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ROLE OF IGF/INSULIN PATHWAY IN THE SKELETAL MUSCLE HYPERTROPHY INDUCED BY FOLLISTATIN

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TABLE OF CONTENTS

SUMMARY

Increasing size and strength of skeletal muscle represents a promising therapeutic strategy for muscular disorders. One possible tool is the inhibition of Myostatin (Mstn), a major inhibitor of skeletal muscle development. Among Mstn inhibitors, Follistatin (FS) induces the most dramatic effect on muscle mass growth. The molecular mechanisms involved in the FS effect are however relatively unknown. An interaction between Mstn and Insulin-like growth factor (IGF) pathways has been suggested by increased expression of IGFs and activation of its downstream pathway Akt/mTOR/S6K in the skeletal muscle when Mstn is inhibited.

The aim of this thesis was to investigate the contribution of IGF-IR/Akt/mTOR/S6K pathway to the FS induced skeletal muscle hypertrophy. Our study shows that the hypertrophic action of FS requires the IGF-IR, Akt and, to some extent, mTORC1 but not S6K. As IGF-IR is activated either by IGFs or by insulin, we tend to delineate the role of each ligand. Our results indicate that the hypertrophic effect of FS requires the presence of insulin but does not seem to require the presence of IGFs.

The present study connects the Mstn and the IGF-IR pathways, the two most important pathways that regulate muscle mass. Defining the key pathway for muscle hypertrophy is a crucial issue for the correct identification of drug targets to mitigate muscle wasting.

LIST OF ABBREVIATIONS

4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1
Act	Activin
ActRIIA/B	Activin Receptor type II A/B
ALK4/5	Activin-Like Kinase-4/5
AMPK	AMP-activated protein kinase
AR	Androgen receptor
BAMBI	BMP and activin membrane-bound inhibitor
BMP	Bone morphogenetic protein
Cdk	Cyclin-dependent kinase
CMV	Cytomegalovirus
CSA	Cross sectional area
CTRL	Control
DAB	Diaminobenzidine
Deptor	Disheveled, egl-10, pleckstrin domain protein interacting with mTOR
dn	dominant negative
DNA	Desoxyribonucleic acid
eEF2 kinase	Eukaryotic elongation factor 2 kinase
eIF 3f/4B/4E	Eukaryotic initiation factor 3f/4B/4E
endofin	endosome associated FYVE domain protein
Erk1/2	Extracellular signal-regulated kinase 1/2
FLRG	Follistatin-like related gene
FKBP12	FK506 binding protein 12
FS	Follistatin
FoxO	Forkhead box O
GAPDH	Glyceraldehyde-3-phosphate deshydrogenase

GASP-1	Growth and differentiation factor-associated serum protein-1
GC	Gastrocnemius
GDF-8/11	Growth and differentiation factor-8/11
GH	Growth hormone
GSK-3β	Glycogen synthase kinase-3β
HA	Hemagglutinin
HS	Heparan sulfate
hSGT	human small glutamin-rich tetratricopeptide repeat-containing protein
IGF-I	Insulin-like growth factor I
IGF-II	Insulin-like growth factor II
IGFBP	IGF binding protein
IGF-IR	Type I IGF receptor
IGF-IIR	Type II IGF receptor
INS	Insulin
IR	Insulin receptor
IRS-1	Insulin receptor substrate-1
JNK	c-Jun N-terminal kinase
kDa	kilodalton
KO	Knock-out
MAPK	Mitogen-activated protein kinase
MEF2	Myocyte enhancer factor 2
MHC	Myosin heavy chain
miR	micro RNA
mLST8	mammalian lethal with SEC13 protein 8
MKR	Murine creatine kinase, muscle promoter directing the expression of the human IGF-IR gene containing the K1003R mutation

MRF	Muscle regulatory factor
mRNA	messenger ribonucleic acid
mSIN1	mammalian stress-activated protein kinase interacting protein 1
Mstn	Myostatin
mTOR	mammalian target of rapamycin
mTORC1/2	mTOR complex 1/2
mTr-FS	muscle transgenic follistatin
MurF-1	Muscle ring finger protein-1
NS	Non significant
Pax-3/7	Paired box gene-3/7
PDK-1	Phosphoinositide-dependent protein kinase 1
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator-1α
PH	Pleckstrin homology
PI3kinase	Phosphoinositide kinase-3
PIP2	Phosphatidylinositol biphosphate
PIP3	Phosphatidylinositol triphosphate
PKB	Protein kinase B or Akt
PP2A	Protein phosphatase 2A
PRAS40	Proline-rich Akt substrate of 40 kDa
pRB	Retinoblastoma protein
Protor-1	Protein observed with Rictor-1
PTEN	Phosphatase and tensin homolog
Rapa	Rapamycin
Raptor	Regulatory associated protein of mTOR
Rictor	Rapamycin-insensitive companion of mTOR
rpS6	ribosomal protein S6
S6K	ribosomal protein S6 Kinase

sActRIIB-Fc	soluble ligand binding domain of the Activin Receptor type IIB fused to the Fc domain IgG
SARA	Smad anchor for receptor activation
SHIP	SH2-domain-containing inositol phosphatase
Smad	Drosophilia mothers against decapentaplegic protein
SMURF	Smad ubiquitination-related factor
STZ	Streptozotocin
TA	Tibialis anterior
TGF-β	Transforming growth factor-β
TSC1/2	Tuberous sclerosis complex 1/2
WT	Wild-type
YAP	Yes-associated protein

Chapter 1

GENERAL INTRODUCTION

1. Skeletal muscle hypertrophy

1.1. DEFINITION AND MECHANISMS

Skeletal muscle hypertrophy is defined as a gain of skeletal muscle mass and occurs during postnatal development or in adult skeletal muscle in response to various stimuli such as exercise or anabolic hormonal stimulation. Muscle growth is obtained by either increase in muscle fiber number (hyperplasia) or an increase in the size of individual myofibers (hypertrophy), or in combination of both.

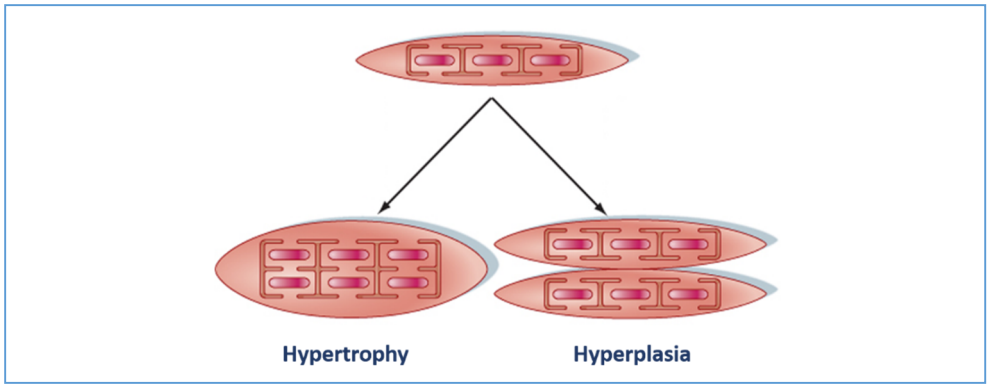

Figure 1. Muscle growth and fiber hypertrophy and hyperplasia
Figure adapted from Berne & Levy Physiology, 6Th Edition
by Bruce M Koeppen and Bruce A. Stanton, 2008 (1)

This increase of skeletal muscle mass results from changes in protein and cell turnover (2). During postnatal muscle growth, satellite cells, the stem cells of skeletal muscle, undergo proliferation and fuse to constitute new muscle growing fibers concomitantly with increased protein synthesis (3). In contrast, in the adulthood, muscle hypertrophy is mostly characterized by fiber hypertrophy and results from changes in protein turnover. This enhancement of muscle fiber size occurs when the rate of new protein synthesis exceeds the rate of degradation of

pre-existing proteins (4). Nutrients and growth factors are primary regulators of net protein balance and myofiber hypertrophy. Moreover, muscle stretching and loading also increase muscle mass and protein synthesis.

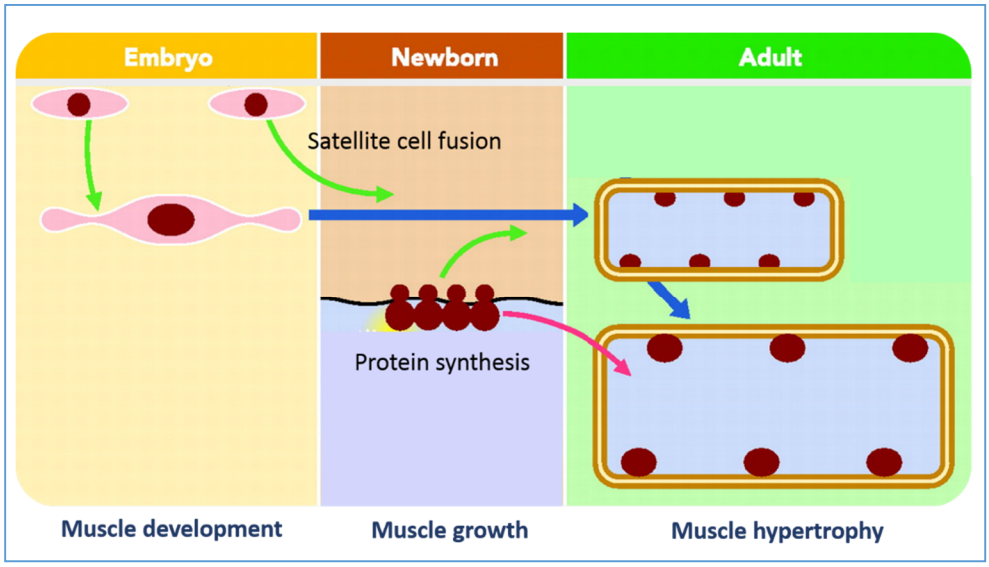

Figure 2. Schematic description
of the different contribution of cellular and protein turnover to muscle growth
Figure adapted from Sandri M, Physiology 23: 160-170, 2008

The role of satellite cells in muscle hypertrophy is still debated. The idea that satellite cells are required for muscle hypertrophy is suggested by the fact that each myonucleus within a muscle fiber is associated with a defined volume of cytoplasm, the myonuclear domain. Therefore, when the amount of cytoplasm enhances during muscle hypertrophy, this hypothesis supposed a parallel increase in the number of myonucleus per fiber to keep the myonuclear domain constant (5-7). So, the addition of new myonuclei to the existing myofiber would come from the fusion of satellite cells (3,8). The role of satellite cells has also been suggested by the increased activation of satellite cells in various models of skeletal muscle

hypertrophy (resistance exercise, overload, hormonal factors such as IGF-I or testosterone) (7 ,8-11). However, hypertrophy in some cases can occur without satellite cell activation (Myostatin inhibition, Akt overexpression, β-adrenergic agonists) (12-14). Moreover, recent studies using mice deficient in syndecan4 or Pax7, two essential proteins for satellite cell function, have shown that muscle hypertrophy can occur without a contribution of satellite cells (15). The discordant conclusions regarding the contribution of satellite cell to muscle hypertrophy may vary according to the nature of the hypertrophic stimulus and the time point of analysis after application of the growth stimulus. Indeed, some researchers proposed that skeletal muscle hypertrophy occurs in successive phases with an initial phase of increased protein synthesis and a later phase of satellite cell activation (16), suggesting that satellite cell addition is necessary only if a certain threshold myofiber size is reached.

1.2. STIMULI OF SKELETAL MUSCLE HYPERTROPHY

1.2.1. Exercise

Resistance or strength training consists of mechanical stimuli which increase skeletal muscle mass and strength. This increase in muscle mass is characterized by an increase of muscle fiber size and results from a stimulation of muscle protein synthesis (17). The rise of muscle fiber size is greater for the fast twitch fiber (type II) compared to the slow twitch fiber (type I). In the long term, resistance training may also induce an increase in fiber number (18). The effect of resistance exercise on protein synthesis is carried out by the activation of mTOR (mammalian target of rapamycin), a key regulator of the mRNA translation into proteins and more globally of the cell size (19). Interestingly, resistance training increases the muscle expression of IGF-I (Insulin-like growth factor I), a stimulus of mTOR and a positive regulator of skeletal muscle mass (20,21). In contrast, endurance training which involves low-resistance work of longer duration, does not increase muscle mass but increase the aerobic capacity of the skeletal muscle by stimulating the shift to slower muscle fiber and by increasing the number of mitochondria (22).

The specific adaptation of skeletal muscle in response to resistance or endurance exercise might be explained by the specific upregulation of PGC-1α (Peroxisome proliferator-activated receptor gamma coactivator-1α) protein variants. Indeed, resistance training induces the expression of PGC-1α4 protein, known to induce skeletal muscle hypertrophy by stimulating the expression of the anabolic hormone IGF-I and downregulating the expression of myostatin (Mstn), a major negative regulator of skeletal muscle growth (23). In contrast, endurance training stimulates the expression of PGC-1α1 protein which mediates many effects of endurance training: mitochondrial biogenesis, fiber-type switching and

angiogenesis (24-26). Moreover, muscle overexpression of PGC-1α1 in transgenic mice has no effect on muscle mass or strength.

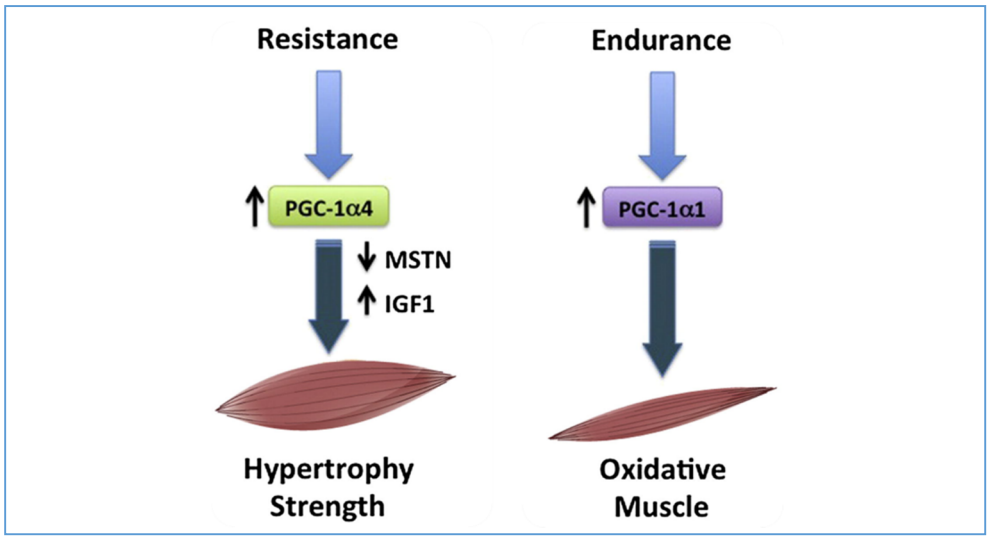

Figure 3. PGC-1α protein variants and the muscle adaptation to exercise
Figure adapted from Ruas JL, Cell 151: 1319-1331, 2012

1.2.2. Nutrition

The anabolic effects of nutrition on skeletal muscle are principally driven by the transfer and incorporation of amino acids captured from dietary protein sources into skeletal muscle proteins. Essential amino acids supplementation, in particular leucine supplementation, is known to stimulate acutely skeletal muscle protein synthesis by activating mTOR (27-29). In contrast, chronic intake of amino acid supplements does not increase skeletal muscle mass (30). However, amino acid supplementation plays a crucial role in skeletal muscle hypertrophy induced by exercise. In fact, the intake of food, in particular amino acids and carbohydrates, after a resistance exercise is necessary for skeletal muscle hypertrophy to occur (31). Dietary amino acids may also control skeletal mass by regulating the IGF-I

production in the liver but also in skeletal muscle (32). Indeed, protein restriction is associated with reduced circulating and muscle IGF-I concentrations which may limit muscle growth (33).

Recently, two compounds naturally present in the food have been shown to promote muscle growth: ursolic acid and tomatidine (34,35). Ursolic acid is a waxy component in apple peels and other edible herbs and fruits. Tomatidine results from the hydrolyzation in the gut of animals of α-tomatine, a glycoalkaloid abundantly present in immature, green tomatoes. Ursolic acid and tomatidine have been shown to induce skeletal muscle hypertrophy in mice by increasing muscle fiber size accompanied by increased strength and exercise capacity (34-36). Ursolic acid-induced hypertrophy results from the activation of Akt/PKB (protein kinase B)/mTOR signaling by enhancing the stimulation of insulin and IGF-I receptors (IR and IGF-IR) (34). In contrast, muscle hypertrophy induced by tomatidine results from activation of mTORC1 independently of Akt (35).

1.2.3. Hormones

1.2.3.1. Testosterone

Testosterone administration increases skeletal muscle mass in young and older men, particularly in case of androgen deficiency (37-40). Testosterone anabolic effects on skeletal muscle are dependent on testosterone dose and circulating concentrations, and often requires supraphysiological doses in healthy subjects (38). Skeletal muscle hypertrophy induced by testosterone is characterized by muscle fiber hypertrophy of both type I and type II (41). This hypertrophy results from an increase in protein synthesis (42) and from a stimulation of satellite cell proliferation and differentiation (11). Moreover, testosterone has the ability to promote myogenic differentiation of pluripotent mesenchymental stem cells (43). The *in vitro* effect of testosterone on muscle cell size is mediated by the nuclear

androgen receptor (AR) and by the PI3K (phosphoinositide-3 kinase)/Akt pathway (44). Interestingly, testosterone stimulates in the skeletal muscle the expression of IGF-I (45) and the activation of S6K (ribosomal S6 kinase) (46), a downstream effector of IGF-I implicated in the activation of protein synthesis. However, IGF-IR signaling is not mandatory for the anabolic effect of testosterone on skeletal muscle (47). Finally, testosterone downregulates the Mstn expression (48) and activity (49). The inhibition of Mstn activity exerted by testosterone is mediated through the upregulation of follistatin (FS) expression, an inhibitor of Mstn.

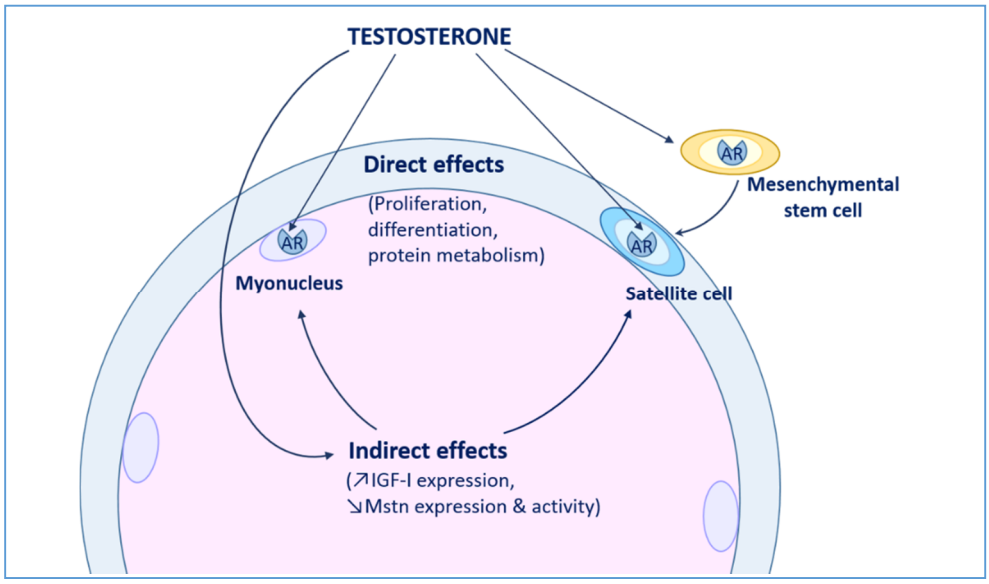

Figure 4. Mechanisms of testosterone action on skeletal muscle
Figure drawn by S Kalista according to Dubois V, Cell Mol Life Sci, 69(10): 1651-67, 2012 (50)

1.2.3.2. Insulin-like growth factors

The Insulin like growth factor (IGF) family is constituted by IGF-I and IGF-II, two proteins playing a critical role for normal growth and development. IGF-I is considered as the major positive regulator of skeletal muscle mass. Indeed, IGF-I administration by infusion (51), transgenic muscle specific overexpression (52) or

viral gene delivery (53) enhances skeletal muscle mass in rodents. In contrast, IGF-I deletion induces a severe muscle hypoplasia (54). It is suggested by *in vitro* (55-57) and *in vivo* (58,59) observations, that IGF-II might also stimulates the development of skeletal muscle. The regulation of skeletal muscle mass by IGFs will be detailed in the second chapter of the general introduction.

1.2.3.3. Insulin

Insulin is synthetized and secreted by pancreatic β cells to maintain homeostasis of blood glucose levels by stimulating glucose influx in skeletal muscle and adipose tissue and by inhibiting its production by the liver. It has been suggested that insulin regulates skeletal muscle development since insulin receptor (IR) null mice present a moderate loss of muscle mass which is exacerbated when both IR and IGF-IR (type I IGF receptor) are invalidated in skeletal muscle (60). Insulin plays an anabolic role in skeletal muscle by enhancing amino acids transport in skeletal muscle (61,62) and by increasing the rate of protein synthesis (63). Furthermore, insulin stimulates myogenesis via PI3K/mTOR/S6K and the p38 MAPK (mitogen-activated protein kinase) pathways (64).

1.2.3.4. Myostatin

Myostatin (Mstn) or GDF-8 (Growth and differentiation factor 8), a growth factor of the TGF-β (Transforming growth factor-β) superfamily, is a strong negative regulator of skeletal muscle mass. Indeed, Mstn gene deletion increases muscle mass and strength (65,66). In contrast, Mstn overexpression in the muscle induces a decrease of skeletal muscle mass (67).

The regulation of skeletal muscle mass by Mstn will be detailed in the third chapter of the general introduction.

1.2.3.5. BMPs

BMPs (Bone Morphogenetic Proteins) are growth factors of the TGF-β superfamily which have recently been shown to promote skeletal muscle growth. Indeed, activation of the BMP signaling (68,69) or muscle administration of BMP-7 (68) induces skeletal muscle and fiber hypertrophy mediated by the activation of Smad (Drosophilia mothers against decapentaplegic protein) 1/5/8. This skeletal muscle hypertrophy is characterized by increased IGF-I expression and by the activation of the Akt/mTOR pathway in muscle (68). Moreover, mTOR is indispensable for the BMP induced skeletal muscle hypertrophy (68). In opposite, inhibition of BMP signaling in muscle is sufficient to induce muscle fiber atrophy (69).

1.3. TRANSDUCTION PATHWAYS OF SKELETAL MUSCLE HYPERTROPHY

1.3.1. Akt/mTOR/S6K pathway

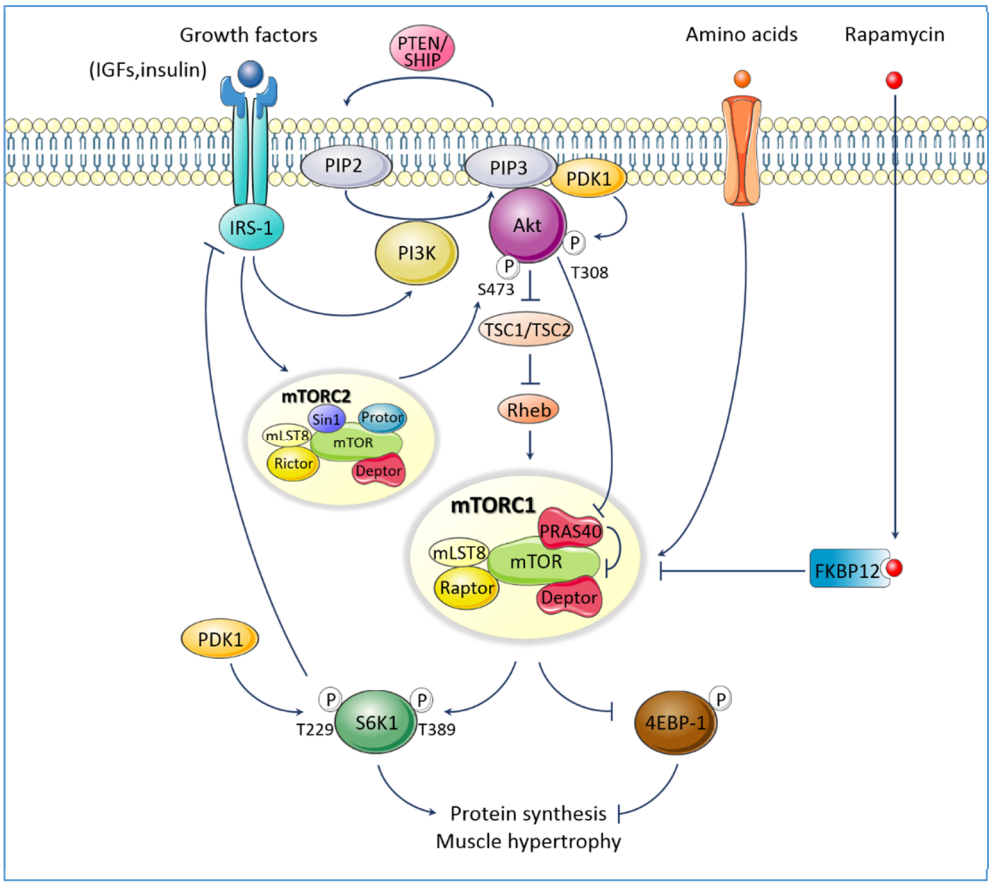

Figure 5. The PI3K/Akt/mTOR/S6K pathway and muscle hypertrophy

Figure drawn by S Kalista

Binding of growth factors such as IGFs or insulin to their receptor induces the activation of PI3K. PI3K catalyses the conversion of PIP2 to PIP3, providing a docking site fot PDK1 and Akt. Akt requires for activation the phosphorylation from PDK-1 and mTORC2 on T308 and S473 respectively. Akt phosphorylates and inhibits TSC2 and PRAS40 allowing mTORC1 activation. Amino acids activates mTORC1 independently of PI3K/Akt. Once activated, mTORC1 stimulates protein synthesis by the activation of S6K1 and by the inhibition of 4EBP-1. Full activation of S6K1 requires phosophorylation by mTORC1 on T389 and by PDK1 on T229. S6K1 is implicated in a negative feedback loop of PI3K/Akt signalling by targeting IRS-1 by phosphorylation and by repression of IRS-1 gene expression. Pharmacological inactivation of mTORC1 by rapamycin is mediated by the binding of the Rapamycin-FKBP12 complex to Raptor.

1.3.1.1. Akt

Definition

Akt, also known as protein kinase B (PKB), is a serine/threonine kinase. Mammals harbor three isoforms of Akt: Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ encoded by separate genes and sharing 80% amino acid sequence identity. In the muscle, Akt1/PKBα and Akt2/PKBβ are expressed at higher levels compared with Akt3/PKBγ, which is mainly expressed in the brain (70). Although Akt1 and Akt2 isoforms are assumed to have identical or similar substrate specificity (71), Akt1 and Akt2 have distinct role in skeletal muscle. Indeed, it is well established that both Akt1 and Akt2 have muscle anabolic action but only Akt2 regulates glucose disposal in muscle (72). While the Akt1 stimulating pathway implicated in the muscle hypertrophy is clearly understood, little is known about the Akt2 stimulating pathways. The next parts of this work explores the role of Akt1 signaling in the muscle hypertrophy and Akt1 is abbreviated as Akt.

