at the gene level. Indeed, several studies support the hypothesis that androgens enhance β-catenin signaling, hereby increasing the expression of target genes including Fst, resulting in Mst inhibition [84]. A downregulation of axin, a negative regulator of β-catenin, is observed in orchidectomized rats treated with testosterone [140]. Moreover, co-immunoprecipitation assays showed direct interaction between AR and β-catenin, which might stabilize β-catenin and prevent it from degradation [84, 141], and might result in β-catenin-mediated tethering of the AR to specific target genes (Fig. 2). In addition, the activation of adenosine monophosphate-activated kinase (AMPK) by androgens might further contribute to the stabilization of β-catenin via phosphorylation at Ser552 [141]. A putative ARE has been identified by in silico analysis in the Mst gene promoter, but no further functional analysis has been presented [142].

Thus, there is some evidence that myogenic androgen action could, at least in part, be mediated through repression of both Mst expression and activity (Fig. 3).

Insulin-like growth factor I

IGF-I and muscle

Insulin-like growth factor I (IGF-I) is a well-characterized muscle growth-promoting factor produced mainly in the liver in response to growth hormone (GH) stimulation. It is also locally expressed in a variety of tissues including skeletal muscle, where it acts as an autocrine/paracrine growth factor under the control of multiple hormones [143]. IGF-I is regarded as an important regulator of muscle mass. Indeed, mice with targeted overexpression of IGF-I in skeletal muscle have a higher muscle mass compared to controls [144]. Stimulation of muscle mass development by IGF-I relies on multiple processes, including increases in protein synthesis and myogenesis and decreases in proteolysis and apoptosis [145, 146]. At the molecular level, IGF-I acts through binding of its specific receptor, the IGF-I receptor (IGF-IR), and subsequent activation of the PI3K/Akt pathway (Fig. 3), resulting in stimulation of protein synthesis and inhibition of FoxO nuclear translocation thereby suppressing the transcription of several atrogenes such as atrogin-1, MuRF-1 and cathepsin L [147]. Importantly, different isoforms of IGF-I can arise through alternative splicing.

Human skeletal muscle expresses two IGF-I variants, namely IGF-IEa, which is similar to the liver type or systemic form, and IGF-IEc, also called mechanogrowth factor (MGF), an autocrine/paracrine and mechanosensitive form [148, 149]. IGF-IEa and MGF are reported to have different myogenic actions. Indeed, MGF increases proliferation and inhibits terminal differentiation in C2C12 myoblast cell line, while the IGF-IEa isoform stimulates myoblast differentiation into myotubes with a smaller effect on proliferation [150]. A recent study suggests that IGF-I enhances β-catenin signaling, as treatment of C2C12 with IGF-IEa or MGF both increased nuclear β-catenin [140]. Thus, inhibition of Mst through enhanced β-catenin signaling could be an additional mechanism resulting in the stimulation of muscle mass development by IGF-I.

Crosstalk between IGF-I and androgens

Several clinical studies have demonstrated that testosterone therapy augments GH secretion [151, 152], which in turn correlates with an increase in serum IGF-I [153]. The androgen-induced stimulation of the GH/IGF-I axis has been studied extensively in animal models. It seems to be mediated centrally, since mice selectively lacking AR in the nervous system show a twofold reduction in serum IGF-I [88]. In addition, the crosstalk between IGF-I and androgens may in part be related to the aromatization of testosterone into the estrogen 17β-estradiol [21]. However, circulating GH and IGF-I may not be essential for the anabolic effects of androgens, as testosterone increases total body weight and LA muscle mass even in hypophysectomized rats that are deficient in GH and low in IGF-I serum levels [154]. Moreover, administration of high doses of dihydrotestosterone to orchidectomized rats did not change serum IGF-I concentrations although LA weight was restored to sham levels [155], and in ARKO male mice, serum IGF-I was not different from wild-type animals [59]. Collectively, these data suggest that circulating GH and IGF-I play only a minor role in mediating the anabolic effects of androgens.