Mechanisms of regulation

Akt acts as a major signal transducer downstream of the IRS-1/PI3K pathway. The binding of growth factors such as IGFs or insulin to the IGF-IR or the IR activates its tyrosine kinase activity. This results in its trans-phosphorylation and the subsequent phosphorylation of IRS (Insulin receptor substrate)-1. Once phosphorylated, IRS-1 binds to the p85 subunit of the PI3K, a critical step in its activation (73). Activated PI3K (p110 subunit) catalyzes the transfer of a phosphate group to the plasma membrane lipid phosphatidylinositol (4, 5)-bisphosphate (PIP2) to produce phosphatidylinositol (3, 4, 5)-trisphosphate (PIP3). PIP3 accumulation in the plasma membrane in response to PI3K activation, leads to the recruitment of Akt and PDK1 to the plasma membrane via the binding of their pleckstrin homology (PH) domain (74). Once translocated to the plasma membrane,

Akt is phosphorylated by PDK1 on Thr308 (75). To complete a full kinase activity, Akt is phosphorylated on Ser473 by mTORC2 (Mammalian target of rapamycin complex 2) and other unknown kinases in the skeletal muscle (76-79). Then, phosphorylated Akt is released from plasma membrane and is translocated to both cytosol and nucleus, whereas it phosphorylates a large number of substrates.

Akt activity can be modulated by directly controlling its phosphorylation state or by altering the levels of PIP3 that it binds to the cell membrane (74). Indeed, protein phosphatase 2A (PP2A) has been shown to dephosphorylate Akt. Moreover, SHIP (SH2-domain-containing inositol phosphatase) and PTEN (Phosphatase and tensin homologous on chromosome 10) are two phosphatases which dephosphorylate PIP3, the membrane binding site for Akt.

Akt and muscle hypertrophy

Akt1 knockout mice present defect in both fetal and postnatal growth, suggesting that Akt1 is required for normal organ growth (80). In contrast, muscle Akt1 overexpression by electrotransfer or transgenesis is sufficient to cause a dramatic skeletal muscle hypertrophy (81-83). Moreover, the activity of Akt increases in several models of skeletal muscle hypertrophy such as functional overload, IGF-I treatment or Mstn inhibition (81,84). The expression of a dominant-negative mutant form of Akt1, which inhibits the endogenous activity of this protein, blocks the myotube hypertrophy induced by IGF-I *in vitro* (85). Taken these observations together, Akt seems to be a major mediator of skeletal muscle hypertrophy.

1.3.1.2. mTOR

Definition

Mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine kinase that integrates signals from nutrients (amino acids and energy) and growth factors to regulate cell growth and cell cycle progression coordinately (86). In mammal cells, mTOR is implicated in two functionally distinct complexes: mTOR complex 1 (mTORC1) and 2 (mTORC2). mTORC1 comprises mTOR, Raptor (Regulatory associated protein of mTOR), mLST8 (mammalian lethal with SEC13 protein 8), PRAS40 (Proline-rich Akt substrate of 40 kDa) and Deptor (Disheveled, egl-10, pleckstrin domain protein interacting with mTOR) while mTORC2 comprises mTOR, Rictor (Rapamycin-insensitive companion of mTOR), mLST8, mSIN1 (mammalian stress-activated protein kinase interacting protein 1), Protor-1 (Protein observed with Rictor-1) and Deptor (87). In skeletal muscle, it has been well established that mTORC1 plays a central role in muscle mass regulation (79).

Mechanisms of regulation

mTORC1 activity can be regulated by multiple signaling pathways. mTORC1 activation in response to growth factors has been linked to the activation of PI3K/Akt1 pathway. Indeed, activated Akt1 is able to inhibit by phosphorylation the TSC (Tuberous sclerosis complex) 1-TSC2 complex, a critical negative regulator of mTORC1 (88-90). The inactivation of TSC1/TSC2 complex allows the activation of mTOR by the GTPase Rheb (91). Moreover, activated Akt1 phosphorylates PRAS40 which results in its dissociation from mTORC1 and an increase in mTORC1 signaling (92). mTORC1 can also be activated by amino acid availability independently of TSC2 (93). mTORC1 activation by amino acids is mediated by the Rag family of GTPases, which interact with Raptor (94).

Rapamycin, a macrolide produced by *Streptomyces hygroscopicus*, is a specific and potent inhibitor of mTORC1, although mTORC2 is also affected during chronic treatment (95,96). Rapamycin binds to FKBP12 (FK506 binding protein 12) and this complex binds to Raptor and results in inactivation of mTORC1.

mTORC1 and muscle hypertrophy

The crucial role of mTORC1 in mediating muscle growth is supported by genetic and pharmacological evidences. Muscle specific knockout of Raptor (79) or mTOR (97) causes reduced muscle mass characterized by a reduction of muscle fiber size and signs of muscle dystrophy. Treatment with rapamycin blocks muscle growth during regeneration (82) and attenuates muscle fiber hypertrophy. Indeed, rapamycin treatment strongly blocks myotube hypertrophy induced by IGF-I (85,98) or testosterone (44) treatment and muscle hypertrophy induced by overload (81) or by Akt overexpression (82). In humans, rapamycin treatment prevents the induction of protein synthesis induced by resistance exercise (19). In contrast, rapamycin treatment blocks only partially muscle hypertrophy induced by Mstn inhibition (99) and does not prevent induction of protein synthesis in this model (100). This suggests that other signaling pathways, independent of mTOR, play a role in the induction of muscle hypertrophy at least in response to Mstn inhibition.

The effect of mTORC1 on the regulation of protein synthesis is mediated by the inactivation of 4EBP-1 (eIF4E-binding protein 1) and by the activation of S6K (ribosomal protein S6 kinase) (87). 4EBP-1 inhibits the translation initiation by interacting with eIF4E (eukaryotic translation initiation factor 4E) preventing eIF4E to associate with eIF4G to form the active eIF4F complex, a necessary component of the 40S initiation complex controlling cap-dependent translation. S6K is known to regulate translation initiation and elongation and ribosome biogenesis.

1.3.1.3. S6 Kinase

Definition

In mammals, S6 kinases (S6Ks) represent a family composed of two distinct genes coding for S6K1 and S6K2 serine/threonine kinase proteins. S6K1 is a major regulator of body and organ size. Indeed, deletion of S6K1 in mice induces a small size at birth and in the adulthood (101). Moreover, deletion of both S6K1 and S6K2 induces the same reduction of size than S6K1 alone suggesting that S6K2 cannot compensate for the growth defect due to S6K1 deletion (102). S6K1 regulates cell size through its ability to regulate protein synthesis via the phosphorylation of several proteins involved in translation initiation and elongation including rpS6 (ribosomal protein S6), eIF4B (eukaryotic translation initiation factor 4B) and eEF2 kinase (eukaryotic elongation factor 2 kinase) and via regulation of ribosome biogenesis (87). In the skeletal muscle, S6K1 plays a crucial role for normal muscle growth (103). Since the most extensive studied isoform of S6K1 is the 70 kDa S6K1 isoform, p70S6K1 is abbreviated as S6K1 in this work.

Mechanisms of regulation

S6K1 C terminal domain has an auto-inhibitory function since its binds to the N terminal domain leading to mask the kinase domain of the protein. The activation of S6K1 begins with the phosphorylation of four serine residues localized on the C terminal domain (Ser 411, 418, 421 and 424). This allows the activation of S6K1 by phosphorylation on a Thr389 residue by mTORC1 and on a Thr 229 residue by PDK1 (104). S6K1 activity can be inhibited via its dephosphorylation by the protein phosphatase 2A (PP2A) (105).

S6K1 controls negatively the activity of the PI3K/Akt pathway by inactivating IRS-1 function via direct phosphorylation on Ser302 of IRS-1 and IRS-1 gene repression (106).

S6K and muscle hypertrophy

Constitutive deletion of S6K1/2 induces a reduction of muscle weight characterized by reduced muscle fiber size with unchanged number of myonuclei (103). Deletion of S6K1 is sufficient to reproduce this atrophic phenotype. However, S6K1 knockout mice show no impairment in protein synthesis and in protein degradation (107). Interestingly, the activation of AMP-activated kinase (AMPK) induced by S6K deletion plays a critical role in the atrophic phenotype (108). In opposite, overexpression of a constitutively active form of S6K1 is sufficient to induce myotube hypertrophy (85). Moreover, IGF-I hypertrophic action is dependent of S6K1 since myotube lacking S6K1 have an impaired hypertrophic response to IGF-I (103).

1.3.2. Smad pathway

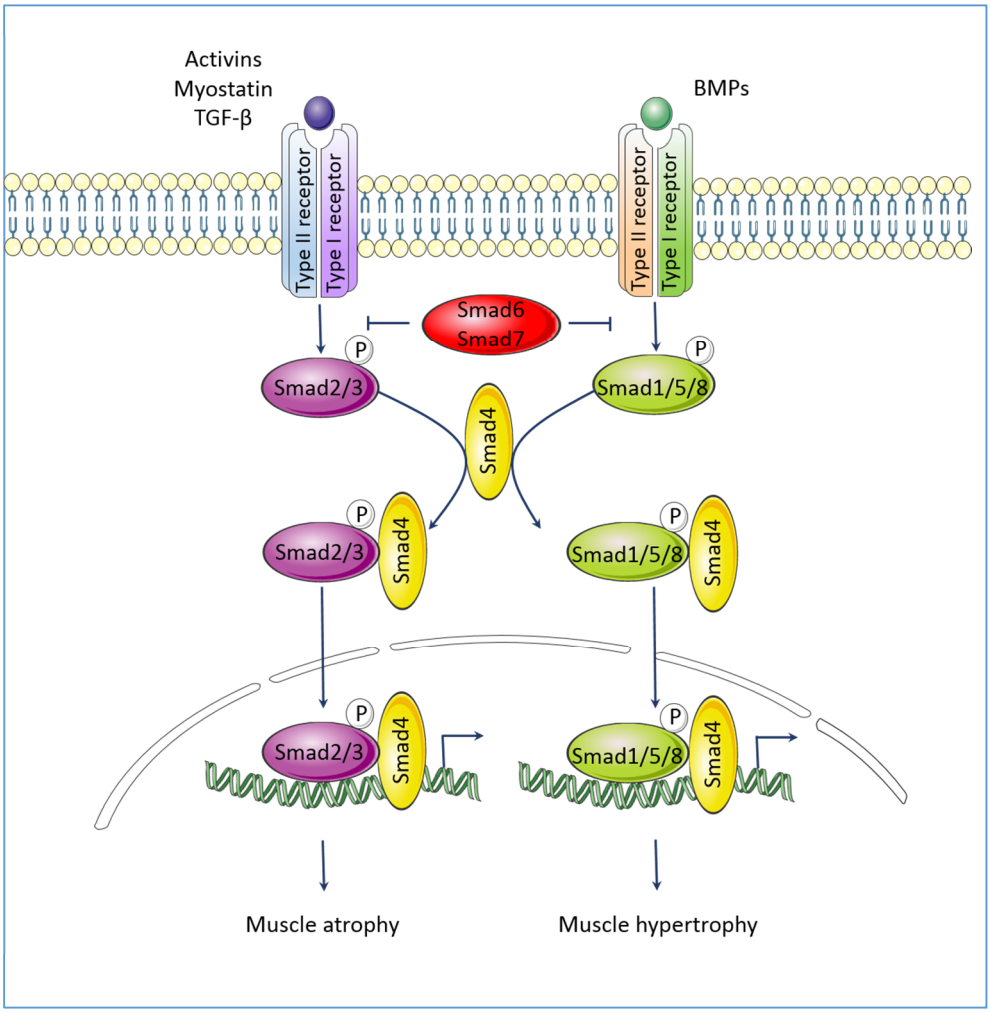

Figure 6. The Smad signaling pathway and skeletal muscle mass

Figure drawn by Kalista S according to

Sartori R, Trends Endocrinol Metab 25: 464-71, 2014 (109)

Increase in TGF-β, activins and myostatin leads to receptor mediated phosphorylation of Smad2 and 3, whereas increase in BMPs results in increased Smad1, 5 and 8 phosphorylation. Phosphorylated Smad2/3 competes with Smad1/5/8 for binding with Smad4 before translocating to the nucleus to regulate gene transcription. Activation of Smad2/3 pathway leads to muscle atrophy, whereas activation of Smad1/5/8 pathway leads to muscle hypertrophy. A balance between these competing pathways is required to maintain muscle mass.

Definition and mechanism of regulation

The Smad (Drosophilia mothers against decapentaplegic protein) proteins are part of the main signaling cascade of TGF-β superfamily proteins. Ligand binding stimulates type I and type II receptor serine/threonine kinases resulting in association and activation of receptor complexes in the cell membrane, which, in turn phosphorylates and activates the receptor regulated Smads (R-Smads). R-Smads form complexes with a common mediator Smad or co-Smad called Smad4. This Smad complex translocates into the nucleus where it cooperate with cofactors to regulate target gene transcription (110,111). The activin/myostatin/TGF-β ligand group activates the R-Smad2/3 (112,113). In contrast, the BMP/GDF group activates the R-Smad1/5/8 (109).

The modulation of the TGF-β superfamily pathways can occur upstream, at the level or downstream of the receptor. Upstream of the receptor, extracellular binding proteins of the TGF-β superfamily ligands such as chordin, noggin or follistatin prevent their binding to the cell surface TGF-β receptors (114). The interaction between the type I and the type II receptors is antagonized by BAMBI (BMP and activin membrane-bound inhibitor) which acts as a pseudoreceptor by binding to the type I receptor (115). At the level of the receptor, the recruitment of the R-Smad and co-Smad to the proximity of the receptor is initiated by the anchor proteins, SARA (Smad enchor for receptor activation) for the activin/myostatin/TGF-β pathway (116) and endofin (endosome associated FYVE domain protein) for the BMP/GDF pathway (117). Downstream of the receptor, the activation of the R-Smads is regulated by the inhibitory Smads (I-Smads), Smad6 and Smad7. Whereas Smad6 primarily inhibits BMP/GDF signaling (118), Smad7 inhibits activin/myostatin/TGF-β signaling (119,120). Moreover, Smad7 associates with the Smad-ubiquitination-related factors, SMURF1 and 2, to target TGF-β

receptor complex for degradation (121,122). The stability of Smad proteins is also controlled by ubiquitination (111).

Smads and muscle hypertrophy

Activation of Smad2/3 signaling in skeletal muscle by overexpression of Smad3 induces muscle fiber atrophy resulting from reduced protein synthesis and Akt/mTOR signaling and enhanced atrogin-1 expression (123). In contrast, knocking down of Smad2 and Smad3 is sufficient to promote skeletal muscle growth independent of type I Activin receptor (99). Moreover, deletion of inhibitory Smad7 which blocks activin/myostatin/TGF-β signaling results in decreased muscle growth (124).

Activation of Smad1/5/8 signaling in skeletal muscle is sufficient to induce massive hypertrophy and requires the presence of Smad4 and mTOR activation (68,69). In opposite, inhibition of Smad1/5/8 signaling in muscle reduces myofiber size (69). Overexpression of inhibitory Smad6 which inhibits the activation of R-Smads 1/5/8 has no influence on basal muscle fiber size but prevents the muscle hypertrophy induced by BMP7 (68).

Muscle specific knock-out co-Smad4 mice present reduced muscle fiber size indicating that Smad4, a shared element of activin/myostatin/TGF-β and BMP/GDF pathways, is required to maintain muscle mass (69).

These observations lead to consider the Smad2/3 axis as a negative regulator of skeletal muscle mass and the Smad1/5/8 axis as a positive regulator of skeletal muscle mass. These two TGF-β signaling compete for interaction with Smad4 and a balance between these competing pathways controls muscle mass (figure 6).

1.4. CLINICAL INTEREST OF THE STUDY OF MUSCLE HYPERTROPHY MECHANISMS

Skeletal muscle atrophy is defined as a loss of skeletal muscle mass resulting principally from reduced muscle fiber size. Decline in muscle mass can have major impact on mobility, whole-body metabolism, disease resistance and quality of life. Muscle atrophy is a major clinical feature of inherited myopathies and of common acquired muscle atrophies associated with aging, sepsis, glucocorticoids and cancer (125).

Preventing muscle wasting by promoting muscle growth has been proposed as a possible therapeutic approach. Currently, the only validated treatment to reduce muscle atrophy is exercise but it is not a practical option for all patients (126-128). Thus, there is clearly a need to develop drug therapies that will prevent the loss of skeletal muscle mass and increase muscle strength to improve patient quality of life and survival. In this context, the blockade of Mstn pathway represents the most promising strategy to combat muscle loss. The study of the molecular signaling implicated in the hypertrophy induced by Mstn seems to be crucial for the development of new, more targeted therapeutic strategies.

2. Regulation of muscle mass by IGFs

2.1. GENERALITIES OVER IGFs

2.1.1. IGF genes and proteins

The Insulin like growth factor (IGF) family contains IGF-I and IGF-II which are coded by two different genes and have a structure closed to pro-insulin. IGF-I gene transcription results in different transcripts (EA and EB in rodents and EA, EB and EC in humans where EB is specific to humans) resulting from different promoter usage, alternative splicing and various polyadenylation site (129). Nevertheless, the coding sequence of the mature peptide is the same throughout all the different transcripts. The IGF-I EA transcript is the more expressed and more conserved isoform throughout species. The IGF-I EB (IGF-I EC in humans) variant is also called Mechano Growth Factor (MGF) since it is produced by damaged or loaded skeletal muscle (130,131).

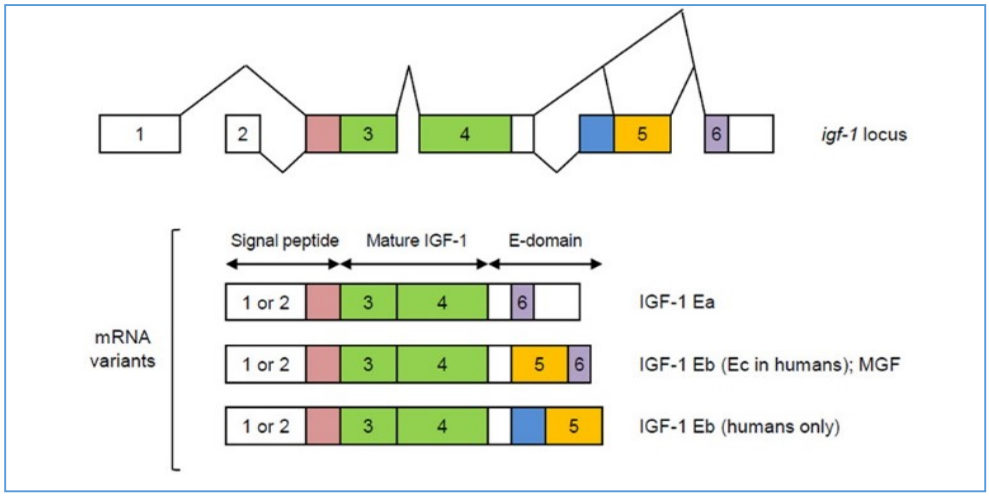

Figure 7. Splicing variants of the IGF-I gene
Figure adapted from Tahimic CGT, Front Endocrinol. 4 (Art 6), 2013

Mature IGFs consist of 70 amino acids for IGF-I and 67 for IGF-II (7.5 kDa) and have 70% homology in their sequences. The full-length precursor of IGFs (pre-pro-IGFs) contains a signal peptide, mature IGFs composed of four domains (B, C, A, D) and a C-terminal E-peptide. While mature IGFs have accepted growth promoting action, the actions of the E-peptides are relatively unknown.

2.1.2. IGF tissue expression and regulation

The main source of circulating IGFs is the liver (132). Hepatic IGF-I is produced in response to pituitary growth hormone (GH) and acts mainly in an endocrine manner. IGF-I is also produced by most of the other tissues and especially skeletal muscle where it acts in a paracrine/autocrine manner (133). The IGF-II expression depends less on GH (134) and is regulated by maternal imprinting. It is expressed from the paternal allele in most tissues except in the liver where both alleles are expressed in human (135,136). In humans, IGF-I and IGF-II are expressed throughout life. Serum IGF-I levels decrease after puberty whereas serum IGF-II levels remain high throughout adulthood (137). In contrast, in rodents IGF-II is expressed predominantly during fetal growth and its expression in adult animals is hardly detectable while IGF-I does not decline in the adulthood (138).

2.1.3. General biological actions

IGF-I is essential for normal development and postnatal growth. It is the major mediator of the postnatal growth promoting actions of GH. Indeed, growth deficiency observed in hypophysectomized rats can be corrected by IGF-I administration (139) and administration of GH does not stimulate growth in IGF-I null mice (140). The necessity of IGF-I for normal growth has also been demonstrated by knockout mouse models. Indeed, IGF-I null mice which survive are born small and grow poorly in postnatal period (54,141). Moreover, mice which do not express the IGF-IR have severe growth retardation and die in neonatal from

respiratory muscle weakness (142). Interestingly, mice carrying a liver IGF-I gene deletion show no major impairment of growth, despite a large decline in circulating IGF-I concentrations (143,144). In the same manner, in the absence of peripheral tissue IGF-I expression, endocrine IGF-I derived from liver is sufficient to maintain normal growth and development (145). Thus, it appears that locally produced IGF-I is also important as circulating IGF-I in promoting postnatal growth. IGF-I is also able to mimic most of the effects of insulin. Indeed, IGF-I induces hypoglycemia by stimulating glucose uptake and inhibiting glucose liver production. Like insulin, IGF-I also inhibits lipolysis and proteolysis (146).

IGF-II, which presents 70% of homology with the IGF-I amino acid sequence, plays a critical role in embryonic growth (147) contrasting with the role of IGF-I in prenatal and postnatal growth.

2.1.4. IGF receptors and binding proteins

IGFs exert their actions mainly by interacting with the type I IGF receptor (IGF-IR), a transmembrane tyrosine kinase heterotretramer which is structurally related to the insulin receptor (IR). The two extracellular α subunits bind IGFs whereas the two transmembrane β subunits have a tyrosine kinase domain with autophosphorylation sites. IGF-IR binds IGF-II with 6-fold lower affinity (148) and insulin with 1000-fold lower affinity compared to IGF-I (149). IGF-II can also be sequestered by the type II IGF receptor (IGF-IIR), which is identical to the mannose-6-phosphate receptor, and functions as a clearance receptor for IGF-II (150). Moreover, IGFs also bind to the IR but with a 200-fold lower affinity than insulin (149). Finally, because IGF-IR and IR are closely related, they can form hybrid receptors. These hybrid receptors retains high affinity for IGF-I, but have a decreased affinity to insulin compared to the IR (151).

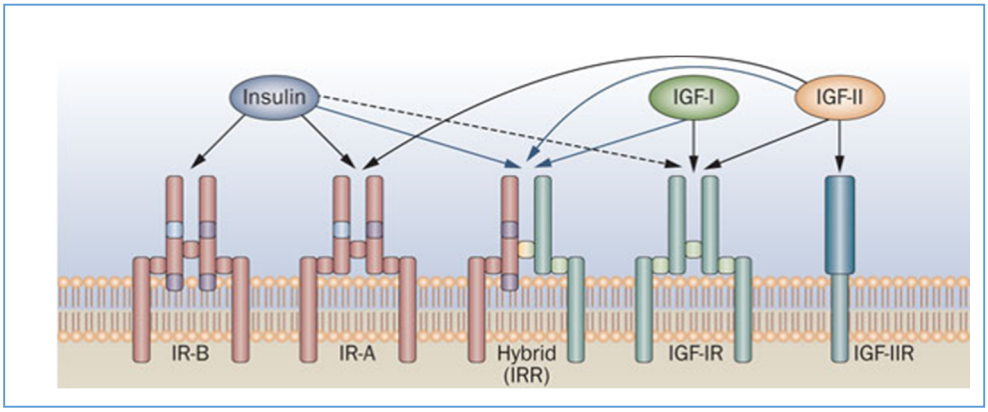

Figure 8. Schematic diagram of IGF and insulin receptors
Figure adapted from Clayton PE, Nat Rev Endocrinol 7:11-24, 2011 (152)

The biological activities of IGFs are regulated by high affinity binding proteins (IGFBPs) which sequester IGFs in the serum, thereby controlling their interaction with specific receptors (150). Adult skeletal muscle expresses IGFBPs (IGFBP-4, 5 and 6) which mostly inhibit the muscle IGF action (153).

2.2. IGF ACTIONS ON SKELETAL MUSCLE

IGF-I is considered as a positive regulator of skeletal muscle mass development. Indeed, IGF-I administration by infusion (51), transgenic muscle specific overexpression (52) or viral gene delivery (53) enhances skeletal muscle mass in rodents. This skeletal muscle hypertrophy is characterized by increased fiber size of both type I and type II and a shift toward more oxidative types (52). IGF-I induces skeletal muscle hypertrophy by increasing protein synthesis and satellite cell proliferation and differentiation (10,154). The signaling pathways implicates in the IGF-I muscle hypertrophic effect are the PI3K/Akt pathway (85), which mediates the increased differentiation and protein synthesis, and the Erk1/2 MAP (mitogen-activated protein) kinase pathway which is involved in the mitogenic effect of IGF-I (155).

The increase of IGF-I expression in skeletal muscle in response to several hypertrophic models suggests that IGF-I may mediate the skeletal muscle hypertrophy in general. The use of mice expressing a dominant negative form of the type I IGF receptor in skeletal muscle (MKR mice) shows that IGF-I signaling is crucial for the hypertrophy induces by GH treatment (156) or exercise (157) but is not crucial for testosterone (47) or overloading (158) induced skeletal muscle hypertrophy.

Several evidences suggest that IGF-II could also play a role in the development of skeletal muscle mass. IGF-II is known to stimulate *in vitro* the myogenesis through its binding and activation of type I IGF receptor (IGF-IR) (55-57). Moreover, a 3-fold increase in muscle IGF-II mRNA resulting from a nucleotide substitution in intron 3 of the IGF-II gene seems sufficient to enhance skeletal muscle mass in pig (58,59). It seems that IGF-II could also play a role in different model of skeletal muscle hypertrophy as suggested by the increased expression of muscle IGF-II in muscle hypertrophy induced by Mstn inhibition (159-161) and in work induced muscle hypertrophy (20).

3. Regulation of muscle mass by myostatin

3.1. GENERALITIES OVER MYOSTATIN

3.1.1. Myostatin gene and protein

Myostatin (Mstn) gene, also called GDF-8 (Growth and differentiation factor 8) has been mapped in several vertebrate species, and is located on chromosome 2 in human, 1 in mouse and 9 in rat (162,163). In all vertebrates, the genomic organization of Mstn gene includes three exons separated by two introns.

The Mstn protein is synthetized as an inactive precursor protein of 375 (human)/376 (mouse and rat) amino acids composed of a signal sequence, a N-terminal inhibitory sequence called propeptide domain and a C-terminal domain that gives rise after dimerization to the active Mstn protein (112,164). The signal sequence is encoded by exon 1, the propeptide domain is encoded by exon 1, 2 and 3 and the active Mstn is encoded by the exon 3. The Mstn protein sequence has been highly conserved across species. Remarkably, the sequence of the mature Mstn peptide is identical in human, rat, mouse, pig and chicken (65). Mstn precursor protein undergoes three proteolytic cleavages to generate biologically active molecule (165). The first cleavage removes the 24-amino acid signal peptide necessary for targeting the protein to the secretory pathway. The second cleavage generates the 38 kDa N-terminal propeptide and the 12.5 kDa C-terminal peptide (164). A disulfide bound between two C-terminal peptides lead to the formation of the homodimeric mature Mstn peptide (26kDa, 218 amino acids). However, Mstn homodimer is held on a tetrameric latent complex via noncovalent interactions with two molecules of propeptide (112). The inhibitory domain of the propeptide is located in the N terminal region between amino acids 42 and 115 (166). This latent complex is disassembled following proteolytic cleavage of the propeptide

between Arg-75 and Asp-76 by a BMP1/Tolloid family enzyme to produce biologically active Mstn (167,168).

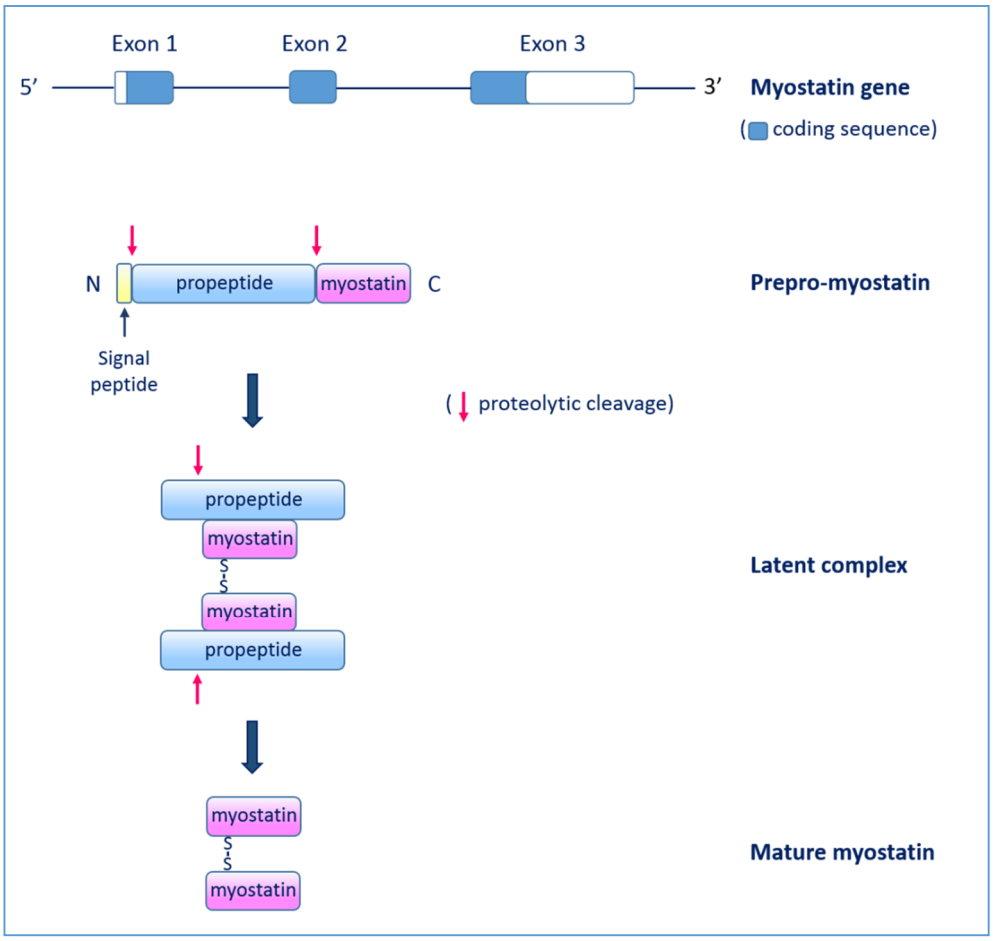

Figure 9. Myostatin gene and protein maturation

Figure drawn by Kalista S according to

Breitbart A, Am J Physiol Heart Circ Physiol 300(6):H1973-82, 2011 (169)

Mstn gene is composed of three exons that code for a precursor protein consisting of three functional domains: signal peptide, N-terminal domain or propeptide and Mstn C-terminal domain. The prepro-Mstn undergoes proteolytic cleavages in order to generate the biologically active peptide. First, the signal peptide is cleaved, a second cleavage separates the N-terminal and the C-terminal domains. Two propeptides link Mstn homodimer by noncovalent binding to form a latent complex. This latent complex is disassembled after proteolytic cleavage at the propeptide to generate mature Mstn.

3.1.2. Myostatin tissue expression

Mstn protein is predominantly expressed in skeletal muscle throughout life. At early stages of development, Mstn expression is restricted to developing somites of the myotome compartment (164). At later stages of development and in adult animals, Mstn expression is almost exclusively restricted to skeletal muscle and decreases with myogenic differentiation (164). In situ hybridization and immunohistochemical staining of skeletal muscle localize Mstn in the muscle fibers but not in the surrounding connective tissue (162,170,171). In rodents, Mstn is more abundant in fast muscle than slow muscles (172,173). Moreover, there is a positive correlation between fast type II fiber content and Mstn mRNA levels in several skeletal muscle (172,174). In contrast, in humans, Mstn is similarly expressed in fast type II and slow type I fibers (162).

Besides skeletal muscle, Mstn is also expressed in adipose tissue (164), heart tissue (cardiomyocytes and Purkinje fibers) (175-177) and lactating mammary gland (170).

3.1.3. Regulation of myostatin secretion and/or activity

The availability of bioactive Mstn is tightly regulated through protein interaction network. After secretion, Mstn is maintained as a latent complex containing Mstn homodimer and two molecules of propeptide that results from the proteolytic processing of Mstn precursor (112). This latent complex prevents Mstn from binding to its receptor (112). Besides the propeptide, several other proteins interact with Mstn to block its secretion or its activity.

In the skeletal muscle, follistatin (FS), a binding protein of a number of TGF-β family members, blocks the activity of Mstn by preventing Mstn binding to its receptor (178,179). Moreover, *in vitro* data suggest that titin-cap (180) and hSGT (human small glutamin-rich tetratricopeptide repeat-containing protein) (181)

proteins associate with Mstn to prevent Mstn secretion from skeletal muscle cell. Finally, decorin, a matrix-associated proteoglycan, has been described to trap Mstn in the extracellular matrix in order to prevent Mstn receptor binding on the skeletal muscle cell surface (182-184).

In the serum, Mstn homodimer is bound either with the propeptide (185) or with two other proteins related to follistatin: FLRG (Follistatin-like related gene) (185) and GASP-1 (Growth and differentiation factor-associated serum protein-1), this last binding protein also binds Mstn propeptide (186,187).

3.2. MYOSTATIN SIGNALING PATHWAY

3.2.1. Myostatin receptor and signaling pathway

Mstn signals into the cell via a receptor complex composed of two type II and two type I serine/threonine kinase receptors. Mstn initiates signaling through Activin Receptor (ActR) type IIB and with lower affinity through ActR type IIA (178). Binding of Mstn to ActRII recruits and activates by phosphorylation the corresponding type I receptor namely ALK (Activin-like kinase) 4 or ALK5, forming an activated heterotetrameric receptor complex (188). The activated receptor complex then phosphorylates Smad2/3 proteins (112,113) which oligomerize with Smad4. The Smads 2/3-Smad4 complex functions as the key intracellular mediator of Mstn signaling by entering the nucleus where it interacts with cofactors to regulate expression of downstream genes. Mstn signaling stimulates the expression of Smad7 which serves to abrogate Mstn signaling by establishing an autoinhibitory feedback loop. Indeed, Smad7 inhibits Mstn gene transcription (189), phosphorylation of Smad2/3 by ALK (120) or interferes with the formation of Smad2/3-Smad4 complex (120).

Aside from the Smad-mediated signaling, Mstn signals through the activation of non Smad pathways such as the p38 and Erk1/2 MAP kinases (190,191) or the c-Jun terminal kinase (JNK) (192).

3.2.2. Cross-talk with other signaling pathways

Several lines of evidence suggest signaling cross-talk between Mstn and PI3K/Akt/mTOR pathways. Indeed, Mstn negatively regulates PI3K/Akt/mTOR signaling in skeletal muscle since Mstn treatment or overexpression inhibits the PI3K/Akt/mTOR signaling (193,194). Moreover, genetic loss of Mstn leads to increase the PI3K/Akt/mTOR signaling in skeletal muscle *in vitro* and *in vivo* (84,195-197). One possible explanation for the inhibition of Akt/mTOR signaling by Mstn is the downregulation of microRNA (miR)-29 and miR-486 expression by Smad2/3 resulting in an increase of PTEN translation, an antagonist of Akt/mTOR pathway (123,198). In opposite, Akt/mTOR signaling can negatively influence Mstn signaling. Indeed, inhibition of mTORC1 by knocking down of Raptor in culture skeletal muscle increases the Mstn-induced Smad2 phosphorylation suggesting that mTOR acts on Mstn pathway by repressing Smad 2/3 signaling (193). Moreover, it is possible that in skeletal muscle Akt inhibits Smad3 activity by physically sequestering it, as observed in other cell types (199).

Recently, a cross-talk between Mstn and BMP signaling involving Smad4 has been described by two different groups (68,69). On one way, Mstn stimulates the binding of phospho Smad2/3 to Smad4 leading to muscle atrophy. On the other way, Mstn inhibition releases Smad4 from Smad2/3 and allows it to be recruited by the BMP-Smad1/5/8 pathway to promote muscle hypertrophy.

In conclusion, Mstn pathway cross-talks with two pathways implicated in the regulation of skeletal muscle mass: the PI3K/Akt/mTOR and the BMP signaling

pathways. This work will focus on the interactions between Mstn and the PI3K/Akt/mTOR pathway.

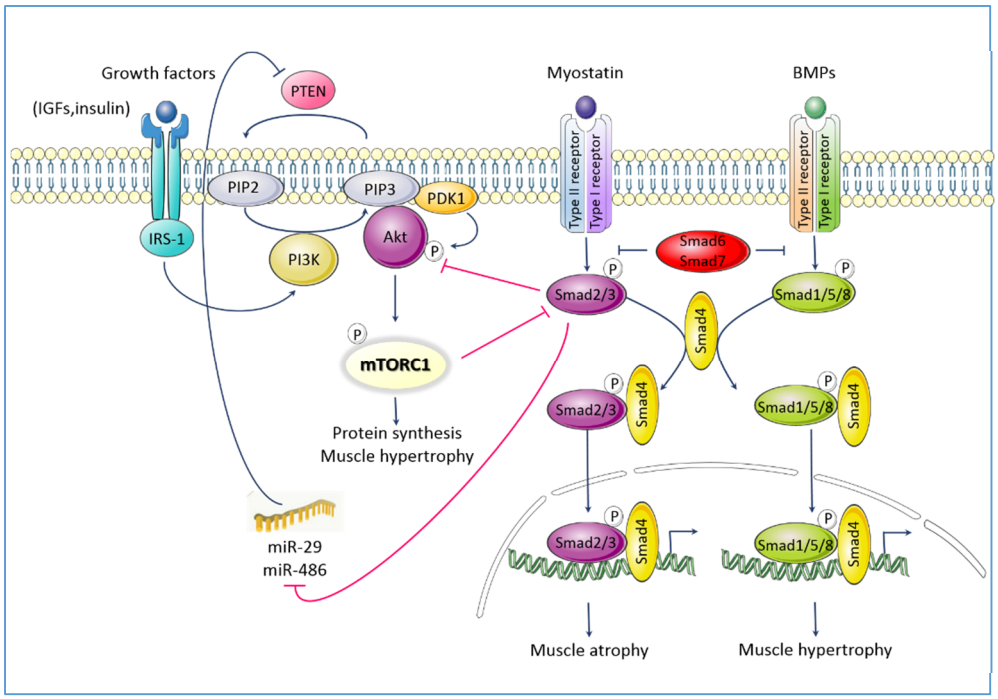

Figure 10. Cross-talks between Mstn signaling pathway and two hypertrophic signaling pathways: the PI3K/Akt/mTOR and the BMP-Smad1/5/8 pathways
Figure drawn by Kalista S according to
Trendelenburg AM, Am J Physiol Cell Physiol 296(6) C1258-70, 2009 (193)
and Sartori R,Trends Endocrinol Metab 25: 464-71, 2014 (109)

3.3. MYOSTATIN ACTIONS ON SKELETAL MUSCLE

3.3.1. Myostatin invalidation and skeletal muscle

The observation of a “double muscling” phenotype in Mstn null mice highlighted a negative role of Mstn in the regulation of muscle growth (164). This skeletal muscle hypertrophy results from increase in the fiber number (hyperplasia) and fiber size (hypertrophia). Naturally occurring mutations in other species such as in certain breeds of cattle, dog, sheep or human lead to a hypermuscular phenotype suggesting that Mstn function is conserved through species.

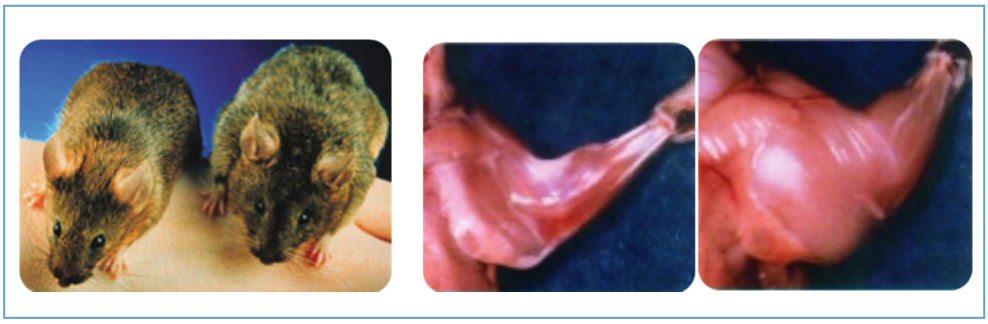

Figure 11. Wild-type and Mstn null mice, and their upper forelimb muscles
McPherron AC *et al*, Nature 387 :83-90, 1997

3.3.2. Mechanisms of regulation of skeletal muscle mass

3.3.2.1. Regulation of satellite cell activation and self-renewal

Satellite cells, mononuclear muscle stem cells localized between the basal lamina and the plasma membrane of myofibers, function as the main stem cell population in the skeletal muscle. Although quiescent in normal adult muscle, they become activated to reenter the cell cycle during muscle fiber growth. The resulting myoblasts, undergo multiple round of division prior to terminal differentiation. Mstn blocks the activation and the renewal of satellite cells *in vitro* via the downregulation of Pax7 (200,201). In contrast, Mstn gene deletion is associated with increased activation and number of satellite cells in mouse (200). Moreover,

satellite cells isolated from Mstn null mice have a higher rate of proliferation in culture than satellite cells from wild-type mice (200). These results suggest that Mstn maintains satellite cells in a quiescent state in adult muscle and inhibits their self-renewal.

3.3.2.2. Inhibition of myoblast proliferation and differentiation

The hyperplasia observed in the muscle of Mstn null mice suggests that Mstn regulates the final number of muscle fibers during development. This action seems to result from the inhibition of myoblast proliferation and differentiation by Mstn.

Mstn prevents the proliferation of myoblasts by inhibiting cell cycle progression from the G1- to S- phase (202). This effect is mediated by the inhibition of the PI3K/Akt/GSK-3β (glycogen synthase kinase-3 beta) signaling by Mstn that lead to cyclin D1 degradation, a key component of cell cycle (203). Mstn also upregulates cyclin-dependent kinase inhibitor 1 (p21), decreasing therefore the level and activity of cdk2 (cyclin dependent kinase 2), resulting in accumulation of hypophosphorylated pRB (retinoblastoma protein) which sequesters some transcription factors necessary for the S phase (202).

In addition to inhibit proliferation, Mstn also impairs myogenic cell differentiation by downregulating the expression of myogenic regulatory factors (MRFs) including Pax3 (204,205), Myf-5 (113,204), myogenin (113,206,207) and MyoD (113,204-206,208) which are directly responsible for muscle differentiation. Mstn inhibits the activity of MyoD via the activation of Smad3 which binds to MyoD and prevents its translocation to the nucleus (113). Moreover, Mstn could exert its negative effect on myogenesis through the activation of Erk1/2 MAPK (191) or the JNK signaling pathways (192). In fact, blocking the JNK activation prevents the Mstn's ability to inhibit differentiation.

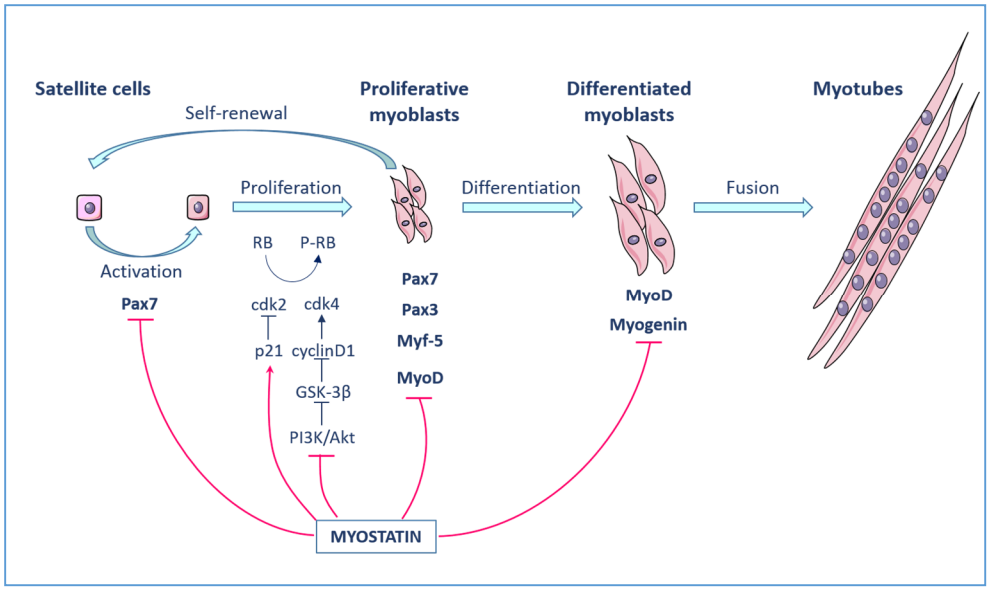

Figure 12. Role of Mstn in the control of myogenesis

Figure drawn by Kalista S

Mstn controls the myogenesis by inhibiting the activation and self-renewal of satellite cells, and the proliferation and differentiation of myoblasts.

3.3.2.3. Inhibition of protein synthesis

A third mechanism by which Mstn inhibits muscle growth is the downregulation of protein synthesis (209). Accordingly, higher protein synthesis rate has been observed in the muscles of Mstn KO mice compared to wild-type mice (210). Furthermore, the postnatal inhibition of Mstn activity by anti-Mstn antibodies (100) or by the binding protein FS (211) stimulates the myofibrillar protein synthesis and the phosphorylation of S6K and ribosomal protein S6 (rpS6), two factors involved in the regulation of protein synthesis. In contrast, the atrophy induced by Mstn overexpression is associated with decrease phosphorylation of Akt, rpS6 and 4EBP-1 (Eukaryotic translation initiation factor 4E-binding protein 1). The data support therefore that the down-regulation of Akt/mTOR/S6K signaling could mediate the negative effect of Mstn on protein synthesis (194).

3.3.2.4. Stimulation of proteolysis

Mstn overexpression in muscle results in skeletal muscle atrophy. However, the mechanisms involved in the catabolic effect of Mstn are not well described. In addition to inhibit protein synthesis, Mstn might also stimulate muscle proteolysis. *In vitro*, Mstn upregulates the expression of genes involved in the ubiquitin proteasome pathway including the atrogenes Atrogin-1, MuRF-1 (Muscle RING finger protein-1) and $E2_{14kDA}$ (Ubiquitin conjugating enzyme ubch2 14kDa) mediated by the activation of FoxO1 (Forkhead box O) via an inhibition of Akt pathway (205). In concordance, muscle from Mstn null mice have a decreased protein degradation associated with decreased Atrogin-1 protein and ubiquitinated myosin heavy chain levels (212). However, the Mstn induced muscle atrophy seems not to be always associated with an upregulation of atrogenes (193,194). Finally, Mstn could trigger protein degradation via the stimulation of the autophagy-lysosomal system. Indeed, Mstn stimulates autophagosome formation and expression of several autophagy-related genes *in vitro* (213). Further works are needed to clarify the role of the proteolysis in the Mstn catabolic action.

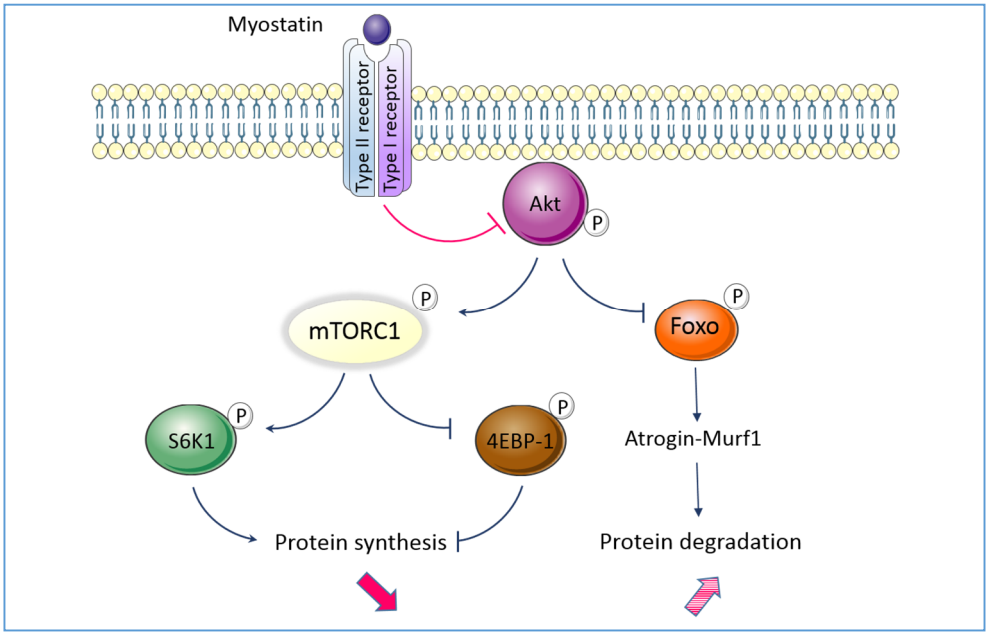

Figure 13. A model for the role of Mstn in the regulation of protein synthesis and degradation
Figure drawn by Kalista S

3.3.2.5. Stimulation of apoptosis

It has been suggested that Mstn might also regulate the balance between survival and apoptosis of skeletal muscle cells. Indeed, the expression of proteins implicated in the protection against apoptosis are upregulated in the muscle of Mstn null mice (196). In the same way, acute Mstn antibody treatment reduces the number of apoptotic myonuclei in the muscles of aged mice (214). These data suggest that Mstn inhibition favors the cell survival which would limit the loss of myonuclei and lead to increased myonuclear to cytosolic ratio, an essential factor in muscle hypertrophy. Nevertheless, opposed results have been observed in cultured myoblasts treated with Mstn which present reduced apoptosis (202,207,215).

3.3.2.6. Regulation of the muscle fiber phenotype

Several observations suggest that Mstn regulates the muscle fiber phenotype. Indeed, the muscles of Mstn null animals contains a larger proportion of fast glycolytic type II fibers (IIb) together with reduced proportion of oxidative fast type II (IIa) and slow type I fibers (216-218). Thus, the absence of Mstn leads to an overall faster and more glycolytic muscle phenotype. It has been shown that Mstn regulates the expression of myosin heavy chain (MHC) during myogenesis. Indeed, Mstn treatment of primary myoblasts upregulates the expression of slow MHC and downregulates the expression of fast MHC (219). Moreover, Mstn regulates fiber-type composition by regulating the expression of MEF2 (Myocyte enhancer factor-2) and MyoD during myogenesis (218). In fact, the muscles of Mstn null mice contain lower levels of MEF2, a transcription factor essential for the formation of type I fibers and increased levels of MyoD, an important factor for the transcription of type IIb MHC, characteristic of fast type II fibers. The effect of Mstn or its inhibition on the muscle phenotype is likely a consequence of developmental process because Mstn inhibition in postnatal does not cause a shift to fast and glycolytic phenotype (217,220,221).

3.4. INTEREST OF MYOSTATIN INHIBITION IN THE TREATMENT OF MUSCLE ATROPHY

The facts that Mstn targets mainly skeletal muscle to limit muscle size has raised the possibility that inhibition of Mstn pathway may be a promising way to counteract muscle atrophy. Moreover, Mstn functions extracellularly and is therefore accessible to pharmacological agents. The inhibition of Mstn pathway seems to be a promising issue in the treatment of muscular dystrophy such as Duchenne disease or in the treatment of muscle loss that occurs in cachexia, a wasting syndrome seen in chronic diseases such as cancer and sepsis. More work will be required to determine if targeting Mstn pathway will have a beneficial effect in human diseases. One therapeutic approach consists of administration of neutralizing antibodies (222) or a vaccine against Mstn (223), both enhance skeletal muscle mass and muscle function. Mstn inhibition may also be achieved by short interfering RNAs targeting Mstn, showing increased muscle mass in rodents (224,225). A more pronounced effect on muscle function is obtained by treatment with Mstn propeptide (226) or with a soluble form of its receptor ActRIIB (227). In addition to propeptide, other binding proteins (FS, FLRG, GASP-1) are able to regulate Mstn activity and then muscle growth (220). Among these binding proteins, Follistatin induces the most dramatic effect on muscle mass growth. The next chapter covers this interesting Mstn binding protein.

4. Regulation of muscle mass by follistatin

4.1. GENERALITIES OVER FOLLISTATIN

4.1.1. Follistatin gene and protein

The follistatin (FS) gene is composed of six exons coding respectively for a signal peptide, a N-terminal domain, three FS domains (FSI, II and III) and a C-terminal domain (228,229). Through an alternative splicing between exons 5 and 6, two mRNAs are produced that encode two pre-proteins of 317 and 344 amino acids. The cleavage of the signal peptide (29 amino acids) results in the mature isoforms of 288 (FS288) and 315 (FS315) amino acids with a molecular weight ranging from 31 to 42 kDa depending of post-translational glycosylation (Fig 5) (230). In addition, the FS315 can be cleaved at the C-terminal to give an isoform of 303 amino acids which is the major form in follicular fluid (231). The two major isoforms FS288 and FS315 differ in their C-terminal sequence (232) and their ability to bind heparan sulfates (HS) of the extracellular matrix. The binding site for HS is located in the FSI domain, at residues 72-86, a region rich in basic amino acids (233,234). In the FS315 isoform, the acidic tail hides the basic region of FSI domain and the FS315 is unable to bind HS. Therefore, FS315 is the main form in serum. In contrast, FS288 is a membrane form of FS and locates at the cell surface.

The FS protein is highly conserved among species with 97% amino acid homology between human and mouse.