There is increasing evidence that, in contrast to the circulating hormone, locally produced IGF-I is an important mediator of androgen action in muscle. Indeed, androgen treatment was found to increase IGF-I mRNA in bovine satellite cells [156] as well as in rat diaphragmatic muscle [19]. In addition, levels of IGF binding proteins (IGFBPs) were dramatically suppressed [155]. The presence of two AREs in the upstream promoter of the IGF-I gene [157] supports this hypothesis. IGF-IEa levels decreased upon orchidectomy both in LA and gastrocnemius muscle, while MGF levels remained constant [158], so IGF-IEa but not MGF expression is androgen-dependent in both perineal and limb muscles. In LA muscle of mice lacking myocytic AR a twofold reduction in IGF-IEa transcript levels was observed compared to control mice, whereas MGF levels were similar [158]. Surprisingly, in gastrocnemius muscle no difference in IGF-IEa expression was detected between mutant and control mice, suggesting that IGF-IEa expression depends on myocytic AR in perineal but not limb skeletal muscles.

A study investigating the effect of androgens on the phosphorylation of $p70^{s6k}$ provided further evidence that the muscular IGF-I system plays an important role in anabolic androgen action. The ribosomal protein kinase $p70^{s6k}$ is a downstream effector of IGF-I participating in the regulation of protein turnover in skeletal muscle [159]. Dihydrotestosterone was shown to induce phosphorylation of $p70^{s6k}$ in LA muscle of orchidectomized rats in a dose-dependent manner [155]. The phosphorylation status of $p70^{s6k}$ was decreased by the AR antagonist flutamide, suggesting that activation of intramuscular IGF-I signaling by androgens is AR-mediated.

Collectively, these data indicate that androgens interfere with the muscular IGF-I system at different levels. Moreover, the fact that IGF-I induces expression, phosphorylation, nuclear translocation and DNA binding activity of the AR in muscle [160, 161] indicates the existence of a feedback-loop between IGF-I and androgens.

Androgens and Notch signaling in muscle

Since the progressive decline of skeletal muscle mass with ageing is reported to be in large part due to a decline in

Notch signaling, Notch regulation by androgens was proposed to be involved in the protective effect of androgens on age-associated muscle degradation [39]. Testosterone-induced muscle hypertrophy in mice is accompanied by an upregulation of the Notch ligand Delta1 and an activation of Notch signaling, as evidenced by the increase in activated forms of Notch1 and Notch2 [162]. Moreover, testosterone treatment inhibited c-Jun NH_2-terminal kinase (JNK) and activated p38 mitogen-activated protein kinase (MAPK), two factors that are critical for the activation of Notch signaling [162]. Enhancement of Notch activation could also play a role in the androgen effects on satellite cells since Notch signaling is essential for satellite cell proliferation and myogenic progression [44, 45]. In addition, a study exploring androgen effects on aged muscle revealed that testosterone treatment can restore Notch signaling in old mice and reverse the age-associated increase in p21, a downstream member of the Notch cascade, which is known to interfere with satellite cell regenerative capacity [163]. As it has been demonstrated that Notch signaling is partly regulated by the PI3K/Akt cascade [164], Notch regulation by androgens could be either direct or through Akt activation (Fig. 3).

In conclusion, several pathways seem to contribute to the myogenic action of androgens. The crosstalk between androgens and other signaling molecules in skeletal muscle is summarized in Fig. 3.

Non-genomic androgen action

Androgens act predominantly through binding of the classical nuclear AR, inducing receptor dimerization, nuclear translocation and coactivator recruitment to promote transcription of target genes [18]. Increasing evidence suggests that, in addition to this genomic mode of action, androgens may also exert fast non-genomic effects within seconds to minutes after hormone administration [165, 166]. Such non-genomic effects may occur (i) through interactions between the AR and the tyrosine kinase c-Src, inducing the MAPK signaling cascade [167, 168], (ii) through interaction of the AR with the sex hormone-binding globulin (SHBG) receptor (SHBGR), increasing protein kinase A (PKA) activity [169] or (iii) by activation of a distinct non-classical receptor associated with the plasma membrane, triggering an increase in intracellular Ca^{2+} levels [170, 171].