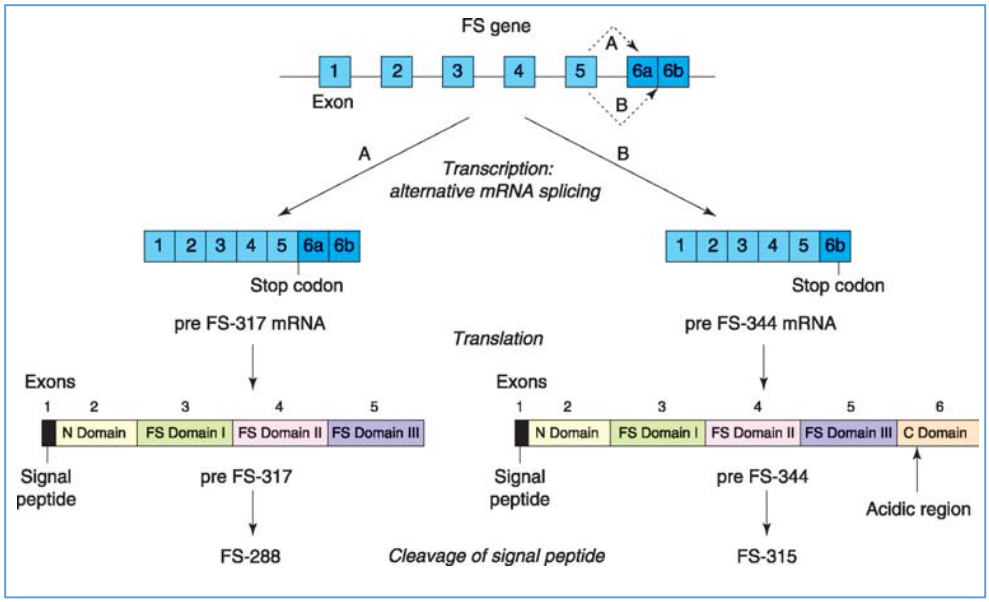

Figure 14. The FS gene and protein
Lin SY, Reproduction 126: 133-148, 2003

4.1.2. Follistatin tissue expression

Although first identified in ovarian follicular fluid (235), FS is widely distributed in embryonic and adult tissues. In addition to ovary and testis, FS is found in cerebral cortex, pituitary, adrenal, thymus, pancreas, gut, kidney, uterus, lung, heart and skeletal muscle (236,237). In the muscular system, FS is abundantly expressed in skeletal tissue and in a lesser extent in heart (238).

4.1.3. Follistatin function

Up to date, the only known biological activity of FS is the neutralization of TGF-β superfamily ligands. FS binds Activin (Act) A with a very high affinity (50 pM), the closely related ActB isoform with 10 fold lower affinity (500 pM) (239) and many

BMPs (2,4,6,7 and 15) with lower affinities (200 to 80000 pM) (240-242). FS binds also Mstn and GDF11 with affinity intermediate between ActA and BMP ligands (500 pM) (178,179,243). Since FS binds Mstn with a higher affinity than its receptor ActIIRB, FS is proposed to block Mstn signaling and action (178).

4.2. FOLLISTATIN ACTION ON SKELETAL MUSCLE

4.2.1. Genetically modified mouse models

Mice lacking FS exhibit growth retardation and defect in skeletal muscle development causing death by asphyxia shortly after birth (244). The characterization of skeletal muscle phenotype of heterozygous FS (FS+/-) mice shows a reduced muscle mass together with a reduced muscle fiber size of both type I and type II fibers, a shift towards more oxidative muscle fibers and no change in fiber numbers (245). These models suggest an important role of FS on skeletal muscle development. In contrast, FS overexpression in skeletal muscle of transgenic (mTr-FS) mice induces a dramatic increase in muscle mass, establishing FS as a potent inducer of muscle growth (178). This skeletal muscle hypertrophy results from a combination of hyperplasia and hypertrophia.

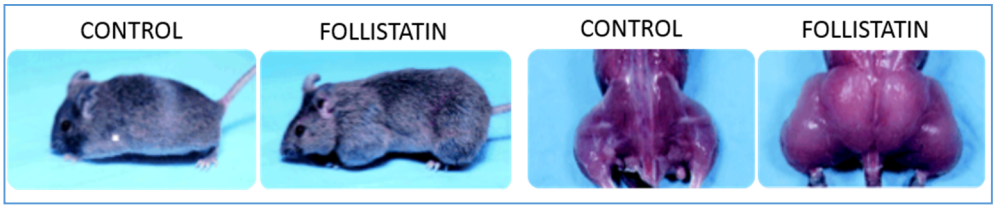

Figure 15. Increased muscling in mice overexpressing FS
Lee SJ, PNAS 98 (16): 9306-11, 2001

4.2.2. Mechanisms of regulation of skeletal muscle mass

4.2.2.1. Follistatin ligands in skeletal muscle

FS is considered to regulate skeletal muscle development through the inhibition of Mstn. Indeed, muscle atrophy of FS+/- mice is lower when Mstn is lacking (245). But, the fact that muscle atrophy is still present when Mstn are invalidated in FS+/- mice suggests that FS regulates skeletal muscle growth through the binding of others TGF-β family members. Moreover, the muscle hypertrophy observed in FS transgenic mice is greater than the increased in muscle mass observed in Mstn null mice. It suggests that at least part of the hypertrophic effect of FS results from inhibition of another ligand besides Mstn (178). This hypothesis is reinforced by the observation of a quadrupling muscle mass in Mstn null mice carrying a FS transgene, compared with the doubling of muscle mass observed in the Mstn null mice (246). Previous work in our laboratory have shown that the hypertrophy induced by FS overexpression results from the inhibition of not only Mstn but also Activin A (161). Up to date, the role of other ligands such as the GDF-11 or BMPs in the FS hypertrophic effect has not been investigated. However, GDF-11 seems to be not crucial for the regulation of skeletal muscle mass as GDF-11 invalidation in skeletal muscle does not affect muscle size (247). Moreover, the affinity of FS towards some BMPs and their potency to inhibit muscle differentiation are lower compared to Mstn and ActA (193).

4.2.2.2. Intracellular mediators of FS hypertrophic effect

FS-induced hypertrophy results from inhibition at least from Mstn and ActA which are known to activate the Smad2/3 signaling pathway. Interestingly, FS overexpression in skeletal muscle decreases phosphorylation of Smad3 which is required for FS to induce skeletal muscle hypertrophy (248). Moreover, recent data have demonstrated that FS-induced hypertrophy is associated with and required

the phosphorylation of Smad1/5 which are known to be activated by BMPs proteins, positive regulators of muscle mass (68). In conclusion, FS acts on the Smad2/3 and the Smad 1/5/8 pathways to regulate skeletal muscle growth.

4.2.2.3. Mechanisms of FS hypertrophic effect

In culture, FS stimulates the proliferation and the differentiation of myoblasts (249-252). Furthermore, FS has been shown to rescue the muscle differentiation inhibited by Mstn. Indeed, in chick limb bud, FS prevents the Mstn-mediated downregulation of Pax-3 and MyoD, two regulators of myogenesis (253). Finally, FS promotes muscle development as measured by increase in the creatine phosphokinase activity and a higher number of nuclei in myotubes (254).

The positive regulation exerted by FS on satellite cell proliferation has been suggested by several observations made in our laboratory (161). Indeed, FS overexpression in muscle is characterized by increased DNA content and PCNA expression attesting of increased cell proliferation. Moreover, FS stimulates the differentiation of satellite cells as suggested by increased neonatal MHC expression. However, the use of γ irradiation to destroy the proliferative capacity of satellite cells in muscle, inhibits only by 50 % the capacity of FS to stimulate muscle growth. This suggests that the muscle hypertrophy induced by FS is in part independent of the proliferation of satellite cells. Nevertheless, our results contrast with other studies showing the absence of satellite cells contribution in the muscle hypertrophy induced by prenatal or postnatal Mstn inhibition. These studies investigated the role of satellite cells either by immunostaining of satellite cells (12) or by using mice lacking syndecan4 or Pax7 (15), two proteins essential for the function of satellite cells. Interestingly, the group of Lee *et al.* showed that muscle FS transgene has a significant muscle hypertrophic effect even in mice severely compromised for satellite cells function (syndecan4 mutants) or number (Pax7

mutants), suggesting that the satellite cells do not play a significant role in the muscle hypertrophy induced by FS. The discrepancies might result from the irradiation model used, which is not specific to satellite cells and has been shown to affect translation (255). These contradictory results are reconciled by the work of Wang *et al.* which shows that Mstn inhibition by a soluble form of its receptor (sActRIIB-Fc) results in satellite cell activation, but which follows and not precedes myofiber hypertrophy, making their role unlikely in this model of hypertrophy (256).

Besides satellite cells, protein synthesis also plays a crucial role in the FS-induced skeletal muscle hypertrophy. Indeed, infusion of FS in neonatal rats stimulates the muscle protein synthesis together with increased phosphorylation of S6K and rpS6 (211). The increase in protein content and MHC2b expression induced by FS overexpression that we previously observed agrees with these results (161). However, further works are needed to clarify the downstream signaling mechanisms responsible for FS's ability to promote skeletal muscle hypertrophy.

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Chapter 2

AIM OF THE WORK

Skeletal muscle loss is a major clinical feature of inherited myopathies and acquired muscle atrophies associated with various diseases such as aging, sepsis, glucocorticoids and cancer. As skeletal muscle accounts for nearly the half of the mass of human body and is essential for locomotion and many metabolic pathways, decrease in skeletal muscle mass leads to reduced quality of life and increased morbidity and mortality. Therefore, the development of strategies to increase size and strength of skeletal muscle represents a significant therapeutic benefit for muscular disorders. In this context, inhibition of Myostatin (Mstn), a major negative regulator of skeletal muscle mass, seems to be a promising therapy. Among Mstn inhibitors, Follistatin (FS) induces the most dramatic effect on muscle mass growth. However, the molecular mechanisms by which FS exerts its hypertrophic action on skeletal muscle mass remains unclear. Therefore, defining the key intracellular mediators of this hypertrophy is a crucial issue for the correct identification of drug targets to mitigate muscle wasting.

The aim of this work was to characterize the mediators of the FS hypertrophic action on skeletal muscle and more particularly to explore:

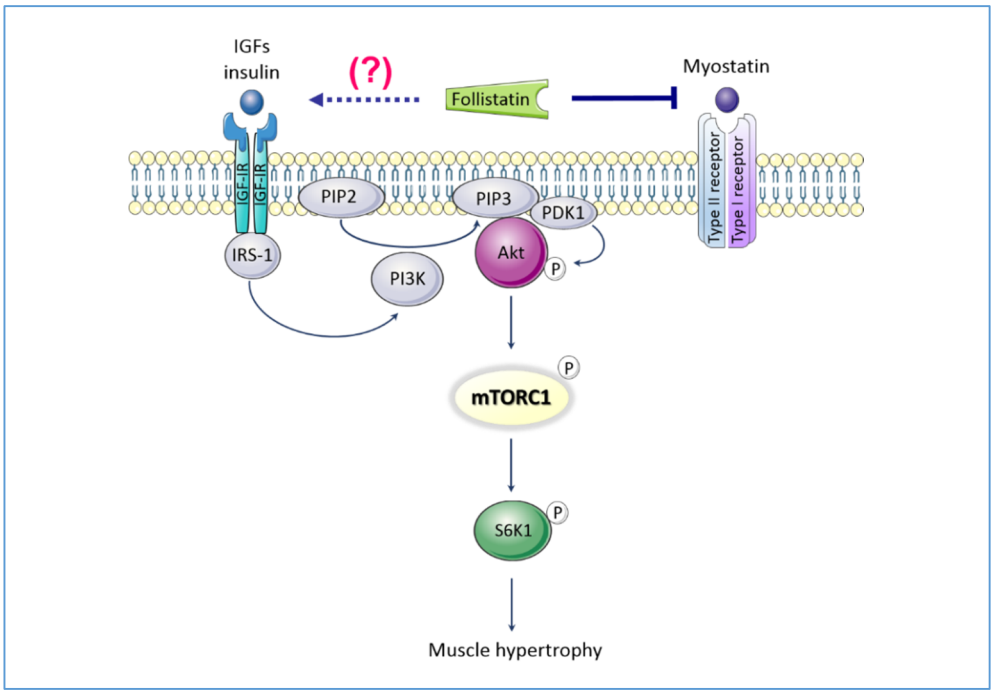
1) The involvement of IGF-II and IGF-IR/Akt/mTOR/S6K pathway.

Mstn inhibition is characterized by increased expression of IGF-II, a growth factor related to IGF-I. By using IGF-II null mice, we investigated its role in the FS induced hypertrophy. As IGFs act via the activation of the IGF-IR, we evaluated the effect of FS overexpression in the skeletal muscle of mice constitutively expressing a dominant negative form of the IGF-IR in skeletal muscle (MKR mice). Moreover, as the activation of IGF-IR exerts its effects through the stimulation of the Akt/mTOR/S6K pathway, we determined the role of this major anabolic pathway for muscle in the FS hypertrophic effect. This pathway is known to be crucial for IGF-I anabolic action and to be stimulated by Mstn inhibition. The role of Akt was

investigated by muscle cotransfection of a dominant negative form of Akt, the role of mTOR by treating mice with rapamycin and the role of S6K by using S6K deficient mice.

2) The role of IGF-I and insulin, two ligands of the IGF-IR.

Since the MKR mice are characterized by a resistance of the skeletal muscle towards IGF-I but also insulin, we tended to evaluate the respective role of IGF-I and insulin in the FS hypertrophic effect. To evaluate the role of IGF-I, we overexpressed FS in the skeletal muscle of hypophysectomized rats characterized by low concentration of circulating and muscle IGF-I. Moreover, we investigated the effect of FS overexpression on muscle sensitivity towards IGF-I. The role of insulin was assessed by overexpressing FS in the skeletal muscle of rats treated with streptozotocin characterized by low insulin levels.



Chapter 3

RESULTS

1. The type 1 insulin-like growth factor receptor (IGF-IR) pathway is mandatory for the follistatin-induced skeletal muscle hypertrophy.

The aim of this first article was to investigate the involvement of the activation of the IGF-IR/Akt/mTOR/S6K pathway in the follistatin (FS) -induced skeletal muscle hypertrophy. In this article, we report that

1) Inhibition of IGF-IR by using mice constitutively expressing a muscle dominant-negative form of IGF-IR severely blunts FS-induced muscle fiber hypertrophy.
2) FS causes the same degree of muscle fiber hypertrophy in IGF-II deficient mice as in wild type mice.
3) Inhibition of Akt by transfection of a dominant-negative form of Akt severely blunts FS-induced muscle fiber hypertrophy.
4) Inhibition of mTOR by rapamycin moderately attenuates FS-induced muscle fiber hypertrophy.
5) FS causes the same degree of muscle fiber hypertrophy in S6K1/2 deficient mice as in wild type mice.

In conclusion, we demonstrate that the IGF-IR/Akt/mTOR pathway plays a critical role in the FS-induced skeletal muscle hypertrophy. We also show that the induction of IGF-II expression and S6K activity by FS are not required for the hypertrophic action of FS on skeletal muscle.

The type 1 insulin-like growth factor receptor (IGF-IR) pathway is mandatory for the follistatin-induced skeletal muscle hypertrophy.

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Collaborations:

M Pende kindly provided the S6K1/2 KO mice and their control littermates.

L Bertrand provided the methodology to perform the measurement of S6K activity

Author contributions:

S Kalista and JP Thissen designed research

S Kalista, O Schakman, H Gilson, P Lause, B Demeulder performed research

S Kalista and JP Thissen analyzed data

S Kalista and JP Thissen wrote the paper

ABSTRACT

Myostatin inhibition by follistatin (FS) offers new approach for muscle mass enhancement. The aim of the present study was to characterize the mediators responsible for the FS hypertrophic action on skeletal muscle in male mice. Since IGF-I and IGF-II, two crucial skeletal muscle growth factors, are induced by myostatin inhibition, we assessed their role in FS action. First, we tested if type 1 IGF receptor (IGF-IR) is required for FS-induced hypertrophy. By using mice expressing a dominant negative IGF-IR in skeletal muscle, we showed that IGF-IR inhibition blunted by 63% fiber hypertrophy caused by FS. Secondly, we showed that FS caused the same degree of fiber hypertrophy in WT and IGF-II KO mice. We then tested the role of the signaling molecules stimulated by IGF-IR, in particular Akt/mTOR/S6K pathway. We investigated whether Akt phosphorylation is required for the FS action. By cotransfecting a dominant negative form of Akt together with FS, we showed that Akt inhibition reduced by 65% fiber hypertrophy caused by FS. Secondly, we evaluated the role of mTOR in FS action. Fiber hypertrophy induced by FS was reduced by 36% in rapamycin-treated mice. Finally, since the activity of S6K is increased by FS, we tested its role in FS action. FS caused the same degree of fiber hypertrophy in WT and S6K1/2 KO mice. In conclusion, IGF-IR/Akt/mTOR pathway plays a critical role in FS-induced muscle hypertrophy. In contrast, induction of IGF-II expression and S6K activity by FS are not required for the hypertrophic action of FS.

Key words: Follistatin, IGFs, Akt/mTOR/S6K pathway, skeletal muscle hypertrophy

INTRODUCTION

Over the last ten years, it has been well established that myostatin (Mstn), a member of the transforming growth factor-beta (TGF-β) superfamily, is a strong negative regulator of skeletal muscle mass. Indeed, Mstn inhibition or gene deletion increases muscle mass and strength both developmentally (1) and in adult animals (2). Conversely, transgenic mice overexpressing Mstn selectively in skeletal muscle have lower muscle mass (3).

Characterization of Mstn-inhibitor binding proteins such as follistatin (FS), follistatin-related gene (FLRG) and growth and differentiation factor-associated serum protein-1 (GASP-1), offers new approaches for muscle mass enhancement (4). Among these, FS seems to be the most efficient to stimulate muscle growth (5;6). In fact, overexpression of FS induces a dramatic increase in muscle mass when expressed as a transgene in mice (5) or delivered by adeno-associated virus (4) or electroporation (7). Muscle hypertrophy induced by FS mainly results from muscle fiber hypertrophy (8). The mechanisms by which FS promotes skeletal muscle fiber growth include activation of muscle satellite cells (7) and stimulation of the protein synthesis (9). However, the downstream signaling mechanisms responsible for FS's ability to promote skeletal muscle hypertrophy are not well characterized.

Interestingly, inhibition of Mstn is characterized by increased expression of insulin-like growth Factor (IGF)-I and II (7;10-14), which are known to be potent stimulus of myogenesis (15-17). Therefore, it is possible that the increased IGFs expression might contribute to skeletal muscle hypertrophy induced by FS. As the anabolic action of IGF-I and IGF-II on skeletal muscle are closely related and are mediated by the same receptor (IGF-IR), it is possible that IGF-IR plays a role in FS-induced muscle hypertrophy. This hypothesis is further supported by the demonstration that the Akt/mTOR/S6K pathway downstream of the IGF-IR is activated by Mstn inhibition (18;19) and is crucial for the hypertrophic action of

IGF-I (20). In this pathway, the striking effect of Akt-1 on muscle size (21-23) suggests a possible role of Akt in the hypertrophy caused by Mstn inhibition. However, the role of Akt in this hypertrophy is controversial, with some groups reporting a crucial role of this kinase (18) and some others not (24). Furthermore, skeletal muscle hypertrophy requires mTOR activity and S6K (21;22;25;26). Indeed, mTOR plays a central role in the control of protein synthesis and muscle fiber size (27;28). Surprisingly, mTOR does not seem so crucial for the hypertrophy caused by Mstn inhibition obtained by the transfection of a dominant-negative ActIIRB, the Mstn receptor (29). The role of S6K in the hypertrophy caused by Mstn inhibition has not been investigated.

Taken together these observations, we hypothesized that the IGF-IR/Akt/mTOR/S6K pathway might be responsible for the skeletal muscle hypertrophy induced by FS. To answer this question, we overexpressed FS288 gene by electrotransfer in TA muscle of mice genetically modified or treated with pharmacological agents.

MATERIALS AND METHODS

1) ANIMALS

To assess the role of IGF-IR signaling in the muscle hypertrophy induced by FS, we used male homozygote MKR transgenic mice (MKR mice, Fernandez AM 2001), overexpressing a dominant-negative IGF-IR specifically in skeletal muscle, and FVB wild type mice (WT mice). To assess the role of IGF-II in the muscle hypertrophy induced by FS, we used male heterozygous IGF-II knock-out mice (IGF-II KO mice, DeChiara TM 1990), harboring a disrupted paternal IGF-II allele, and 129/Sv//Ev wild type mice (WT mice). To assess the role of S6K in the muscle hypertrophy induced by FS, we used male homozygous S6K1/2 knock-out mice (S6K1/2 KO mice,

Pende M 2004), harboring a combined disruption of S6K1 and S6K2, and C57Bl/6-129Ola wild type mice (WT mice). To assess the role of Akt and mTOR in the muscle hypertrophy induced by FS, we used male FVB mice provided by Janvier Breeding (Le-Genest-Saint-Isle, France).

All mice analyzed were male of 8 weeks of age at the start of the experiment, excepted for the experiment on S6K1/2 KO mice in which mice were male from 8 to 10 week-old. The mice were housed individually under controlled conditions of lighting (12-h light, 12-h dark cycle) and temperature (22 $\pm$ 2°C). The animals were allowed to free access to chow and water. The experiments were approved by the Animal Ethics Committee of the Catholic University of Louvain, Brussels.

2) EXPRESSION PLASMIDS AND DNA PREPARATION

pM1-hFS288 plasmid, coding for the human FS containing 288 amino acids, was constructed as previously described (7) . A c-myc tag was added by PCR to the hFS288 cDNA and the cDNA was inserted in pM1 Expression Vector (Roche Molecular Biochemicals) to produce pM1-hFS288-c-myc. The pM1-hFS288-c-myc plasmid retained the full hypertrophic action on skeletal muscle of the pM1-hFS288 plasmid. pM1-dnAkt plasmid (gift from P Delafontaine, Tulane University School of Medicine, New Orleans) codes for hemagglutinin (HA) tagged dominant negative (dn) Akt form. Empty pM1 was used as a control plasmid. Plasmids were amplified in *Escherichia coli* top 10 F' (Invitrogen, Carlsbad, CA) and purified with an EndoFree Plasmid Giga Kit (QIAGEN). Plasmids were stocked at -80°C. The day before injection, 30 µg of plasmid were lyophilized and resuspended in 30 µl 0.9% NaCl solution.

3) DNA ELECTROTRANSFER AND SACRIFICE

Each animal was anesthetized with a mixture of 75 mg/kg ketamine (Ketalar®, Pfizer) and 15 mg/kg xylazine hydrochloride (Rompun®, Bayer) administered by intraperitoneal injection. 30 µl of plasmid solution (1 µg/µl) was injected into each tibialis anterior (TA) muscle using a Hamilton syringe with a 30-gauge needle, and the muscles were then electroporated using the electroporation conditions described by Bloquel *et al*. (8 pulses of 200 V/cm and 20 ms per pulse at 2 Hz) (30). The mice were sacrificed by decapitation 17 days after electroporation. TA muscles were dissected, and a transverse slice of 0.5-cm thickness was fixed with buffered formol for 48h and embedded in paraffin for morphological analysis. The remaining ends of the muscles were frozen in liquid nitrogen for mRNA analysis.

4) EXPERIMENTAL DESIGN

4.1. Role of IGF-IR signaling in the FS-induced muscle hypertrophy

To confirm the inhibition of the IGF-IR signaling in MKR mice, muscle Akt phosphorylation was assessed after IGF-I IV injection. Briefly, the WT and the MKR mice were anesthetized with a mixture of ketamine/xylazine. Under sterile conditions, 20 µl of a solution of recombinant human IGF-I (14 µg/animal; Genentech) or 20 µl saline solution (vehicle, 0.9% NaCl) were injected in the inferior vena cava after abdomen opening. The mice were sacrificed by decapitation 15 min after the injection. Gastrocnemius muscles were removed and frozen in liquid nitrogen for western blot analysis.

To assess the role of IGF-IR signaling in FS-induced muscle hypertrophy, TA muscles of MKR mice (n=8) and WT mice (n=8) were transfected with the plasmid pM1-FS288-c-myc (left leg) and the control plasmid pM1 (right leg).

4.2. Role of IGF-II in the FS-induced muscle hypertrophy

The TA muscles of IGF-II KO mice (n=14) and WT mice (n=11) were transfected with the plasmid pM1-FS288 (left leg) and the control plasmid pM1 (right leg).

4.3. Role of Akt in the FS-induced muscle hypertrophy

FVB mice were divided in three groups: FS (n=6), dnAkt (n=6) and FS+dn Akt (n=6). The left TA muscles of FS group were transfected with a plasmid solution of 15 µg pM1-FS288-c-myc and 15 µg pM1. The left TA muscles of dnAkt group were transfected with a plasmid solution of 15 µg pM1-dnAkt and 15 µg pM1. The left TA muscles of FS+dnAkt group were transfected with a plasmid solution of 15 µg pM1-FS288-c-myc and 15 µg pM1-dnAkt. The right TA muscles of all the mice were transfected with the control plasmid pM1.

4.4. Role of mTOR in the FS-induced muscle hypertrophy

The TA muscles of FVB mice were transfected with the plasmid pM1-FS288 (left leg) and the control plasmid pM1 (right leg). Mice were divided in two groups: vehicle (n=12) and rapamycin (Rapa, n=12). Four days after transfection and until the sacrifice, mice received daily intra-peritoneal injection of mTOR inhibitor rapamycin (2 µg/g of BW/day; LC Laboratories) or vehicle. The concentrated rapamycin solution in ethanol (40 µg/µl), stored at -20°C, was diluted at 0.4 µg/µl on the day of injection with 0.2% sodium carboxymethylcellulose/0.25% Tween®80 in water.

To ensure the efficacy of rapamycin injections, rapamycin was dosed in whole blood by Antibody Conjugated Immunoassays (ACMIA) method (RXL® Dimension, Siemens) as described by Ventura E (31).

4.5. Role of S6K in the FS-induced muscle hypertrophy

The TA muscles of S6K1/2 KO mice (n=15) as well as WT mice (n=13) were transfected with the plasmid pM1-FS288 (left leg) and the control plasmid pM1 (right leg).

5) mRNA ANALYSIS BY REAL TIME QUANTITATIVE (RTQ)-PCR

Total RNA was isolated from the TA muscle using TRI Reagent® as described by the manufacturer. Recovery was 1 µg/mg of TA muscle. Reverse transcription and RTQ-PCR were done as previously described (32). Accession number for the sequences and primers used were: FS: NM_008046 (GGCAGATCCATTGGATTAGCC – CCGCCACACTGATATCTTCA), IGF-II: NM_031511 (GTCGATGTTGGTGCTTCTCATCT – CGGTCCGAACAGACAAACTGAA), IGF-IR: X044434.1 (TCATGCCTTGGTCTCCTTGTCCTT – GTCACTTCCTCCATGCGGTAAATTTCG) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): AF106860 (TGCACCACCAACTGCTTA – GGATGCAGGGATGATGTTC) used as reporter gene. Primers were tested in order to avoid primer dimers, self-priming formation or unspecific amplification. Primers were designed to have standardized optimal PCR conditions.

6) HISTOLOGICAL ANALYSIS OF MUSCLE

Serial sections (5 µm thick) were cut and mounted on glass slides (Superfrost Plus; Menzel-Glaser, Germany). FS, FS-c-myc and HA-dnAkt were detected by immunohistochemistry with goat polyclonal anti-FS or rabbit polyclonal anti-c-myc or rabbit polyclonal anti-HA respectively, as previously described (7;33). Fiber cross-sectional areas (CSAs) were measured with a microscope Axio-Star (Carl Zeiss) coupled to a Zeiss Axiocam digital camera MRc and to image analyzer software (Axiovision software version 4.7, Carl Zeiss). To evaluate the effect of FS on muscle

fiber CSA, all the positive muscle fibers in the TA transfected with FS gene were measured. To evaluate the effect of cotransfection of FS and dnAkt, only the muscle fibers co-expressing the two proteins were measured and considered as positives. Two hundred negative fibers, randomly chosen in the contralateral TA transfected with insert-less plasmid (pM1), were measured and considered as controls.

7) WESTERN BLOTS

Muscle proteins of gastrocnemius muscle were extracted as previously described (7). Equal amounts of proteins were resolved by SDS-polyacrylamide gel 10% electrophoresis and transferred to hydrophobic PVDF membranes (Amersham Hybond-P, GE Healthcare). Membranes were probed with anti-phospho-Akt (Ser473, 1:1000; Cell Signaling), anti-Akt (1:1000, Upstate), anti-S6K (1:1000, Santa Cruz Technology) and anti-GAPDH (1:3000, Cell Signaling) followed by horseradish peroxidase-coupled secondary antibody, anti-mouse or anti-rabbit (1:40000, Amersham) and developed by a chemiluminescence-based detection system (Amersham ECL Plus, GE Healthcare). Developed film was scan and analyzed as previously described (33).

8) ASSAY OF S6K ACTIVITY

The TA muscles of FVB mice (n=12) were transfected with the plasmid pM1-FS288 (left leg) and the control plasmid pM1 (right leg). The muscles were removed 17 days after transfection and frozen in liquid nitrogen. Whole TA muscles were homogenized with Ultraturrax (IKA-Labortechnik) in ice-cold lysis buffer (50 mM HEPES [pH7.5], 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% NP40, 1 mM DTT, 50 mM β-glycerophosphate, 50 mM NaF, 5 mM NaPPi, 1 mM vanadate, anti-protease cocktail). Homogenates were then centrifuged 10 min at 10 000 rpm (Sorvall SS-34 rotor) and the supernatant muscle protein content was determined by using Bradford's protein assay (Bio-Rad). S6K activity was assayed after

immunoprecipitation of 400 µg protein with anti-total S6K antibody (Santa Cruz Technology) as described by C Sanchez Canedo (34). Briefly, the immunoprecipitates were incubated with the appropriate buffer with 0.2 mM peptide substrate and 0.1 mM [γ- ^{32} P] ATP-Mg (specific radioactivity 1000 cpm/pmol) for 20min at 30°C. One unit of S6K activity corresponds to 1 nmole of product formed per minute under the assay conditions.

9) STATISTICAL ANALYSIS

Results are presented as means ± standard error of the mean (SEM). Statistical analyses were performed using a one-way ANOVA followed by a Bonferroni Multiple comparison test or unpaired t-test to compare muscles from different animals undergoing different experimental conditions, or a paired t-test to compare muscles undergoing different experimental conditions within the same animal. Interactions between FS and genetic background or pharmacological treatment were assessed using a two-way ANOVA followed by a Bonferroni post-test. Fiber cross-sectional area distribution statistical analysis was performed using χ^2 Pearson test. Statistical significance was set at $P < 0.05$.

RESULTS

1) FS-INDUCED MUSCLE FIBER HYPERTROPHY IS SEVERELY BLUNTED IN MICE CONSTITUTIVELY EXPRESSING A MUSCLE DOMINANT NEGATIVE FORM OF IGF-IR.

Inhibition of Mstn is characterized by increased expression of both IGF-I and IGF-II (7;10-14). Therefore, it is possible that the increased IGFs expression might contribute to skeletal muscle hypertrophy induced by FS. As the action of IGF-I and IGF-II are closely related and are mediated by the same receptor (IGF-IR), it is possible that IGF-IR plays a role in FS-induced muscle hypertrophy. Therefore, we

tested whether the IGF-IR mediated-signaling is required for FS to promote muscle hypertrophy. This hypothesis was assessed by overexpressing FS in the muscle of MKR transgenic mice expressing a dominant-negative form of IGF-IR specifically in skeletal muscle. The skeletal muscles of these mice are resistant to the actions of IGF-I and insulin (35). To confirm the resistance of skeletal muscle of MKR mice to IGF-I, we studied the phosphorylation of Akt, a key intracellular mediator of IGF-I action, after IV injection of IGF-I. As shown in figure 1A, the phospho-Akt signal is increased in the muscle of MKR compared to WT mice. Nevertheless, IGF-I treatment failed to increase Akt phosphorylation (Ser473) in MKR mice in contrast to WT mice.

To investigate the role of IGF-IR in FS-induced muscle hypertrophy, mouse TA muscles of WT and MKR mice were transfected with pM1-hFS288-c-myc or pM1 as a control condition and collected 17 days after. As expected, real-time quantification of IGF-IR mRNA by using primers designed to quantify both the endogenous IGF-IR and the transgene showed that IGF-IR is highly increased in muscles of MKR mice (Fig 1B). FS overexpression in TA of WT mice caused muscle hypertrophy characterized by increased muscle mass (+37%, 52.3 ± 1.6 vs. 38.3 ± 1.0 mg, n=8; $P<0.001$) and fiber CSA (+147%, $P<0.001$). Muscle hypertrophy obtained by FS overexpression, as assessed by the measurement of the fiber CSA, was severely blunted in MKR mice (+54%, 3089 ± 184 vs. 2011 ± 52 µm², n=8; NS) compared to WT mice (+147%, 7445 ± 794 vs. 3011 ± 56 µm², n=8; $P<0.001$) (Fig 1C). Furthermore, the analysis of size distribution of FS tranfected fibers showed a marked shift to the left in MKR mice compared to WT mice ($p<0.001$) (Fig 1D). These results support the crucial role of the IGF-IR in the FS-induced hypertrophy.

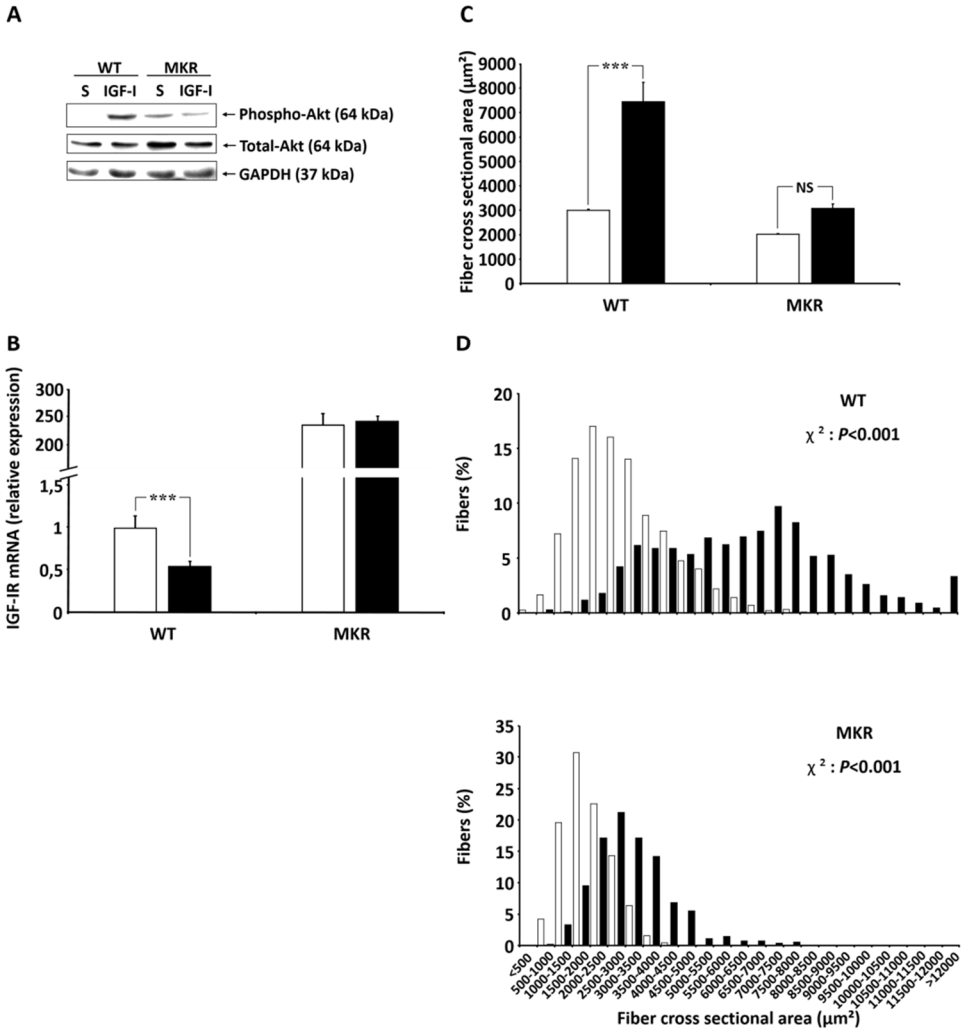

Figure 1. Role of the IGF-IR in the FS-induced muscle fiber hypertrophy. The fiber hypertrophy caused by overexpression of FS was severely blunted in mice constitutively expressing a muscle dominant negative form of IGF-IR (MKR mice) compared to WT mice. (A) IV injection of IGF-I (14 µg/animal vs. saline (S)) failed to increased muscle Akt phosphorylation (Ser473) in MKR mice compared to WT mice. Muscle IGF-IR mRNA (B) and fiber CSAs (C) were determined 17 days after transfection of pM1 (white column) or pM1-FS288 (black column) in WT and in MKR mice. The results are expressed as mean ± SEM. Statistical analysis was performed using 2-way ANOVA test (Muscle IGF-IR mRNA: FS effect, *P*<0.01; MKR effect, *P*<0.001; Interaction, *P*<0.01; Fiber CSAs: FS effect, *P*<0.001; MKR effect, *P*<0.001; Interaction, *P*<0.001) and Bonferroni post-test (***, *P*<0.001). (D) The proportion of large fibers in WT (up panel) and MKR (bottom panel) mice was greater in the FS288-transfected (black columns) than in the pM1-transfected muscle fibers (white columns) (*P*<0.001). The distribution of FS288-transfected muscle fibers (black columns) was shifted to the left in MKR mice (bottom panel) compared to WT mice (up panel) (*P*<0.001). Statistical analysis was performed using χ^2 Pearson test.

2) FS-INDUCED MUSCLE FIBER HYPERTROPHY IS MAINTAINED IN THE ABSENCE OF IGF-II.

To define the role of IGF-II in the FS-induced muscle hypertrophy, KO mice harbouring a constitutive deletion of the first IGF-II exon in the paternal IGF-II allele were used. Mouse TA muscles were transfected with pM1-hFS288 or pM1 as a control condition and collected 17 days after. TA muscle mass of IGF-II KO mice was reduced in comparison with WT mice (19.8 ± 1.2 mg vs. 38.3 ± 1.0 mg, $P<0.001$). As shown in figure 2A, FS overexpression increased muscle mRNA levels of IGF-II in WT mice (2-fold, $P<0.001$) but not in IGF-II KO mice, as expected. Despite the absence of IGF-II induction, muscle hypertrophy obtained by FS overexpression, as assessed by the measurement of the fiber CSA, reached a similar magnitude in WT (+118%, 3020 ± 237 vs. 1385 ± 97 µm², n=11; $P<0.001$) and IGF-II KO mice (+106%, 3432 ± 293 vs. 1665 ± 123 µm², n=14; $P<0.001$) (Fig 2B). The increase in the muscle fiber CSA induced by FS overexpression was also supported by the analysis of fiber size distribution showing a greater proportion of large fibers among the FS transfected fibers than among control fibers in both IGF-II KO and WT animal groups ($P<0.001$) (Fig 2C). These observations therefore suggest that FS can exert its hypertrophic effect in the absence of IGF-II.

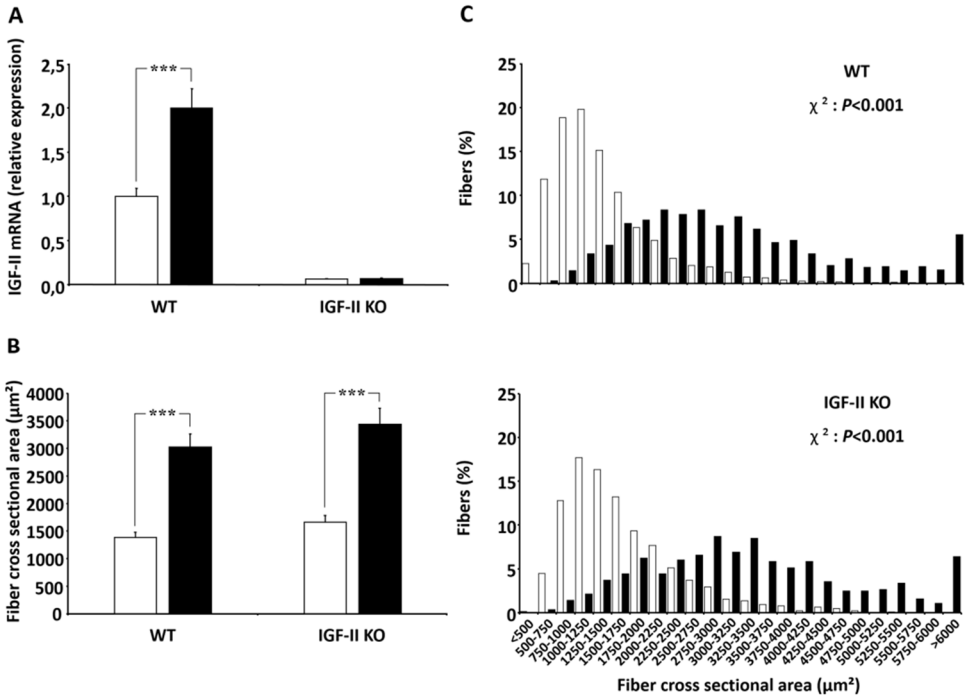

Figure 2. Role of IGF-II in the FS-induced muscle fiber hypertrophy. Overexpression of FS stimulated the expression of IGF-II only in WT mice but induced similar muscle fiber hypertrophy in WT and IGF-II KO mice. Muscle IGF-II mRNA (A) and fiber CSAs (B) were measured 17 days after transfection of pM1 (white column) or pM1-FS288 (black column) in the TA muscle of WT and IGF-II-KO mice. The results are expressed as mean ± SEM. Statistical analysis was performed using 2-way ANOVA test (Muscle IGF-II mRNA: FS effect, *P*<0.001; KO effect, *P*<0.001; Interaction, *P*<0.01; Fiber CSAs: FS effect, *P*<0.001; KO effect, NS; Interaction, NS) and Bonferroni post-test (***, *P*<0.001). (C) The proportion of large fibers in WT (up panel) and IGF-II-KO (bottom panel) mice was greater in the FS288-transfected (black columns) than in the pM1-transfected muscle fibers (white columns). Statistical analysis was performed using χ^2 Pearson test (*P*<0.001).

3) INHIBITION OF AKT BY TRANSFECTION OF A DOMINANT NEGATIVE FORM SEVERELY BLUNTS MUSCLE FIBER HYPERTROPHY INDUCED BY FS OVEREXPRESSION.

We then tested the role of the signaling molecules stimulated by IGF-IR, in particular the Akt/mTOR/S6K pathway, in the FS action. This pathway is known to be central for IGF-I anabolic action on skeletal muscle (20) and appears activated when Mstn action is inhibited such as in Mstn KO mice (19;36).

To investigate whether Akt phosphorylation is required for the FS hypertrophic effect, we cotransfected a dominant negative form of Akt together with FS. Mouse TA muscles of mice were transfected with pM1-FS288-c-myc or pM1-dnAkt or the two plasmids and collected 17 days after. Expression of FS or/and dnAkt was detected by immunohistochemistry in TA muscles electroporated with the pM1-FS288 or/and pM1-dnAkt using antibodies raised against the tag c-myc of FS and the tag HA of dnAkt (Fig 3A). Muscle hypertrophy obtained by FS overexpression, as assessed by the measurement of the fiber CSA, was severely blunted in cotransfected fibers (+37%, 3487 ± 231 vs. 2543 ± 119 μm^2, n=6; $P<0.05$) compared to fibers transfected by FS only (+105%, 5146 ± 313 vs. 2505 ± 114 μm^2, n=6; $P<0.001$) (Fig 3B). Fiber CSA was not changed in fibers transfected only by dnAkt compared to pM1-transfected fibers (Fig 3B). The analysis of size distribution of FS transfected fibers showed a marked shift to the left in cotransfected fibers compared to fibers transfected by FS only ($p<0.001$) (Fig 3C). These results suggest that Akt plays an important role in the FS hypertrophic action on muscle fibers.

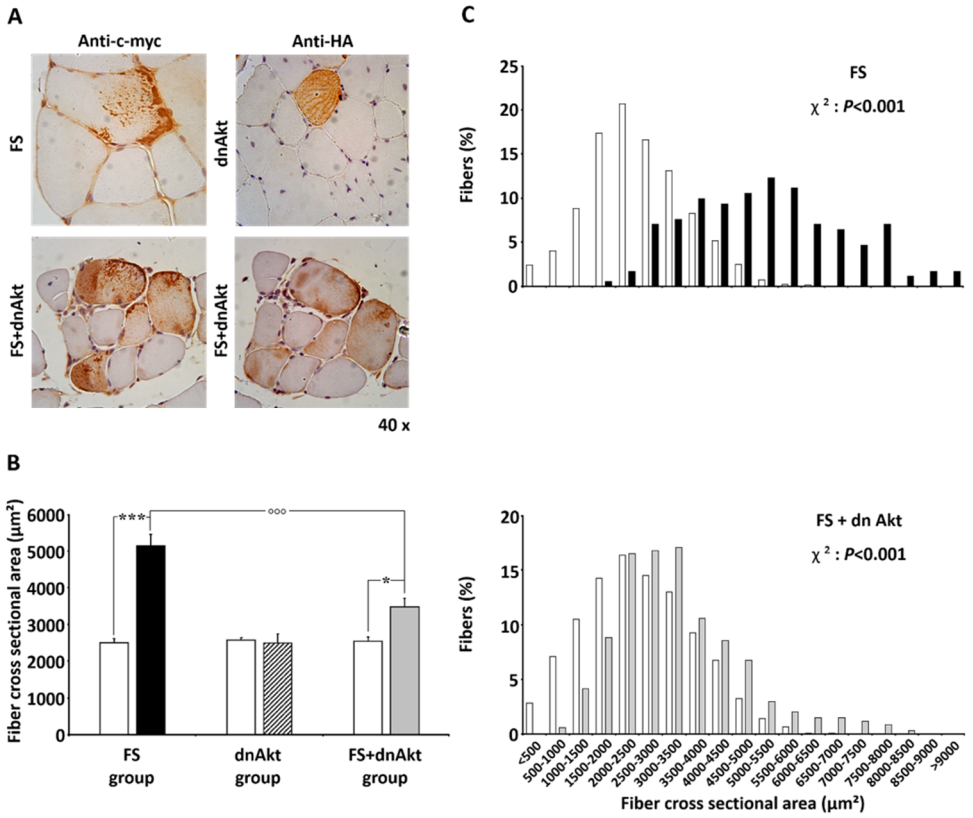

Figure 3. Role of Akt in the FS-induced muscle fiber hypertrophy. The fiber hypertrophy caused by overexpression of FS was severely blunted in muscle co-transfected with a dominant negative form of Akt (dnAkt) compared to muscle transfected with FS only. (A) Fibers overexpressing FS or/and dnAkt were respectively detected by anti-c-myc and anti-HA antibody. (B) Fiber CSAs were determined 17 days after transfection of pM1 (white column) or pM1-dnAkt (lined column) or pM1-FS288 without (black column) or with pM1-dnAkt (grey column). The results are expressed as mean ± SEM. Statistical analysis was performed using 1-way ANOVA test followed by a Bonferroni Multiple Comparison Test (*, p<0.05; ***, ooo; *P*<0.001). (C) The proportion of large fibers was greater in the muscle fibers transfected with FS288 without (black column) or with dnAkt (grey column) than in the pM1-transfected muscle fibers (white columns) (***, *P*<0.001). The distribution of FS288 and dnAkt cotransfected muscle fibers (grey column, bottom panel) was shifted to the left compared to the distribution of FS288 only transfected muscle fibers (black column, up panel) (*P*<0.001). Statistical analysis was performed using χ^2 Pearson test.

4) INHIBITION OF mTOR BY RAPAMYCIN ATTENUATES MUSCLE FIBER HYPERTROPHY INDUCED BY FS OVEREXPRESSION.

To evaluate the role of mTOR, a major target of Akt, mice transfected with FS were treated with rapamycin (2 µg/g BW/day), a specific inhibitor of mTOR. Mouse TA muscles were transfected with pM1-hFS288 or pM1 as a control condition and collected 17 days after.

Rapamycin dosage on whole blood showed an increase of circulating rapamycin concentrations in rapamycin-treated mice compared to vehicle-treated mice (62.3 ± 1.2 vs. 0.5 ± 0.1 ng/ml, n=12/group). These rapamycin blood levels are known to block mTOR activity (37). Rapamycin treatment was found to block the specific activity of S6K, a target of mTOR (-71%, 0.015 ± 0.002 vs. 0.052 ± 0.019 pmol/min/mg prot in pM1-transfected muscles, n=4/group; $p<0.05$) (Fig 4A). These data confirm that the inhibition of mTOR was effectively obtained by our rapamycin treatment.

Muscle hypertrophy obtained by FS overexpression, as assessed by the measurement of the fiber CSA, was reduced in rapamycin-treated mice (+62%, 4761 ± 296 vs. 2945 ± 137 µm², n=8; $P<0.001$) compared to vehicle-treated mice (+106%, 5972 ± 229 vs. 2893 ± 87 µm², n=8; $P<0.001$) (Fig 4B). Rapamycin treatment by itself did not affect myofiber size. The analysis of size distribution of FS tranfected fibers showed a shift to the left in rapamycin-treated mice compared to vehicle-treated mice ($p<0.001$) (Fig 4C). These results suggest that mTOR plays a moderate role in the FS hypertrophic action on muscle fibers.

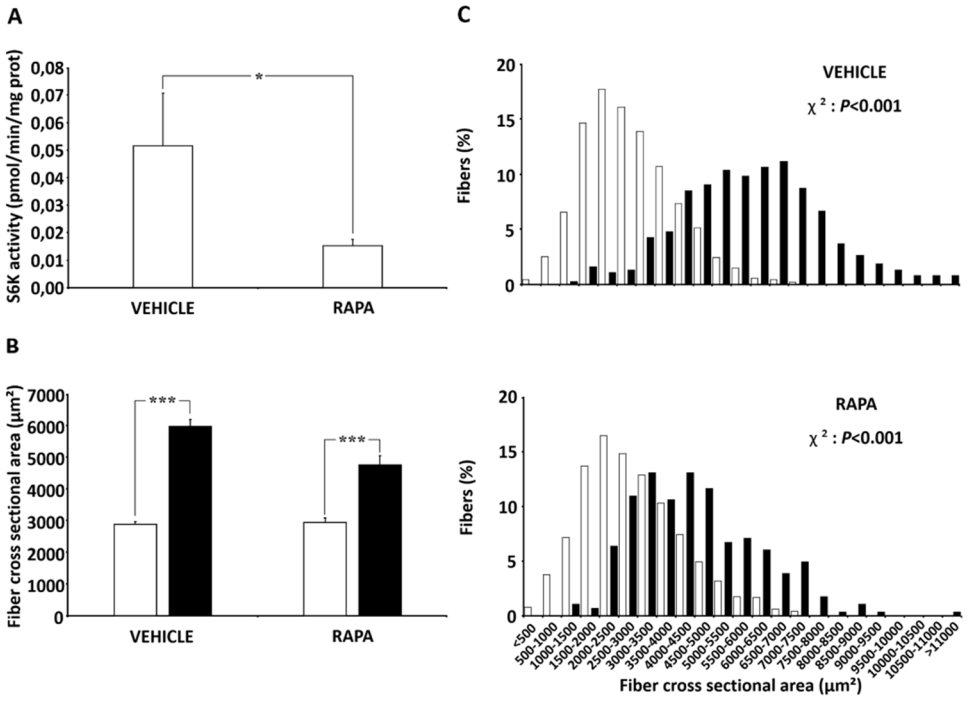

Figure 4. Role of mTOR in the FS-induced muscle fiber hypertrophy. Inhibition of mTOR by rapamycin attenuated muscle fiber hypertrophy induced by FS overexpression compared to vehicle treated mice. (A) The activity of S6K, a target of mTOR, was measured in the TA muscle 17 days after transfection of pM1 (white column). The results are expressed as mean ± SEM. Statistical analysis was performed using unpaired t-test (*, $P<0.05$). Fiber CSAs (B) were determined after transfection of pM1 (white column) or pM1-FS288 (black column) in vehicle- or rapamycin-treated (2 µg/g BW.day) mice. Statistical analysis was performed using 2-way ANOVA test (FS effect, $P<0.001$; Rapa effect, $P<0.01$; Interaction, $P<0.01$) and Bonferroni post-test (***, $P<0.001$). (C) The proportion of large fibers in vehicle- and in rapamycin-treated mice was greater in the FS288-transfected (black columns) than in the pM1-transfected muscle fibers (white columns) (***, $P<0.001$). The distribution of FS288-transfected muscle fibers was shifted to the left in rapamycin-treated mice (black column, bottom panel) compared to vehicle-treated mice (black column, up panel) ($P<0.001$). Statistical analysis was performed using χ^2 Pearson test.

5) FS-INDUCED MUSCLE FIBER HYPERTROPHY IS MAINTAINED IN THE ABSENCE OF S6K.

To investigate the role of S6K, a target of mTOR, in FS-induced muscle hypertrophy, TA muscles of WT and S6K1/2 KO mice were transfected with pM1-hFS288 or pM1 as a control condition and collected 17 days after. Western blot analysis of S6K1/2 KO mice revealed a strong reduction of the S6K protein in gastrocnemius muscle (Fig 5A). The specific activity of S6K was increased in muscle transfected with FS compared to pM1-transfected muscle (+60%, 0.086 ± 0.028 vs. 0.053 ± 0.014 pmol/min/mg, n=12/group; $P<0.01$) (Fig 5B). Muscle hypertrophy obtained by FS overexpression, as assessed by the measurement of the fiber CSA, reached a similar magnitude in WT (+101%, 4418 ± 398 vs. 2200 ± 176 µm², n=13; $P<0.001$) and S6K1/2 KO mice (+107%, 4204 ± 264 vs. 2034 ± 100 µm², n=15; $P<0.001$) (Fig 5C). The similar increase in the muscle fiber CSA induced by FS overexpression was also supported by the analysis of fiber size distribution showing a greater proportion of large fibers among the FS transfected than among control fibers in both S6K1/2 KO and WT animal groups ($P<0.001$) (Fig 5D). These observations therefore suggest that FS can exert its hypertrophic effect in the absence of S6K1/2.