AR–c-Src interaction and MAPK signaling

Stimulation of the MAPK pathway through interaction of the AR with c-Src may contribute to myogenic androgen action in several ways. Firstly, it is possible that this non-genomic action of the AR ultimately influences AR transcriptional activity in skeletal muscle. Indeed, AR phosphorylation by extracellular signal-regulated kinase (ERK), a downstream member of the MAPK signaling cascade, is associated with enhanced AR transcriptional activity and an increased ability to recruit the coactivator ARA70 [172]. In addition, phosphorylation of the steroid receptor coactivators (SRCs) by MAPK results in an increased ability of these coactivators to recruit additional coactivator complexes to the DNA-bound receptor [173]. Secondly, the c-Src-mediated activation of MAPK is involved in multiple cellular processes, including myoblast proliferation and differentiation [174, 175]. A recent study suggests an AR-independent mechanism of MAPK activation by androgens [176]. Dihydrotestosterone treatment of isolated intact mammalian skeletal muscle fiber bundles increased both twitch and tetanic contractions in fast twitch fibers, and these changes were accompanied by an increase in MAPK/ERK phosphorylation. Interestingly, these effects were insensitive to inhibitors of c-Src and AR, but abolished by an inhibitor of the epidermal growth factor receptor (EGFR), suggesting that the non-genomic effects of androgens on skeletal muscle involve the EGFR.

AR–SHBGR interaction and PKA activity

The majority of testosterone and dihydrotestosterone in human serum is complexed to SHBG [177]. Its action as a steroid transporter is well known, but it could also affect target cells via a specific cell surface receptor for SHBG which has been reported in a number of tissues including skeletal muscle [178]. The intracellular interaction of the AR complex with this SHBGR was proposed to increase PKA activity [169] and this could influence AR-mediated transcription by altering the phosphorylation of the AR and its coactivators [179–181]. However, whether SHBG has similar effects on the skeletal muscle has not been demonstrated yet [181, 182].

Activation of a non-classical plasma membrane receptor

Testosterone induces a rapid increase in intracellular Ca^{2+} level in several cell types [171]. Possibly, this involves a membrane binding site which is saturable and selective for androgens but immunologically and functionally different from the classical intracellular AR [170]. This transient Ca^{2+} increase is sensitive to the G-protein coupled receptor (GPCR) inhibitor pertussis toxin, suggesting that this membrane androgen-binding protein is either a GPCR or that its function is closely linked to one [183].

A rapid increase in intracellular Ca^{2+} in response to androgens has also been observed in primary cultures of rat

myotubes treated with testosterone and this Ca^{2+} increase was preceded by an increase in inositol 1,4,5-triphosphate (IP3) [20]. In addition, exposure of these myotubes to androgens produced an IP3-Ca^{2+} dependent and pertussis toxin-sensitive increase in ERK phosphorylation [184]. Indeed, the increase in intracellular Ca^{2+} is followed by the activation of several signal transduction cascades, including PKA and MAPK [185]. As already mentioned, PKA and MAPK/ERK activity influence AR-mediated transcription by altering the phosphorylation of the AR and its coactivators.

Androgens have also been reported to exert AR-independent effects on skeletal muscle. In the AR-negative rat L6 myoblast cell line, testosterone promotes both proliferation and differentiation. Bovine serum albumin (BSA)-linked testosterone, which does not cross the plasma membrane, has similar effects as free testosterone. The inhibition of these effects by pertussis toxin further suggests the involvement of a GPCR. Using specific inhibitors, it is shown that the stimulation of L6 proliferation by testosterone involves the MAPK/ERK pathway, whereas PKA signaling plays a role in androgen-mediated stimulation of L6 differentiation [186].