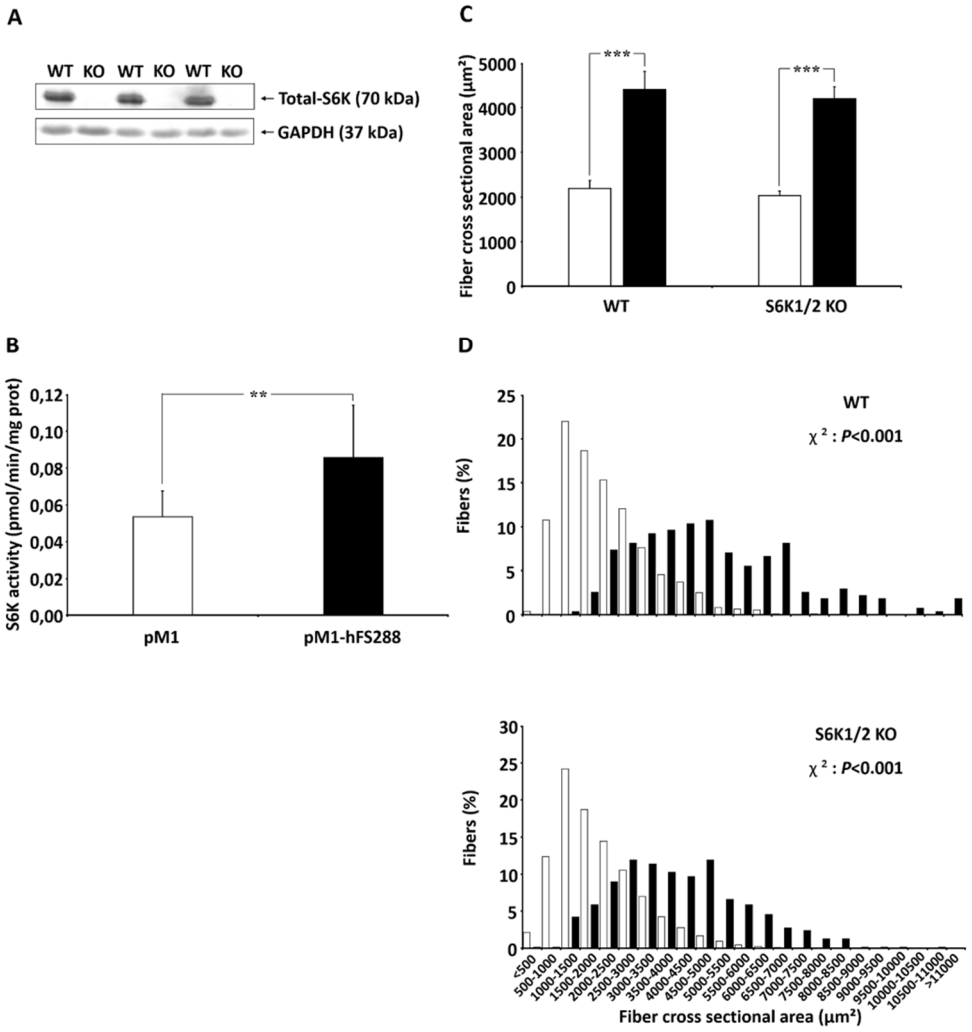

Figure 5. Role of S6K in the FS-induced muscle fiber hypertrophy. Overexpression of FS induced a similar muscle fiber hypertrophy in WT and S6K1/2 KO mice. (A) Western blot of total-S6K in muscle of WT and S6K1/2 KO mice. (B) Overexpression of FS increased the activity of S6K. The activity of S6K was measured in the TA muscle 17 days after transfection of pM1 (white column) or pM1-FS288 (black column). The results are expressed as mean ± SEM. Statistical analysis was performed using paired t-test (**, *P*<0.01). (C) Fiber CSAs were determined 17 days after transfection of pM1 (white column) or pM1-FS288 (black column) in WT and in S6K1/2 KO mice. The results are expressed as mean ± SEM. Statistical analysis was performed using 2-way ANOVA test (FS effect, *P*<0.001; KO effect, NS; Interaction, NS) and Bonferroni post-test (***, *P*<0.001). (D) The proportion of large fibers in WT (up panel) and S6K1/2 KO (bottom panel) mice was greater in the FS288-transfected (black columns) than in the pM1-transfected muscle fibers (white column). Statistical analysis was performed using χ^2 Pearson test (***, *P*<0.001).

DISCUSSION

In the present study, we explored the signaling mechanisms of the skeletal muscle hypertrophy induced by FS. To answer this question, we overexpressed FS288 gene by electrotransfer in TA muscle of mice genetically modified or treated with pharmacological agent. We demonstrate that the hypertrophic action of FS on skeletal muscle requires the activation of the IGF-IR/Akt/mTOR pathway. In contrast, induction of IGF-II expression and S6K activity by FS seems to be not required for the hypertrophic action of FS. Taken together, our results show, for the first time *in vivo*, the role of IGF-IR/Akt/mTOR pathway in the skeletal muscle hypertrophy caused by FS.

IGF-I is known to be a positive regulator of skeletal muscle mass. In fact, IGF-I overexpression specifically in skeletal muscle increases skeletal muscle mass (38;39). In contrast, IGF-I or IGF-IR knock-out mice exhibit significant muscle hypoplasia and die soon after birth (40). The role of IGF-I in the hypertrophy caused by Mstn inhibition is suggested by the increased circulating levels of IGF-I observed in KO Mstn mice (14). Furthermore, Mstn inhibition stimulates the Akt/mTOR/S6K pathway, the pathway mainly responsible for the muscle hypertrophy caused by IGF-I (18-20). Finally, Mstn inhibition by FS induces muscle hypertrophy by increasing protein synthesis and satellite cell activation, as IGF-I does (7;9;41;42). The molecular mechanisms by which signaling pathways integrating IGFs and Mstn action on muscle cells have not been previously identified *in vivo*. Here, we showed that IGF-IR-mediated signaling is required for FS to promote muscle hypertrophy by using MKR transgenic mice expressing a dominant-negative form of IGF-IR specifically in skeletal muscle. Indeed, muscle hypertrophy obtained by FS overexpression, as assessed by the muscle fiber diameter, was severely blunted in MKR mice compared to WT mice. These results are in the line of previous data demonstrating the crucial role of IGF-IR in some models of muscle hypertrophy such

the one induced by GH treatment (43) or by exercise (44). In contrast, IGF-IR does not seem to be crucial for muscle hypertrophy induced by overloading (45) and testosterone (46). Insulin receptor (IR) in the muscle of MKR mice is also dysfunctional due to the formation of heterodimers with the mutated IGF-IR, as illustrated by the diabetic phenotype of these animals (35). Besides IGF-IR, IR contributes also to the maintenance of the skeletal muscle mass (47). So, we cannot exclude the possibility that the IR is required for the FS-induced hypertrophy or metabolic disorders contribute to blunt the FS action. The fact that either IR or IGF-IR activation is required for muscle hypertrophy caused by FS suggests that the hypertrophy caused by Mstn inhibition might be impaired in situations characterized by low insulin or IGF-I such as in malnutrition and diabetes (48).

Muscle hypertrophy induced by Mstn inhibition either by Mstn gene deletion or by FS overexpression is accompanied by increased muscle IGF-II expression (7;10-13). This IGF-II up-regulation might contribute to the muscle hypertrophy caused by Mstn inhibition. This hypothesis is indeed supported by several observations. First, IGF-II is known to be a potent stimulator of both mitogenesis and myogenesis through its binding and activation of IGF-I receptor (IGF-IR) (15-17). Second, overexpression of Mighty, a promyogenic factor negatively regulated by Mstn, results in myotube hypertrophy partially mediated by increased IGF-II expression (12). Conversely, deletion of IGF-II gene causes a severe impairment of muscle development, as we showed. In rodents, circulating IGF-II decreases with maturation in contrast to IGF-I but the muscle expression levels of IGF-I and IGF-II are very similar in the skeletal muscle of adult mice (data not shown). Although all these observations suggest that IGF-II might be a putative mediator of the muscle hypertrophy caused by FS, our results demonstrate that IGF-II is not mandatory for this muscle hypertrophy. Indeed, muscle hypertrophy obtained by FS overexpression, as assessed by the muscle fiber diameter, reached a similar

magnitude in WT and IGF-II KO mice. All together, these observations suggest therefore that IGF-II should be considered as a marker of the activation of specific cell pathways more than a crucial player in FS-induced muscle hypertrophy. Indeed, IGF-II induction might reflect the activation of mammalian target of rapamycin complex 1 (mTORC1), a key regulator of myogenesis (49). Indeed, mTORC1 has been demonstrated to control myoblast differentiation by stimulating IGF-II expression (50). Furthermore, mTORC1 was directly responsible for the increased IGF-II expression in a model of muscle regeneration (51). Otherwise, the increase of IGF-II might reflect satellite cell activation. Indeed, it has been shown that the increase of IGF-II induced by Mstn inhibition is localized in activated satellite cells (10). Since previous works have demonstrated the role of satellite cells (7) and protein synthesis (9) in the muscle hypertrophy caused by FS, our work suggests that these processes can take place in the absence of IGF-II at least during postnatal life. As the model of IGF-II invalidation was constitutive and global, an adaptation to favour the action of IGF-I may still occur. However, this adaptation seems not sufficient to compensate the absence of IGF-II for the development of muscle mass.

A central component of the cascade activated by IR and IGF-IR is the Akt kinase. Several evidences indicate that this kinase may play a crucial role in the FS-induced muscle hypertrophy. Akt -1 overexpression by transgenesis or electrotransfer is sufficient to cause a dramatic muscle hypertrophy (21-23). In addition, myostatin inhibition is characterized by an increase in expression and activity of Akt in skeletal muscle (18;19;52;53). Here, we demonstrated that Akt activation is required for the hypertrophic effect of FS *in vivo* by cotransfecting a dominant negative form of Akt together with FS. In fact, muscle hypertrophy obtained by FS overexpression, as assessed by the muscle fiber diameter, was severely blunted in cotransfected fibers compared to fibers transfected only by FS. These results extend previous *in vitro* observations. Indeed, in C2C12 cells, inhibition of Akt completely blocks myotube

hypertrophy induced by Mstn inhibition (18). In contrast, previous results show that neither Akt1 nor Akt2 isoforms are required for the hypertrophic response to inhibition of Mstn by treatment with a soluble form of its receptor ActIIRB (ActIIRB-mFC) (24). The discrepancy between this last report and ours might result from differences in the choice of the tool to inhibit Akt-1. In our conditions, Akt inhibition was local and postnatal while, in the conditions used by Goncalves *et al* (24), the Akt inhibition was systemic and developmental.

Because mTOR, a kinase downstream of Akt, is an integrator of growth factor signal that controls protein synthesis and cell size (27;28), we evaluated its role in the FS hypertrophic action by treating mice with rapamycin, a specific inhibitor of mTORC1. Our data demonstrated that mTORC1 plays a modest role in FS-induced muscle hypertrophy. Indeed, muscle hypertrophy obtained by FS overexpression, as assessed by the muscle fiber diameter, was reduced in rapamycin-treated mice compared to vehicle-treated mice. The circulating concentrations of rapamycin, and more importantly, the dramatic decrease in S6K activity, a downstream target of mTOR, confirm that mTOR activity was indeed inhibited in skeletal muscle of rapamycin-treated mice. Our results are in agreement with those of Sartori *et al.* showing that muscle fiber hypertrophy induced by the transfection of a dominant-negative ActIIRB is inhibited by 33% when mice are treated with rapamycin (29). Our observations suggest therefore that the muscle hypertrophy induced by Mstn inhibition is only partially dependent on the protein synthesis stimulated by mTORC1 signaling. This interpretation is supported by the observation of Welle *et al.* showing that rapamycin does not prevent the acute stimulation of protein synthesis induced by anti-Mstn antibody (54). The moderate role of mTOR suggests that other molecules phosphorylated by Akt such as GSK-3β might be implicated in FS-induced muscle hypertrophy. In fact, it is well established both *in vitro* and *in vivo* that GSK-3β kinase and some of its targets play a crucial role for skeletal muscle

growth (20;33). Moreover, Mstn treatment in C2C12 cells activates GSK-3β through an inhibition of the PI3K/Akt pathway (55). Further studies will have to decipher the role of this pathway in the muscle hypertrophy caused by Mstn inhibition. Interestingly, the relatively modest role of mTOR in FS-induced fiber hypertrophy contrasts with what has been observed in other models of muscle hypertrophy. For example, myotube hypertrophy induced by IGF-I is strongly prevented by rapamycin in C2C12 cells (20;56). Furthermore, the muscle hypertrophy induced by overload of the plantaris muscle (21) or caused by clenbuterol in tibialis anterior muscle is fully prevented by rapamycin and therefore mTOR-dependent (57). Finally, the contraction-induced increase in muscle protein synthesis in humans is also blocked by rapamycin (37). Despite the modest effect of rapamycin treatment on FS-hypertrophic action, we cannot exclude a possible mTOR raptor-independent effect. In fact, rapamycin inhibits only partially mTOR activity and a large fraction of mTOR substrates is insensitive to rapamycin (58). The use of novel highly selective compounds that block the kinase activity of mTORC1 and mTORC2 (59) will allow to decipher more completely the role of mTOR in hypertrophy induced by Mstn inhibition.

It is well established that Myostatin inhibition is characterized by an increase in activity of S6K1, a target of mTOR, in skeletal muscle (9;29;54). The increase in S6K activity after FS overexpression that we observed agrees with these previous observations. S6K is known to play a role in skeletal muscle growth. In fact, deletion of S6K1/2 in mice results in decreased skeletal muscle mass (26). In contrast, overexpression of S6K1 is sufficient to increase the diameter of myotube (20;26). Here, we demonstrated that S6K is not mandatory in FS-induced muscle hypertrophy. Indeed, muscle hypertrophy obtained by FS overexpression, as assessed by the muscle fiber diameter, reached a similar magnitude in WT and S6K1/2 KO mice. These results are in line with those Mc Mullen *et al.* showing that

deletion of S6K1/2 have no impact on the development of physiological, pathological or IGF-IR-PI3K-induced cardiac hypertrophy (60). The absence of effect of S6K deletion in FS action suggests that other molecules phosphorylated by mTOR such as 4E-BP1 might be implicated in FS-induced muscle hypertrophy. Moreover, we cannot rule out the possibility that the activation of a compensatory pathway in S6K1/2 KO mice may mask the role of S6Ks in muscle hypertrophy induced by FS. In fact, it has been shown that the activity of Akt, a crucial mediator of FS-induced skeletal muscle hypertrophy, is upregulated in S6K1/2 KO mice (61). In contrast to FS-induced hypertrophy, myotube hypertrophy induced by IGF-I is severely attenuated by rapamycin (20) or S6K1/2 deletion (26). This divergence suggests that, although the stimulation of the IGF-IR is mandatory for the FS-induced hypertrophy, mTOR and S6K activities seem less crucial for the hypertrophy induced by FS, supporting the role of other pathways in this hypertrophy model.

In conclusion, our results show that IGF-IR signaling plays a critical role in FS-induced skeletal muscle hypertrophy. The present study connects the Mstn and the IGF-IR pathways, the two most important pathways that regulate muscle mass. Defining the key pathway for muscle hypertrophy is a crucial issue for the correct identification of drug targets to mitigate muscle wasting.

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2. Role of IGF-I in the Follistatin-induced muscle hypertrophy

The aim of this second article was to investigate the role of IGF-I and insulin, two ligands of the IGF-IR, in the Follistatin (FS) hypertrophic action on skeletal muscle. In this article, we report that

1) Muscle hypertrophy induced by Myostatin inhibition is associated with a down regulation of muscle IGF-I expression.
2) FS retains its full hypertrophic effect towards muscle despite very low concentrations of circulating and muscle IGF-I in a model of hypophysectomized animals.
3) FS does not increase muscle sensitivity to IGF-I but decrease it once hypertrophy is present
4) FS muscle hypertrophic effect is attenuated in a model of low insulin obtained by streptozotocin injection.
5) The full anabolic response to FS is restored in the streptozotocin-treated animals by insulin but also by IGF-I infusion.

In conclusion, FS-induced muscle hypertrophy requires the activation of the Insulin/IGF-I pathway either by insulin or IGF-I. When insulin or IGF-I alone is missing, FS retains is full anabolic effect. But, when both are deficient, as in streptozotocin-treated animals, FS fails to simulate muscle growth.

Role of IGF-I in the Follistatin-induced muscle hypertrophy

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Collaborations:

O Ritvos kindly provided soluble Activin receptor type IIB fused to Fc

Author contributions:

C Barbé and S Kalista contributed equally to this work

C Barbé, S Kalista, A Loumaye and JP Thissen designed research

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ABSTRACT

Follistatin, a physiological inhibitor of Myostatin, induces a dramatic increase in skeletal muscle mass requiring the type 1 IGF-I receptor/Akt/mTOR pathway. The aim of the present study was to investigate the role of IGF-I and insulin, two ligands of the IGF-I receptor, in the Follistatin hypertrophic action on skeletal muscle. In a first step, we showed that Follistatin increases muscle mass while being associated with a down regulation of muscle IGF-I expression. In addition, Follistatin retained its full hypertrophic effect towards muscle in hypophysectomized animals despite very low concentrations of circulating and muscle IGF-I. Furthermore, Follistatin did not increase muscle sensitivity to IGF-I in stimulating phosphorylation of Akt but, surprisingly, decreased it once hypertrophy is present. Taken together, these observations indicate that increased muscle IGF-I production or sensitivity does not contribute to the muscle hypertrophy caused by Follistatin. Unlike low IGF-I, low insulin, as obtained by streptozotocin injection, attenuated the hypertrophic action of Follistatin on skeletal muscle. Moreover, the full anabolic response to Follistatin was restored in this condition by insulin but also by IGF-I infusion. Therefore, Follistatin-induced muscle hypertrophy requires the activation of the insulin/IGF-I pathway either by insulin or IGF-I. When insulin or IGF-I alone is missing, Follistatin retains its full anabolic effect. But, when both are deficient, as in streptozotocin-treated animals, Follistatin fails to stimulate muscle growth.

Key words: IGF-I, insulin, Follistatin, Myostatin, skeletal muscle hypertrophy

INTRODUCTION

Myostatin (Mstn), a member of the TGF-β family primarily expressed in skeletal muscle, is a negative regulator of skeletal muscle mass as shown by the increase in skeletal muscle mass and strength following Mstn inhibition or gene deletion (28, 44). Among Mstn inhibitors, Follistatin (FS) and the soluble ligand binding domain of the Activin Receptor type IIB fused to the Fc domain IgG (sActRIIB-Fc) have been shown to bind and antagonize Mstn leading to a dramatic increase in muscle mass (3, 30, 37). The muscle fiber hypertrophy induced by FS is due to increased protein synthesis and requires the activation of Smad1/5 (63) and the type I IGF-I receptor (IGF-IR)/Akt/mTOR pathway (34, 64).

IGF-I is known to be a major positive regulator of skeletal muscle mass. Indeed, IGF-I overexpression specifically in skeletal muscle increases skeletal muscle mass (13, 47), while IGF-I knock-out mice exhibit significant muscle hypoplasia (42). Increased muscle IGF-I expression has been reported in several models of muscle hypertrophy such as exercise, overloading, GH and testosterone treatment (1, 20, 27, 39, 40). The role of IGF-I in the muscle hypertrophy caused by Mstn inhibition is suggested by several evidences. First, muscle mRNA expression and circulating concentrations of IGF-I have been reported to be increased following Mstn inhibition (35, 62, 64). Moreover, Mstn inhibition causes muscle hypertrophy by increasing protein synthesis and satellite cell activation, as IGF-I does (5, 6, 24, 58, 64). Finally, muscle hypertrophy induced by both IGF-I overexpression and Mstn inhibition requires the IGF-IR/Akt/mTOR pathway (8, 34, 51, 64). Taking these observations together, we hypothesized that IGF-I might contribute to the muscle hypertrophy caused by Mstn inhibition. To answer this question, we overexpressed hFS288 gene by electrotransfer in tibialis anterior (TA) muscle of animals either deficient in IGF-I and/or insulin and treated or not by

insulin or IGF-I. This work allowed us to delineate the role of IGF-I and insulin in the skeletal muscle hypertrophy caused by FS-induced Mstn inhibition.

MATERIALS AND METHODS

1) ANIMALS

To assess the effect of Mstn inhibition on skeletal muscle mass and IGF-I expression, we used C57BL/6 transgenic mice (mTr-FS) overexpressing a human FS288 (hFS288) short form specifically in skeletal muscle (37) and their control wild-type littermates (WT); FVB Mstn knock-out mice (Mstn KO) harboring a constitutive deletion of the third Mstn exon (28) and their control wild-type littermates (WT); and FVB mice (Janvier Breeding, Le-Genest-Saint-Isle, France) treated by intraperitoneal (ip) injection with the sActRIIB-Fc (31) at a dose of 10 mg/kg twice a week during 14 days or with PBS (CTRL). The sActRIIB-Fc was prepared as described by Hoogaars et al. (30). All the mice used were male between 6 and 8 week-old, except if otherwise stated. To assess the role of IGF-I in the muscle hypertrophy induced by FS, we used hypophysectomized female Wistar rats purchased from Charles River (Charles River Laboratories, France). Hypophysectomy was performed at 5 weeks of age and hypophysectomized (HYPOX) animals were delivered to our animal quarters 7 days after surgery with their control intact littermates (CTRL). To assess the effect of FS on IGF-I sensitivity, we used mTr-FS 8 week-old male mice and their control wild-type littermates (WT). To assess the effect of the sActRIIB-Fc on IGF-I sensitivity, we treated 8 week-old C57Bl/6 male mice with sActRIIB-Fc during a time period of 5 days. To explore the role of insulin in the muscle hypertrophy induced by FS, we used streptozotocin (STZ)-injected male Wistar rats. Animals (100-124 g) were provided by Janvier Breeding (Janvier Labs, France) at 4 weeks of age and treated by STZ injection a

week later. All the animals were housed individually under controlled conditions of lighting (12 h light, 12 h dark cycle) and temperature (22 ± 2°C). They received standard chow pellets and water ad libitum. The study was conducted in accordance with the directives of and approved by the Animal Ethics Committee of the Catholic University of Louvain (Brussels, Belgium).

2) EXPERIMENTAL DESIGN

2.1. Role of IGF-I in FS-induced muscle hypertrophy

To assess the role of IGF-I in FS-induced muscle hypertrophy, we used HYPOX rats. The experiment was started after a 9-day adaptation period in our animal quarters. To attest the success of hypophysectomy, changes in body weight was recorded during this period. As expected, the body weight gain of HYPOX rats was severely blunted compared to intact animals (+18.0±2.1 *vs.* +94.9±7.4 g, n=6/group, $P < 0.001$). During the adaptation period, water was supplemented by 5% glucose and 0.9% NaCl. Throughout the remainder of the experiment, the rats received water containing 0.9% NaCl only and a hormonal replacement. HYPOX rats (n=6) were given replacement therapy with L-thyroxine (10 µg/kg/day; Sigma-Aldrich, Diegem, Belgium) and hydrocortisone hemisuccinate (500 µg/kg/day; Solu-Cortef, Pfizer, Oslo, Norway) diluted in saline by daily subcutaneous injection (08.00 AM) while CTRL rats (n=6) were injected with saline solution. One week after the beginning of the hormonal treatment, TA muscles of HYPOX and CTRL rats were transfected with the plasmid pM1-hFS288-c-myc (left leg) and the control plasmid pM1 (right leg). TA muscles and serum were collected 17 days after electroporation for assessment of muscle hypertrophy and IGF-I concentrations.

2.2. Effect of FS and sActRIIB-Fc on skeletal muscle IGF-I sensitivity

To investigate the effect of FS on IGF-I sensitivity, mTr-FS and WT mice were fasted overnight and muscle and liver Akt phosphorylation (pAkt) was assessed after ip injection of recombinant human IGF-I (Genentech, South San Francisco, CA). One hundred µl of a solution of IGF-I (200 or 400 µg/kg of body weight) or 100 µl of saline solution (vehicle, 0.9% NaCl) was injected 15 min before the sacrifice. To investigate the effect of the sActRIIB-Fc on IGF-I sensitivity, mice treated with two ip injections of 10 mg/kg of the sActRIIB-Fc (or same volume of PBS for control animals) during a time period of 5 days and their controls were fasted overnight and muscle pAkt was assessed after ip injection of recombinant human IGF-I (Genentech, South San Francisco, CA). One hundred µl of a solution of IGF-I (400 µg/kg of body weight) or 100 µl of saline solution (vehicle, 0.9% NaCl) was injected 15 min before the sacrifice. Liver and gastrocnemius (GC) muscles were collected for assessment of pAkt by western blotting analysis. Therefore, muscle IGF-I sensitivity was explored in a transgenic model characterized by a marked hypertrophy and in a non-transgenic model at the early stages of hypertrophy.

2.3. Role of Insulin in FS-induced muscle hypertrophy

To explore the role of Insulin in FS-induced muscle hypertrophy, we used a model of STZ-induced diabetes. After a 7-day adaptation period in our animal quarters, TA muscles of rats were transfected with the plasmid pM1-hFS288-c-myc (left leg) and the control plasmid pM1 (right leg). Three days after electroporation, diabetes was induced by injecting STZ (Sigma-Aldrich Corp., St. Louis, MO) freshly prepared in 0.01M citrate buffer, pH 4.5 at the dose of 60 mg/kg of body weight into the tail vein. The CTRL group was injected with an equivalent volume of 0.01M citrate buffer, pH 4.5. Only rats showing polydipsia, polyuria, glycosuria and glycemia over 400 mg/dl were included in the experiment. Two days after STZ

injection, diabetic animals were randomized in two groups: one was infused with insulin (STZ+INS group) and the second group was not treated (STZ group). Insulin treatment was achieved by two insulin implants Linplant® (Linshin Canada INC., Ontario, Canada) placed subcutaneously in the interscapular region of STZ+INS rats. For implantation, the three groups of rats (CTRL, STZ, STZ+INS) were anesthetized with a mixture of 100 mg/kg ketamine (Anesketin®; Pfizer, Oslo, Norway) and 10 mg/kg xylazine hydrochloride (Rompun®; Bayer, Fernwald, Germany) administered by ip injection. Two implants were placed in STZ+INS animals while CTRL and STZ animals were sham operated. Each implant delivered an average daily dose of 2 units of insulin. Growth was assessed by body weight measurement. TA muscles and serum were collected 14 days after electroporation for assessment of muscle hypertrophy and IGF-I concentrations.

2.4. Role of low IGF-I concentrations in the attenuation of FS-induced muscle hypertrophy by insulinopenia

To explore the role of low IGF-I concentrations in the attenuation of FS-induced muscle hypertrophy by insulinopenia, we used the model of STZ-induced diabetes described in experimental design 3. In this experiment, IGF-I was infused in place of insulin. Two days after STZ injection, diabetic animals were randomized in two groups: one was infused with IGF-I (STZ+IGF-I group) and the second group was not treated (STZ group). Recombinant human IGF-I Increlex®, generously given by IPSEN, (Ipsen Biopharmaceuticals, Inc., Merelbeke, Belgium) was administrated at a dose of 3 mg/kg/day by an Alzet® osmotic pump (model 2M2L, DURECT Corp., Cupertino, CA) implanted subcutaneously in the interscapular region of STZ+IGF-I rats. For implantation, the three groups of rats (CTRL, STZ, STZ+IGF-I) were anesthetized with a mixture of 100 mg/kg ketamine (Anesketin®; Pfizer, Oslo, Norway) and 10 mg/kg xylazine hydrochloride (Rompun®; Bayer, Fernwald,

Germany) administered by ip injection. One pump filled with IGF-I was placed in STZ+IGF-I animals while CTRL and STZ animals received a pump filled with vehicle (0.05M acetate buffer, pH 5.4). Growth was assessed by body weight measurement. TA muscles were collected 14 days after electroporation for assessment of muscle hypertrophy.

3) EXPRESSION PLASMIDS AND DNA PREPARATION

A pM1-hFS288 c-myc plasmid coding for the human FS containing 288 amino acids was constructed as previously described (24, 34). Empty pM1 was used as a control plasmid. Plasmids were amplified in Escherichia coli top 10 F' (Invitrogen, Carlsbad, CA) and purified with an EndoFree plasmid giga kit (QIAGEN, Valencia, CA). Plasmids were stocked at -80 °C. The day before injection, 100 µg of plasmid were lyophilized and resuspended in 100 µl 0.9% NaCl solution.

4) DNA ELECTROTRANSFER

Each animal was anesthetized with a mixture of 100 mg/kg ketamine (Anesketin®; Pfizer, Oslo, Norway) and 10 mg/kg xylazine hydrochloride (Rompun®; Bayer, Fernwald, Germany) administered by ip injection. Ten microliters of plasmid solution (1 µg/µl) were injected into 10 different sites (total volume per muscle = 100 µl) in each TA muscle and the muscles were then electroporated using the electroporation conditions described previously (53). The animals were killed 14-17 days after electroporation.

5) ANIMAL SACRIFICE AND BIOLOGICAL SAMPLE COLLECTION

All the animals were killed by decapitation after CO_2 euthanasia and blood was collected from the trunk vessels. TA muscles were dissected and a transverse slice of 0.5-cm thickness was fixed with buffered formalin for 48 h and embedded in paraffin for morphological analysis. The remaining ends of the TA muscles were frozen in liquid nitrogen for Elisa and mRNA analysis. GC muscles were removed and frozen in liquid nitrogen for Elisa, mRNA and western blot analysis.

6) mRNA ANALYSIS BY REAL TIME QUANTITATIVE (RTQ)-PCR

Total RNA was isolated from the TA muscle using TRI reagent® as described by the manufacturer. Recovery was 1 µg/mg of TA muscle. Reverse transcription and real-time quantitative PCR were done as previously described (18). Accession numbers for the sequences and primers used were: IGF-I: AH002176 (CAGGCTATGGCTCCAGCAT- GGAAGCAACACTCATCCACA) IGF-IR: X044434.1 (TCATGCCTTGGTCTCCTTGTCCTT – GTCACTTCCTCCATGCGGTAAATTTCG), IGFBP5: NM_010518.2 (TCCGAACAAGGCCCCTGCCG-GCTGTCGAAGGCGTGGCACT), MAFbx/Atrogin-1: NM_026346.3 (CCATCAGGAGAAGTGGATCTATGTT-GCTTCCCCCAAAGTGCAGTA), REDD1: NM_029083.2 (AGACTCCTCATACCTGGATGGG-AGCTGCATCAGGTTGGCAC) and glyceraldehyde-3-phosphate dehydrogenase, AF106860 (TGCACCACCAACTGCTTA GGATGCAGGGATGATGTTC) used as the reporter gene. Primers were tested to avoid primer dimers, self-priming formation, or unspecific amplification. The primers were designed to have standardized optimal PCR conditions. We have confirmed that the expression values of GAPDH normalized to RNA were not affected by Mstn inhibition in our different animal models. This was confirmed at the protein level, as illustrated on the blots presented on figure 3 panel A, showing

that the abundance of GAPDH protein is not affected by FS overexpression, making GAPDH a valuable control.

7) MUSCLE AND CIRCULATING IGF-I CONCENTRATIONS

One hundred mg of TA muscle, previously pestled in liquid nitrogen, was homogenized with Ultraturrax (IKALabortechnik, Staufen, Germany) in 1 ml of pH 7.4 1X PBS (130 mM NaCl, 17 mM Na_2HPO_4 and 3 mM NaH_2PO_4) and stored overnight at ≤ -20 °C. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 g. The supernatant was removed and stored at ≤ -80 °C until the assay. Blood was allowed to clot for 15 minutes at room temperature before centrifuging for 10 minutes at 2000 g. Serum was removed and stored at ≤ -80 °C until the assay. Muscle and circulating levels of IGF-I peptide were determined using Quantikine® ELISA Mouse/Rat according to the manufacturer's instructions (R&D, Minneapolis, MN).

8) WESTERN BLOTS

Muscle proteins of GC muscle were homogenized in ice-cold and pH 7.0 buffer containing 20 mM Tris, 270 mM sucrose, 5 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM DTT (1,4-dithiothreitol), 1% (v/v) Triton X-100 and 10% protease inhibitor cocktail (Roche Applied Science, Belgium). Homogenates were centrifuged at 10,000 g for 10 min at 4 °C and supernatants were immediately stored at -80 °C. Equal amounts of proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel 10% electrophoresis and transferred to PVDF membranes. Membranes were probed with the following primary antibodies: anti-phospho-Akt (Ser473, 1:2000; Cell Signaling Technology, Leiden, Netherlands), anti-Akt (1:2000; Millipore, Overijse, Belgium) and anti-glyceraldehyde-3- phosphate

dehydrogenase (1:40000; Cell Signaling Technology). Then membranes were incubated with a horseradish peroxidase-coupled secondary antibody (Cell Signaling Technology) and developed using Enhanced Chemiluminescence (ECL) Western blotting Detection System Plus (GE Healthcare, Belgium). Developed film was scan and analyzed as previously described (54). All results were normalized for the signal to GAPDH protein.

9) HISTOLOGICAL ANALYSIS OF MUSCLE

Serial sections (5 µm thick) were cut and mounted on glass slides (Superfrost Plus; Menzel-Glaser, Braunschweig, Germany). FS-c-myc was detected by immunohistochemistry with rabbit polyclonal anti-c-myc as previously described (24). Fiber cross-sectional areas (CSA) were measured with a microscope Axio-Star (Carl Zeiss, Okerkochen, Germany) coupled to a Zeiss Axiocam digital camera MRc and to image analyzer software (Axiovision software version 4.7; Carl Zeiss). To evaluate the effect of FS on muscle fiber CSA, all the positive muscle fibers in the TA transfected with FS gene were measured. Two hundred negative fibers, randomly chosen in the contralateral TA transfected with insertless plasmid (pM1), were measured and considered as controls.

10) STATISTICAL ANALYSIS

Results are presented as means ± standard error of the mean (SEM). Statistical analyses were performed using a one-way ANOVA followed by a Bonferroni Multiple comparison test or unpaired t-test to compare muscles from different animals undergoing different experimental conditions. Interactions between FS and hypophysectomy, as well as between FS/sActRIIB and IGF-I injection, were assessed using a two-way ANOVA followed by a Bonferroni post-test. Fiber CSA

distribution statistical analysis was performed using χ^2 Pearson test. Statistical significance was set at $P < 0.05$.

RESULTS

1) THE MUSCLE HYPERTROPHY CAUSED BY MSTN INHIBITION IS ASSOCIATED WITH DECREASED MUSCLE IGF-I mRNA AND PEPTIDE LEVELS.

The IGF-IR mediated-signaling is required for FS to promote muscle hypertrophy (34). Since the IGF-IR is primarily activated by IGF-I, we investigated whether the muscle hypertrophy induced by Mstn inhibition is mediated by increased muscle IGF-I expression. First, muscle IGF-I mRNA levels were assessed in TA and GC muscles from different animal models of muscle hypertrophy induced by Mstn inhibition: mTr-FS, Mstn KO and sActRIIB-Fc-treated mice. Surprisingly, we found that muscle IGF-I expression was systematically reduced in mTr-FS, KO Mstn and sActRIIB-Fc-treated mice compared to their control littermates (Figure 1B) in contrast to muscle mass (Figure 1A). Interestingly, muscle IGF-I mRNA levels were already decreased (-44%; $P < 0.05$) in 4-week-old mTr-FS mice, namely before any muscle hypertrophy. Then, the IGF-I peptide concentrations were determined in the TA muscle of mTr-FS. We confirmed that FS overexpression decreased not only IGF-I mRNA but also peptide in the skeletal muscle (mTr-FS: -41%; 9±1 *vs.* WT: 16±2 ng/g, n=8/group, $P < 0.05$) (Figure 1C). In the other animal models of Mstn inhibition, we are confident that changes in muscle IGF-I mRNA translate in parallel changes in muscle IGF-I peptide. Indeed, in several animal models, we observed parallel changes in IGF-I mRNA and peptide in skeletal muscle (data not shown). Circulating IGF-I concentrations were not affected by FS overexpression (Figure 1D).

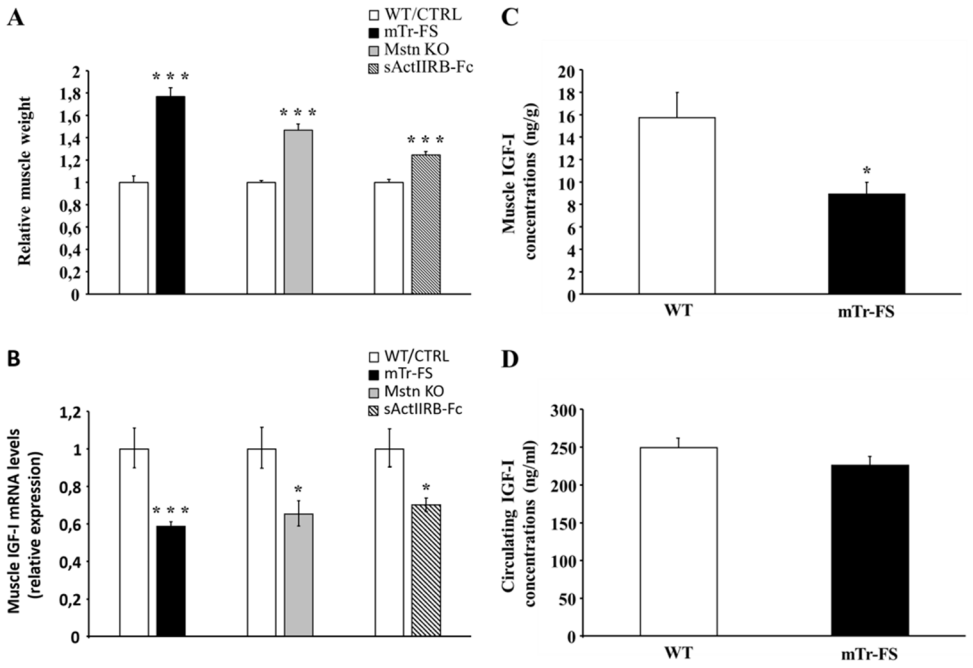

Figure 1. The muscle hypertrophy caused by Mstn inhibition is associated with decreased muscle IGF-I mRNA and peptide levels. (A, B) Muscle weight and muscle IGF-I mRNA levels were assessed in TA muscle of C57Bl/6 mTr-FS *vs.* WT mice (n=11/group) and FVB sActRIIB-Fc treated *vs.* saline-treated mice (n=6/group), and in GC muscle of FVB Mstn KO *vs.* WT mice (n=7/group). (C, D) IGF-I peptide concentrations were assessed in the TA muscle (C) and in the circulation (D) of WT (white column) and mTr-FS (black column) mice (n=8/group). Results are expressed as means ± SEM. Statistical analysis was performed using unpaired t-test ($P > 0.05$; *, $P < 0.05$ and ***, $P < 0.001$).

2) FS INDUCES MUSCLE HYPERTROPHY IN HYPOX ANIMALS DESPITE VERY LOW CONCENTRATIONS OF CIRCULATING AND MUSCLE IGF-I.

To investigate the role of IGF-I in FS-induced skeletal muscle hypertrophy, we overexpressed FS by DNA electrotransfer in the TA muscle of HYPOX rats characterized by very low concentrations of IGF-I. The IGF-I mRNA levels in the TA muscle were modestly decreased after hypophysectomy (-22%; $P < 0.01$). However, both muscle and circulating concentrations of the IGF-I peptide were severely reduced, respectively by 90% (pM1-transfected muscle: 2±1 *vs.* 15±1 ng/g, n=6/group, $P < 0.001$) and by 95% (52±3 *vs.* 1097±57 ng/ml, n=6/group, $P < 0.001$) after hypophysectomy (Figure 2A and 2B). Interestingly, despite very low concentrations of muscle and circulating IGF-I, FS overexpression caused the same increase of muscle mass in HYPOX rats (+23%, 217±6 *vs.* 177±5 mg, $P < 0.001$) and in their intact CTRL littermates (+28%, 448±27 *vs.* 351±25 mg, $P < 0.001$) (n=6/group) 17 days after electroporation (Figure 2C). Furthermore, FS overexpression induced an even larger rise of fiber CSA in HYPOX (+102%, 1766±166 *vs.* 877±39 μm^2, $P < 0.001$) than in CTRL rats (+57%, 2420±175 *vs.* 1552±102 μm^2, $P < 0.001$) (n=6/group) (Figure 2D). These results demonstrate that FS retains its full hypertrophic effect towards muscle despite very low concentrations of circulating and muscle IGF-I.

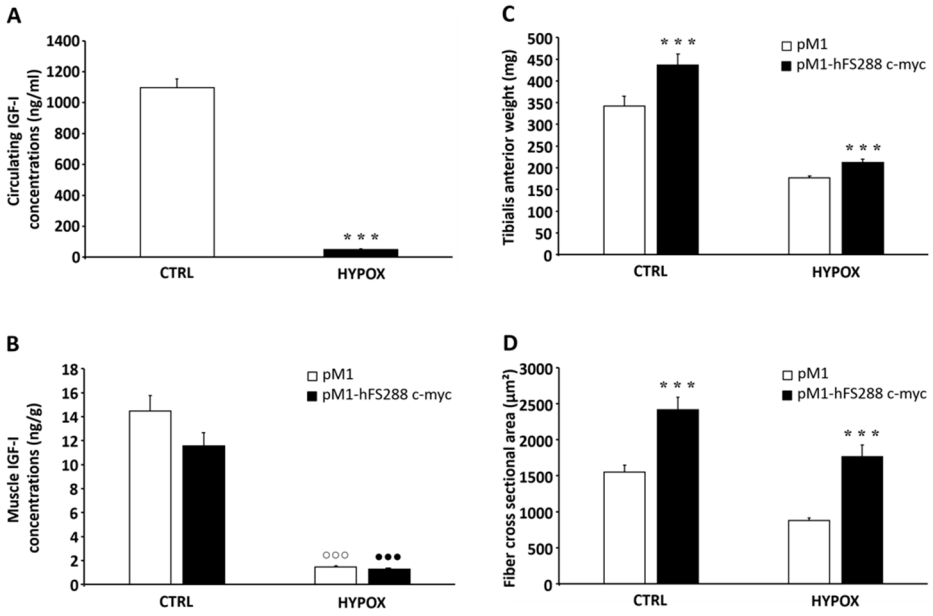

Figure 2. FS induces muscle hypertrophy in HYPOX animals despite very low concentrations of circulating and muscle IGF-I. (A) Circulating concentrations of IGF-I were assessed in CTRL (white column) and in HYPOX (black column) rats (n=6/group). Results are expressed as mean ± SEM. Statistical analysis was performed using unpaired t-test (***, $P < 0.001$). (B, C, D) Muscle concentrations of IGF-I peptide (B), TA muscle weight (C) and fiber CSA (D) were assessed 17 days after transfection of pM1 (white column) or pM1-hFS288 (black column) in CTRL and in HYPOX rats (n=6/group). Results are expressed as mean ± SEM. Statistical analysis was performed using 2-way ANOVA test (Muscle IGF-I concentrations: FS effect, *NS*; HYPOX effect, $P < 0.001$; Interaction, *NS*; TA weight: FS effect, $P < 0.01$; HYPOX effect, $P < 0.001$; Interaction, *NS*; Fiber CSA: FS effect, $P < 0.001$; HYPOX effect, $P < 0.001$; Interaction, *NS*) and Bonferroni post-test (***, $P < 0.001$ *vs.* contralateral muscle; °°°, $P < 0.001$ *vs.* CTRL pM1 muscle; •••, $P < 0.001$ *vs.* CTRL pM1-hFS288 c-myc muscle).

3) FS OVEREXPRESSION DOES NOT INCREASE SKELETAL MUSCLE IGF-I SENSITIVITY.

The ability of FS to exert its full anabolic effect, even in the presence of very low IGF-I concentrations, suggests two hypotheses: either FS overexpression increases the muscle sensitivity to the anabolic effect of IGF-I or, alternatively, IGF-I is not required for FS to promote muscle hypertrophy. The first hypothesis is suggested by the demonstration made previously that Mstn inhibition increases the insulin-stimulated activation of Akt and glucose uptake in skeletal muscle (29, 45, 66, 67). To investigate whether FS overexpression enhances IGF-I sensitivity in

skeletal muscle, we assessed the activation of protein kinase Akt, a downstream target of the IGF-IR crucial for the anabolic action of FS (34), after injection of graded doses of IGF-I into mTr-FS and WT mice (Figure 3). Activation of the IGF-I signaling normally increases the phosphorylation state of Akt as seen in GC muscle from WT mice (4.4±0.4-fold, IGF-I 400 µg/kg *vs.* 1±0.4-fold, Saline, $P < 0.001$) (n=3-4/group). Unexpectedly, the increased phosphorylation of Akt in response to IGF-I was almost abolished in GC muscle of mTr-FS mice (1.8±0.6-fold, IGF-I 400 µg/kg *vs.* 1.1±0.1-fold, Saline, $P > 0.05$) (Figure 3A, B, C). In contrast, IGF-I injection (400 µg/kg) activated Akt to a similar extent in the liver from mTr-FS and WT mice (3.0±0.6-fold *vs.* 3.1±0.5-fold; *NS*) (n=3-4/group) (Figure 3D, E, F). Levels of the total Akt protein were increased in response to Mstn inhibition, as already reported (26, 45, 66). Taken together, our observations unravel a muscle specific decrease of the IGF-I signaling in mTr-FS mice. In order to examine the mechanisms for this resistance, we assessed the expression of potential candidate proteins that might limit IGF-I responsiveness of the muscle in mTr-FS mice. The muscle hypertrophy caused by FS overexpression was associated with an increase in muscle IGF-Binding Protein (IGFBP)-5 mRNA levels (+64%, $P < 0.001$) together with a decrease in IGF-IR mRNA levels (-24%, $P < 0.01$) (Fig S1), supporting the hypothesis of a decreased muscle sensitivity to IGF-I. Although muscle IGF-I sensitivity is decreased in adult mTr-FS mice, the possibility still exists that an increase in muscle IGF-I sensitivity might play a role at the early stages of hypertrophy. To test this hypothesis, we assessed the muscle IGF-I sensitivity caused by Mstn inhibition using mice treated with the sActRIIB-Fc during a brief period as a model of incipient hypertrophy. In these conditions, the increase of Akt phosphorylation induced by IGF-I was not affected by the sActRIIB-Fc pretreatment (7.9±10.7-fold vs. 7.2±1.4-fold; NS) (n=3-4/group) (Figure 4A, B, C). This last observation supports the view that the decreased muscle IGF-I sensitivity observed in mTr-FS is secondary to muscle

hypertrophy. Taken together, our observations indicate that the muscle hypertrophy caused by Mstn inhibition does not result from increased IGF-I sensitivity.

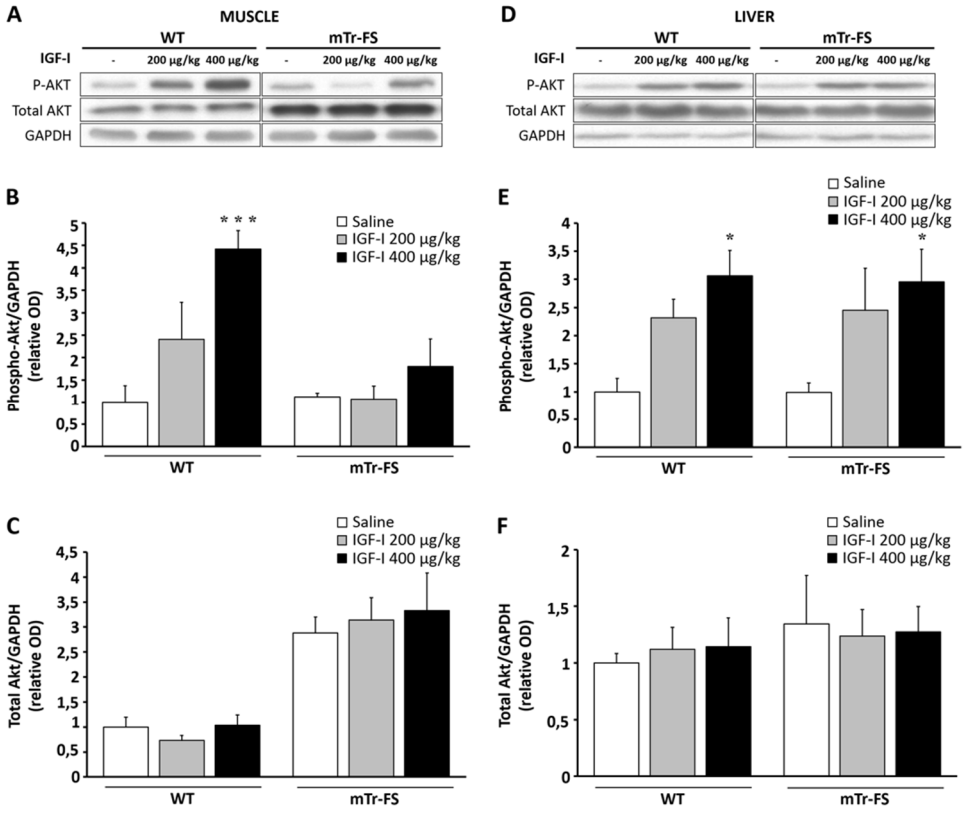

Figure 3. FS does not increase muscle IGF-I sensitivity. (A, B, C) The increase of muscle pAkt (Ser473) induced by acute IGF-I injection (200 µg/kg, grey column or 400 g/kg, black column *vs.* saline, white column) is severely blunted in mTr-FS mice (n=3-4/group). Muscle activation of Akt was assessed by western blot analysis (A) and densitometric analysis of pAkt/GAPDH (B) and total-Akt/GAPDH (C). The results are expressed as mean ± SEM. Statistical analysis was performed using 2-way ANOVA test (pAkt/GAPDH: IGF-I effect, $P < 0.01$; mTr-FS effect, $P < 0.01$; Interaction, $P < 0.05$; total-Akt/GAPDH: IGF-I effect, *NS*; mTr-FS effect, $P < 0.001$; Interaction, *NS*) and Bonferroni post-test (***, $P < 0.001$ *vs.* saline). (D, E, F) The increase in liver pAkt (Ser473) induced by acute IGF-I injection (200 µg/kg, grey column or 400 g/kg, black column *vs.* saline, white column) is similar in WT and mTr-FS mice (n=3-4/group). Liver activation of Akt was assessed by western blot analysis (D) and densitometric analysis of pAkt/GAPDH (E) and total-Akt/GAPDH (F). The results are expressed as mean ± SEM. Statistical analysis was performed using 2-way ANOVA test (pAkt/GAPDH: IGF-I effect, $P < 0.01$; mTr-FS effect, *NS*; Interaction, *NS*; total-Akt/GAPDH: IGF-I effect, *NS*; mTr-FS effect, *NS*; Interaction, *NS*) and Bonferroni post-test (*, $P < 0.05$ *vs.* saline).

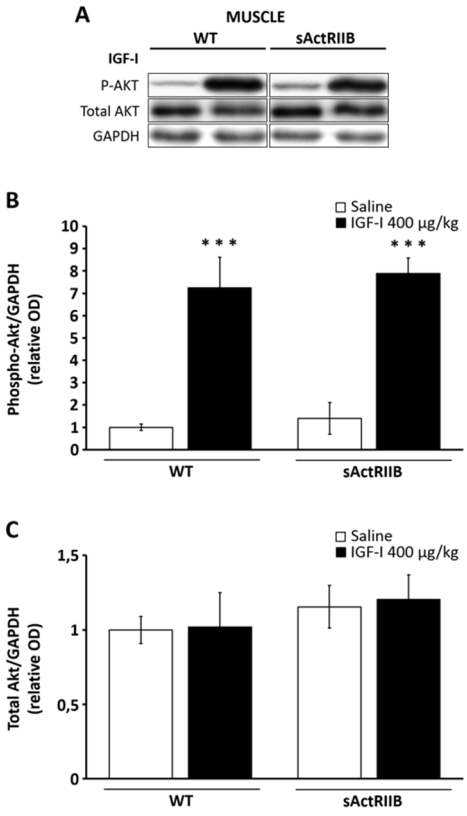

Figure 4. sActRIIB-Fc does not increase muscle IGF-I sensitivity.(A, B, C) The increase of muscle pAkt (Ser473) induced by acute IGF-I injection (400 g/kg, black column vs. saline, white column) is not affected in sActRIIB-Fc-treated mice (n=3-4/group). Muscle activation of Akt was assessed by western blot analysis (A) and densitometric analysis of pAkt/GAPDH (B) and total-Akt/GAPDH (C). The results are expressed as mean ± SEM. Statistical analysis was performed using 2-way ANOVA test (pAkt/GAPDH: IGF-I effect, $P < 0.001$; sActRIIB effect, *NS*; Interaction, *NS*; total-Akt/GAPDH: IGF-I effect, *NS*; sActRIIB effect, *NS*; Interaction, *NS*) and Bonferroni post-test (***, $P < 0.001$ *vs.* saline).

4) THE FS-INDUCED MUSCLE HYPERTROPHY IS ATTENUATED IN STZ-DIABETIC ANIMALS.

Since the IGF-IR can be activated either by IGF-I or insulin, we investigated the role of insulin in skeletal muscle hypertrophy induced by FS overexpression. To investigate the role of insulin in FS-induced skeletal muscle hypertrophy, we

overexpressed FS by DNA electrotransfer in the TA muscle of STZ-induced diabetic rats, characterized by low insulin concentrations. To attest the success of STZ injection, glycemia was determined two days after STZ injection and at the end of the experiment. As expected, the glycemia of STZ rats was markedly increased compared to controls (581±11 *vs.* 116±5 mg/dl, n=8/group, $P < 0.001$) and restored to the normal in STZ rats treated with insulin implants (115±7 *vs.* 116±5 mg/dl, n=8/group, *NS*) (Figure 5A). Concentrations of IGF-I peptide were significantly decreased in STZ rats compared to CTRL rats both in the circulation (-38%, 920±73 *vs.* 1476±69 ng/ml, n=8/group, $P < 0.001$) and in the muscle (-51%, pM1-transfected muscle: 14±1 *vs.* 30±3 ng/g, n=8/group, $P < 0.001$) (Figure 5C and D). They were restored to the normal levels in STZ rats treated with insulin implants both in the circulation (1518±54 vs. 1476±69 ng/ml, n=8/group, *NS*) and in the muscle (pM1-transfected muscle: 28±3 *vs.* 30±3 ng/g, n=8/group, *NS*) (Figure 5C and D). In contrast to hypophysectomy, insulinopenia decreased the FS-induced hypertrophy. Indeed, FS overexpression during 17 days induced a lower rise of fiber CSA in STZ rats (+33%, 2440±166 *vs.* 1835±80 µm², $P < 0.05$) than in CTRL rats (+57%, 3191±150 *vs.* 2037±56 µm²; $P < 0.001$) (Figure 5E). In order to examine some potential mechanisms for this attenuation, we assessed the expression of MAFbx/Atrogin-1 and REDD1, two factors which inhibit muscle protein synthesis (14, 19) and might thus limit the responsiveness to FS. The expression of MAFbx/Atrogin-1 (Fig S2A) and REDD1 (Fig S2B) was significantly increased in the muscle of STZ animals (respectively in pM1-transfected muscle: 2.7-fold, $P < 0.001$ and 1.9-fold, $P < 0.001$), supporting the idea of a decreased protein synthesis in response to FS in case of insulinopenia. Moreover, insulin implants in STZ rats restored the full hypertrophic effect of FS as assessed by the fiber CSA (+60%, 3501±199 *vs.* 2181±97 mg, $P < 0.001$) as well as the baseline expression levels of MAFbx/Atrogin-1 and REDD1.