Thus, myogenic androgen effects are partly mediated through non-genomic pathways (Fig. 2), including activation of a non-classical G-protein linked binding site on the plasma membrane of myoblasts resulting in Ca^{2+}-dependent activation of several kinases. However, the relative contribution as well as the exact mechanisms through which these non-genomic effects impact skeletal muscle growth and maintenance have to be further elucidated.

Selective androgen receptor modulators

As discussed in the previous sections, it is now well established that androgen administration increases muscular and lean body mass. Thus, testosterone could potentially be exploited in the treatment of muscle wasting caused by various underlying diseases. However, these therapies may have severe side-effects, including the stimulation of prostate hyperplasia in men, as well as virilization in women [187]. Therefore, therapeutic agents that could achieve anabolic effects on skeletal muscle without androgenic activities such as prostatic effects and virilization are of great clinical interest. A first approach to achieve tissue selectivity is to elucidate the mechanisms of androgen action in skeletal muscle and prostate, and to identify signaling molecules that are downstream of the AR and which activate pathways involved in skeletal muscle hypertrophy but not in prostatic growth. From this point of view, Mst is an ideal target molecule, since β-catenin and TGF-β/SMAD signaling play essential roles in mediating testosterone effects on myogenic differentiation (see previous sections). This strategy is currently being explored [188, 189].

A second approach to dissociate anabolic and androgenic activities of androgens is the development of tissue-selective AR-ligands, also called selective androgen receptor modulators (SARMs). A well-established body of evidence supports their in vivo tissue selectivity in animal models [190]. However, the mechanisms by which SARMs achieve the observed tissue selectivity are not fully elucidated. Several hypotheses have been proposed, although these hypotheses are not mutually exclusive. Firstly, most SARMs are nonsteroidal and are therefore not substrates for reduction by 5α-reductase, an enzyme highly expressed in androgenic tissues including the prostate and responsible for amplification of testosterone action in these organs by its conversion to the more potent dihydrotestosterone [191]. Secondly, several studies have shown that SARMs induce a conformational change of the AR that is distinct from testosterone, thus recruiting different coregulator complexes [192]. Finally, the earlier mentioned non-genomic pathways may play a role in the mode of action of SARMs [193].

Although the mechanisms by which SARMs work at the molecular level are still debated, the immense interest regarding the therapeutic potential of SARMs in humans has culminated in the development of several compounds being evaluated in phase I clinical trials [190]. Recently, Dalton et al. [194] have reported a phase II clinical trial where GTx-024, an orally available nonsteroidal SARM, has been tested in healthy elderly men and postmenopausal women. GTx-024 treatment significantly increased total lean body mass and improved physical function, whereas no increased adverse effects were observed compared to placebo. The effects of SARMs on body composition and muscle strength are not only promising for future muscle-wasting treatment strategies, but also illuminate the mechanisms of anabolic androgen action. Indeed, unlike testosterone, nonsteroidal SARMs are not substrates for 5α-reductase or for aromatase, indicating that, although reduction to dihydrotestosterone and aromatization to estradiol may contribute to myogenic androgen action to some extent, these conversions are not essential for mediating androgen response in skeletal muscle.

Outlook

The effects of androgens on skeletal muscle are very diverse and are mediated via different cellular targets as well as biochemical pathways, as summarized in Figs. 1 and 2. Clinical studies complemented with animal models and in vitro cell cultures continue to enhance our

understanding of these processes. However, despite growing clinical interest in anabolic action of androgens, many research questions remain largely unresolved. What is the relative importance of the many pathways that may cross talk with androgen action in skeletal muscle? How large is the non-genomic contribution of androgen action? What are the main cellular targets under normal physiological as well as clinical conditions? What are the direct AR targets in the different cell types? Which pathways can safely be used in therapeutic strategies for the treatment of disease or age-related muscle wasting? It is expected that the development of cell- and stage-specific knockout or knockin approaches combined with recently developed techniques like ChIP-seq, transcriptomics, proteomics and metabolomics in model organisms as well as human subjects will provide new insights which will serve as inspiration for the development of clinical applications.

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