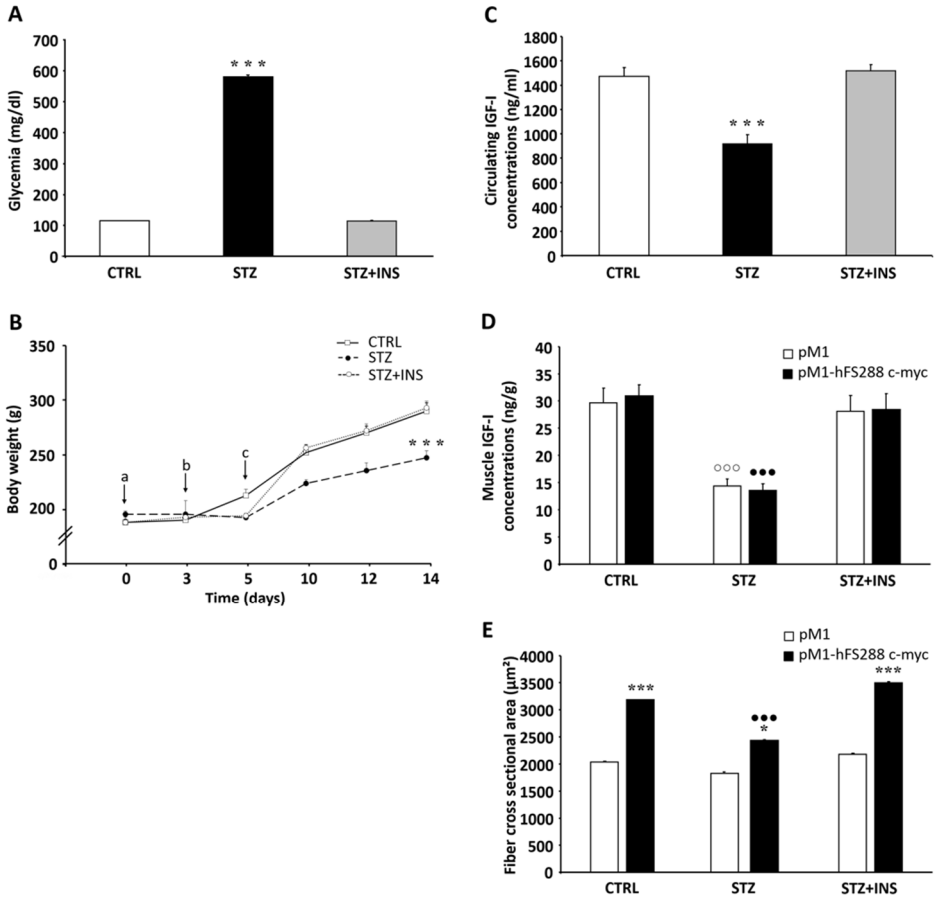

Figure 5. The FS-induced muscle hypertrophy is attenuated in STZ-diabetic animals. (A) Glycemia is significantly higher in STZ-treated rats (black column) compared to CTRL rats (white column) and is restored to normal levels in STZ rats treated with insulin implants (grey column) (*** $P < 0.001$ *vs.* CTRL) (n=8-10/group). (B) Evolution of body weight of the CTRL (–), STZ-treated rats (---) and STZ rats treated with insulin (·····). Three days after DNA injection (a), rats were treated with STZ (60 mg/kg) (b) and two days later rats were treated with insulin (4U/day) (c) (*** $P < 0.001$ *vs.* CTRL). (C, D) Circulating (C) and muscle (D) IGF-I concentrations are significantly decreased in STZ rats compared to CTRL rats and are restored to the normal levels in STZ rats treated with insulin implants. Fiber CSA (E) were assessed 14 days after transfection of pM1 (white column) or pM1-hFS288 (black column) in CTRL, STZ and STZ+INS rats (n=8-10/group). Results are expressed as mean ± SEM. Statistical analysis was performed using 1-way ANOVA test and Bonferroni post-test (* $P < 0.05$, *** $P < 0.001$ *vs.* contralateral muscle; °°° $P < 0.001$ *vs.* CTRL pM1 muscle; ••• $P < 0.001$ *vs.* CTRL pM1-hFS288).

5) IGF-I TREATMENT RESTORES THE FULL HYPERTROPHIC EFFECT OF FS IN STZ-DIABETIC ANIMALS.

To investigate whether restoration of the full anabolic effect of FS was due to insulin infusion or to normalization of IGF-I or glycemia, we administrated IGF-I to STZ rats electroporated with FS. IGF-I infusion restored the body growth of STZ rats without normalizing glycemia (Figure 6A and B). Furthermore, we showed that IGF-I treatment also restored to normal the FS-induced muscle hypertrophy as assessed by the measurement of the fiber CSA (STZ+IGF-I: +59%; $P < 0.001$ *vs.* STZ: +28%; $P < 0.05$) (Figure 6C). Accordingly, we demonstrated that FS does not exert its full anabolic effect in the absence of insulin associated with a decrease of IGF-I. Interestingly, in case of insulinopenia, IGF-I infusion could restore the FS hypertrophy and therefore compensate for the lack of insulin, despite persistence of hyperglycemia.

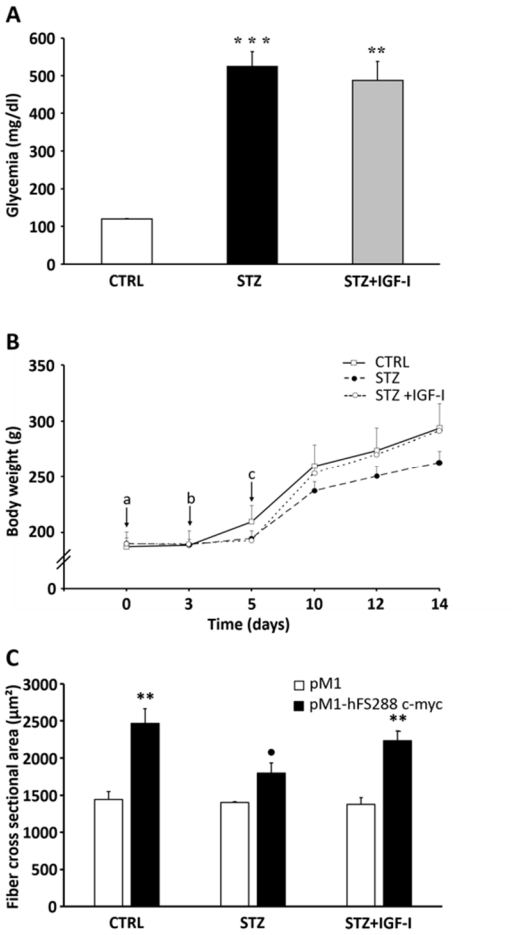

Figure 6. IGF-I treatment restores the FS-induced muscle fiber hypertrophy in STZ-diabetic animals. (A) Glycemia is significantly higher in STZ-treated rats and STZ rats treated with IGF-I compared to CTRL rats (** $P < 0.01$, *** $P < 0.001$ *vs.* CTRL) (n=3/group). (B) Evolution of body weight of the CTRL (–), STZ-treated rats (---) and STZ rats treated with IGF-I (⋯). Three days after DNA injection (a), rats were treated with STZ (60 mg/kg) (b) and two days later rats were treated with IGF-I (3mg/kg/day) (c). (C) Fiber CSA were determined 14 days after transfection of pM1 (white column) or pM1-hFS288 (black column) in CTRL, STZ and STZ+IGF-I rats (n=3/group). Results are expressed as mean ± SEM. Statistical analysis was performed using 1-way ANOVA test and Bonferroni post-test (** $P < 0.01$ *vs.* contralateral muscle; • $P < 0.05$ *vs.* CTRL pM1-hFS288).

DISCUSSION

Our study investigated the role of IGF-I and insulin in the muscle hypertrophy caused by FS overexpression. Indeed, we showed in a previous experiment that the hypertrophic action of FS on skeletal muscle requires the activation of the IGF-IR/Akt/mTOR pathway (34). Since the IGF-IR can be activated either by IGF-I or insulin and because the insulin signaling could also contribute to the regulation of the skeletal muscle mass (48), we specifically investigated the role of these ligands in the FS-induced muscle hypertrophy. Our results show that FS causes a decrease in muscle IGF-I expression which contrasts with the marked muscle hypertrophy. Moreover, FS retains its full anabolic effect towards muscle even in the presence of very low concentrations of IGF-I. Finally, FS does not increase muscle sensitivity to IGF-I in stimulating phosphorylation of Akt but, surprisingly, decreases it once hypertrophy is present. Taken together, our observations indicate that increased IGF-I production or sensitivity does not contribute to the muscle hypertrophy caused by FS. In contrast, insulinopenia induced by STZ injection attenuates the hypertrophic action of FS on skeletal muscle. Moreover, the full anabolic response to FS can be restored in this condition by insulin but also by IGF-I infusion. Therefore, our results indicate that insulin is required for the anabolic action of FS, but can be substituted by IGF-I in case of insulinopenia.

The regulation of IGF-I during the muscle hypertrophy caused by Mstn inhibition remains debated. Such regulation has been suggested by the observation of increased muscle mRNA expression and circulating concentrations of IGF-I in response to Mstn inhibition (35, 62, 64). However, some authors have also reported a decrease in the muscle IGF-I mRNA expression in the same conditions (12, 23, 62). The reasons of this discrepancy are unclear. They might result from the choice of primers for RTq-PCR which may assess different IGF-I mRNA transcripts (7). In our work, we used specific primers located in exons 3 and 4 to measure the expression

levels of all IGF-I mRNA transcripts, and not only the Mecano-Growth Factor (MGF) (65). Moreover, the approaches to inhibit Mstn differ from one study to another. It is known that FS and the sActRIIB-Fc inhibit both Mstn and Activin A (ActA) (24, 38, 57) in contrast to a KO Mstn model in which only Mstn is inactivated. Finally, the mechanism of muscle hypertrophy caused by Mstn inhibition may result from fiber hypertrophy and/or hyperplasia according to the stage of development at which the inhibition occurs (28, 44). In this study, we assessed the IGF-I mRNA expression in different Mstn inhibition models: prenatal deletion of the Mstn gene (KO Mstn) resulting in fiber hyperplasia (23), postnatal inhibition of Mstn and ActA by sActRIIB-Fc resulting in fiber hypertrophy (11) and prenatal inhibition of Mstn and ActA by FS overexpression resulting in fiber hyperplasia and hypertrophy (37). In all conditions, we showed that muscle IGF-I expression is systematically reduced regardless of the approach used to inhibit Mstn. Moreover, we demonstrated that this decrease of muscle IGF-I mRNA levels is correlated with low muscle IGF-I peptide concentrations. The possibility that this decrease in muscle IGF-I peptide results from a decrease in extracellular matrix as observed in response to Mstn inhibition (9, 10, 60) is unlikely. Indeed, immunohistochemical studies showed that IGF-I protein is mainly localized in muscle fiber and not in extracellular matrix (40).

The role of IGF-I during the muscle hypertrophy caused by Mstn inhibition has never been investigated. Not only IGF-I by itself increases skeletal muscle mass (2, 13, 47), but its increased muscle expression in response to several anabolic stimuli suggests that it may contribute to muscle hypertrophy in many conditions (overloading, testosterone, overexpression of PGC1α4, …) (1, 20, 39, 40, 52). Evidence suggests that IGF-I might contribute to FS-induced muscle hypertrophy. Indeed, FS induces muscle hypertrophy by increasing protein synthesis and satellite cell activation, as IGF-I does (5, 6, 24, 58). In addition, Mstn inhibition stimulates the Akt/mTOR/S6K pathway, the pathway mainly responsible for the muscle

hypertrophy caused by IGF-I (41, 45, 51). Finally, the MKR mice, which are characterized by down regulation of muscle IGF-IR, are resistant to the anabolic effect of FS (34). To investigate the role of IGF-I in FS-induced hypertrophy, we used a model of HYPOX rats. Indeed, this model has already been successfully used to assess the role of IGF-I in testosterone- and load-induced skeletal muscle hypertrophy (25, 56). Furthermore, this animal model is probably the only non-lethal model characterized by a profound and global IGF-I deficiency (4, 42, 49). This model allowed us to demonstrate that the GH-IGF-I axis is not required for FS to exert its anabolic action. Since HYPOX animals still present very low concentrations of IGF-I, we cannot exclude the possibility that these low IGF-I concentrations were sufficient to allow the FS-induced muscle hypertrophy. However, these concentrations were clearly not sufficient to support body and muscle growth. Except if FS increases the muscle sensitivity to the anabolic action of IGF-I, the role of IGF-I in the FS-induced muscle hypertrophy is not supported by our observations.

The possibility for FS to increase muscle sensitivity to IGF-I is suggested by some reports showing that Mstn inhibition increases the insulin-stimulated activation of Akt and glucose uptake in skeletal muscle (29, 45, 66, 67). Although IGF-I and insulin signaling pathways share multiple intracellular mediators, no study has investigated the muscle IGF-I sensitivity in case of Mstn inhibition. It has been demonstrated that IGF-I and FS promote muscle hypertrophy via the Akt/mTOR signaling pathway leading to the stimulation of protein synthesis (34, 51). Therefore, since Akt is an important mediator in the hypertrophy process, we assessed its activation after injection of IGF-I to investigate the IGF-I sensitivity of the muscle from mTr-FS mice. For the first time, our results demonstrated a decreased IGF-I sensitivity of muscle overexpressing FS. This absence of Akt activation in response to IGF-I contrasts with the increased pAkt in response to insulin observed in Mstn KO mice (29). The mechanisms operational in this

reduction of muscle sensitivity to IGF-I are unknown. The possibility has been suggested for Mstn to interact with the IGF signaling by modulating the local expression of IGF binding proteins (IGFBPs) (16, 62). Interestingly, we observed an increase of muscle IGFBP-5 expression in response to Mstn inhibition. Since IGFBP-5 has been shown to inhibit the IGF-I action towards muscle cells (33, 46, 59), its induction by FS could reduce the local bioavailability of IGF-I and attenuate the IGF-I action specifically in the skeletal muscle and not in the liver. Furthermore, as previously described (34), we also showed a decrease in the muscle IGF-IR mRNA expression in response to Mstn inhibition. Therefore, we cannot exclude that the decrease of muscle IGF-I sensitivity observed in mTr-FS mice might result from a down regulation of the muscle IGF-IR. Finally, alterations in muscle vascularization in mTr-FS might also impair the access of IGF-I to the membrane IGF-IR. Indeed, Mstn inhibition has been shown to decrease muscle capillary density together with VEGF expression (31, 50). However, this hypothesis is unlikely as this decrease in muscle capillary density does not seem to impair the stimulation of muscle Akt and glucose uptake by insulin (29). Collectively, these results lead thinking that in response to FS, muscle cells put in place mechanisms inhibiting the IGF-I actions as a negative feedback loop to prevent greater hypertrophy. This view is supported by the normal muscle IGF-I sensitivity observed at early stages of hypertrophy induced by the sActRIIB-Fc, suggesting that decreased muscle IGF-I sensitivity observed in mTr-FS mice is secondary to muscle hypertrophy. Therefore, neither increased IGF-I production nor increased IGF-I sensitivity contribute to the muscle hypertrophy caused by FS.

Since the IGF-IR can be activated by IGF-I but also insulin, we investigated the role of these ligands in the skeletal muscle hypertrophy induced by FS. Our results show that low insulin, unlike low IGF-I, severely blunts the FS-induced muscle hypertrophy. This conclusion is also suggested by the results of Wang Q *et al.*

reporting a reduced hypertrophic effect of sActRIIB-Fc treatment in mice treated with STZ (61). Since muscle hypertrophy induced by FS mainly results from a stimulation of protein synthesis via the Akt/mTOR pathway (34, 58, 64), insulinopenia could impair the effect of FS through an altered protein synthesis. This hypothesis is supported by several observations. First, STZ decreases the activation of the Akt/mTOR pathway (32) required for the anabolic effect of FS (34). Second, insulinopenia stimulates the muscle expression of REDD1, a well-recognized translational repressor inhibiting the Akt/mTOR signalling (21, 32), and MAFbx/Atrogin-1(17), a muscle-specific ubiquitin ligase which targets for degradation the protein initiation translation factor eIF3f (14, 15, 36). As we showed an increase in REDD1 and MAFbx/Atrogin-1 mRNA expression, it seems that the anabolic effect of FS towards muscle requires the action of insulin probably to allow stimulation of protein synthesis.

The restoration of the full anabolic effect of FS by IGF-I in STZ animals, despite the persistence of hyperglycemia, indicates that IGF-I may substitute for the anabolic properties but not for the hypoglycemic effect of insulin. Similar observation was already made for the bone growth. Indeed, IGF-I treatment restored the longitudinal bone growth of STZ-treated rats without normalization of the glycemia (55). The fact that FS retains its hypertrophic action in IGF-I-treated STZ animals despite the presence of hyperglycemia suggests that the failure of FS to stimulate muscle hypertrophy in STZ animals is not due to glucose toxicity. However, the observation that IGF-I restores growth in STZ-diabetic animals without restoring normoglycemia cannot be interpreted as the result from the IGF-I binding to the IGF-IR. Indeed, our work did not investigate which of IGF-IR or insulin receptor (IR) is required for FS to exert its anabolic action. While the ligands insulin and IGF-I can substitute one to the other, this phenomenon does not seem to apply for their receptors. Indeed, deletion of the IR (48) as the IGF-IR (43) is

associated with decreased muscle growth, suggesting that both receptors are required for proper physiological muscle growth. Moreover, expression of the dominant-negative form of IGF-IR or MKR, which inhibits the FS–induced muscle hypertrophy (34), impairs both insulin and IGF-I signaling in muscle due to hybrid receptors formation (22). Therefore, since the two receptors are mandatory for proper physiological muscle growth, we cannot specify which of IR or IGF-IR is involved in the FS-induced muscle hypertrophy. Ultimately, our study establishes the need of insulin or IGF-I for the full anabolic effect of FS towards skeletal muscle. When insulin or IGF-I alone is missing, FS retains its full anabolic effect. But, when both are deficient, as in STZ animals, FS fails to simulate muscle growth. In physiological conditions, both hormones probably allow FS to promote muscle hypertrophy. Thus, IGF-I is not absolutely required for FS to induce skeletal muscle hypertrophy except in case of insulinopenia.

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SUPPLEMENTAL FIGURES

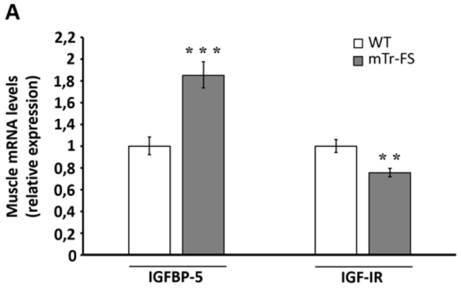


Figure S1. The muscle hypertrophy caused by FS is associated with increased muscle IGFBP-5 and decreased IGF-IR mRNA levels. (A) Muscle IGFBP-5 and IGF-IR mRNA levels were assessed in TA muscle of C57Bl/6 mTr-FS (grey column) vs. WT mice (white column) (n=11/group). Results are expressed as means ± SEM. Statistical analysis was performed using unpaired t-test (**, P < 0.01 and ***, P < 0.001).

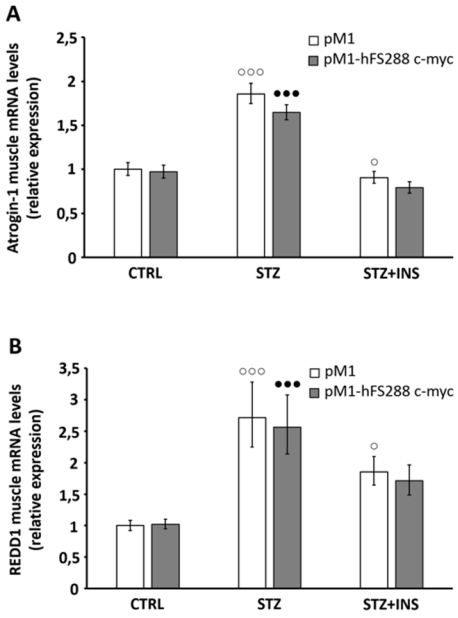


Figure S2. STZ treatment enhances the expression of MAFBX/Atrogin-1 and REDD1 mRNAs in muscle. Muscle MAFBX/Atrogin-1 mRNA (A) and REDD1 mRNA (B) levels were assessed in TA muscle 14 days after transfection of pM1 (white column) or pM1-FS288 (grey column) in CTRL, STZ and STZ+INS rats (n=8-10/group). Results are expressed as mean ± SEM. Statistical analysis was performed using 1-way ANOVA test and Bonferroni post-test (○ P<0.05, ○○○ P<0.001 vs. CTRL pM1 muscle; ●●● P<0.001 vs. CTRL pM1-hFS288 c-myc muscle).

Chapter 4

GENERAL DISCUSSION AND PERSPECTIVES

1. General discussion

Although Mstn and IGFs are two growth factors regulating skeletal muscle mass, their relationships in the control of muscle development are not well characterized. A cross-talk between the Mstn and the IGF signaling pathways is suggested by the increased IGF-IR/Akt/mTOR/S6K signaling observed with Mstn inhibition (1,2), as this pathway is known to be essential for the hypertrophy induced by IGF-I (3). Therefore, the aim of this thesis was to identify the molecular mechanisms connecting Mstn and IGF-I pathways involved in the hypertrophic effect of FS on skeletal muscle. In this work, we have tested the hypothesis that the activation of the IGF-IR/Akt/mTOR/S6K pathway might contribute to the muscle hypertrophy induced by FS, a potent inhibitor of Mstn action. Moreover, we have investigated the role of IGFs and insulin, the ligands of IGF-IR, in the skeletal muscle hypertrophy caused by FS.

Our work shows that FS-induced skeletal muscle hypertrophy requires IGF-IR, Akt and, to some extent, mTORC1 but not S6K. Furthermore, our results indicate that the hypertrophic effect of FS requires the presence of insulin but does not seem to require the presence of IGFs.

Role of the IGF-IR/Akt/mTOR/S6K pathway in the skeletal muscle hypertrophy

The role of the IGF/IGF-IR/Akt/mTOR/S6K pathway in the control of muscle mass has been suggested by several evidences in many models of muscle hypertrophy. Firstly, the activation of this pathway by IGF-I infusion or overexpression induces consistently skeletal muscle hypertrophy (4-6). Secondly, the increase in muscle IGF-I expression in response to several hypertrophic stimuli (GH, exercise, testosterone) suggests that IGF-I may mediate the skeletal muscle hypertrophy in many models of hypertrophy. In addition, increased Akt/mTOR/S6K signaling is observed in skeletal muscle hypertrophy caused by Mstn inhibition (1,2), but also by BMP administration (7), resistance exercise (8) or testosterone treatment (9,10). Finally, genetic deletion of IGF ligands and receptors is almost always associated with muscle atrophy, and often with muscle fiber atrophy (11) (table 1). Nevertheless, the contribution of IGF-I and its signaling to muscle hypertrophy seems to vary accordingly to the growth stimulus.

Genotype	Viability	Muscle phenotype	References
GHR null	viable	↘ fiber size nl fiber number	(12,13)
GHR null in muscle	viable	↘ fiber size ↘ fiber number	(14)
IGF-I null	neonatal lethality	↘ fiber size ↘ fiber number	(15-18)
IGF-I null in muscle	viable	normal growth	(14)
IGF-II (+/-) null	viable	↘ fiber size	(19,20)
IGF-IR null	neonatal lethality	muscle atrophy	(17)
IGF-IR null in muscle	viable	↘ fiber size ↘ fiber number	(14)
dnIGF-IR in muscle (MKR)	viable	↘ fiber size ↗fiber number	(21)
IR null	neonatal lethality	muscle atrophy	(22,23)
IR null in muscle	viable	↘ fiber size nl fiber number	(24,25)
IGF-IR/IR null in muscle (MIGIRKO)	viable (premature death)	↘ fiber size nl fiber number	(25)
Akt 1/2 null	neonathal lethality	↘ fiber size nl fiber number	(26)
mTOR null	embryonic lethality		(27 ,28)
mTOR null in muscle	viable (premature death)	↘ fiber size nl fiber number (fast-twitch) severe myopathy	(29)
S6K1/2 null	perinathal lethality	↘ fiber size nl fiber number	(30)

Table 1: Genetic models of IGF/IGF-IR/Akt/mTOR/S6K pathway components: effect on muscle growth. *(nl: normal)*

Although muscle IGF-I expression is increased in most models, the presence of IGF-I does not appear mandatory for the muscle hypertrophy caused by most stimuli (Table 2). Surprisingly, muscle IGF-I expression was even down regulated in response to Mstn inhibition, either constitutively or postnatally (31). Therefore, the down regulation of muscle IGF-I induced by Mstn inhibition seems specific to this muscle hypertrophy model. Furthermore, while the signaling by IGF-IR, the main IGF receptor in the muscle, is crucial for the muscle hypertrophy induced by FS (20), exercise (21), it is not the case for testosterone (32) or overload (33)-induced skeletal muscle hypertrophy. As IGF-I is known to exert its hypertrophic effect mainly through the stimulation of protein synthesis, the role of mTOR, a key regulator of protein synthesis, was investigated. It appears that mTOR is a common mediator of the muscle anabolic effect of FS (20), exercise (34), loading (35) or BMPs (7). Nevertheless, mTOR seems less crucial for the FS hypertrophic effect compared to the other stimuli. Moreover, the activation of mTOR observed in these muscle hypertrophy models seems to be not always dependent of the activation by the Akt kinase. Indeed, the activation of mTOR is Akt dependent in the muscle hypertrophy induced by testosterone (10) and FS (20), but Akt independent in exercise (36). Therefore, although the IGF-I pathway is clearly activated in muscle hypertrophy, the role of IGF-I and its signaling may vary according to the model.

	Follistatin	Exercise	Loading	Testosterone	BMPs
IGF-I	–	–(circ.) (37)	– (38)	– (32)	nd
IGF-II	–	nd	nd	nd	nd
INS	+	nd	– (39)	nd	nd
IGF-IR	+	+ (21)	– (33)	– (32)	nd
Akt	+	– (36)	nd	+ (10)	nd
mTOR	+	+ (34)	+ (35)	nd	+ (7)
S6K	–	nd	nd	nd	nd

Table 2: Role of the IGF-I/INS/IGF-IR/Akt/mTOR/S6K pathway components in the skeletal muscle hypertrophy induced by various stimuli.

(–: dispensable; +: indispensable; nd: not determined; circ.: circulating IGF-I)

Limitations of the animal models used

To decipher the role of the mediators and the pathways involved in the muscle hypertrophy caused by FS, we used several gain and loss of function approaches. These studies were carried out in different species of rodent (rat/mouse) and in different mouse strains. Nevertheless, the hypertrophic action of FS is comparable in these different species and strains. While of great interest, all these animal models suffer from limitations.

To investigate the role of the *IGF-I R*, mice expressing a dominant negative form of the type I IGF receptor (IGF-IR) specifically in skeletal muscle (MKR mice) were used. This model allowed to demonstrate that IGF signaling is crucial for the hypertrophy induced by FS (20), exercise (21) but not for testosterone (32) or overload (33) induced skeletal muscle hypertrophy. However, expression of the dominant-negative form of IGF-IR impairs both insulin and IGF-I signaling in muscle due to hybrid receptor formation (40). Moreover, deletion of the insulin receptor (IR) as the IGF-IR is associated with decreased muscle growth, suggesting that both receptors are required for proper physiological muscle growth (17,22,23).

Therefore, we cannot specify which from IR or IGF-IR is involved in these models of hypertrophy. A definitive answer should be obtained by using mice with muscle specific deletion of IGF-IR or IR, as recently described by R Kahn's group (25) to identify which of these two receptors plays the major in muscle hypertrophy caused by FS. Nevertheless, the fact that either IGF-IR or IR is required in FS-induced hypertrophy suggests that this hypertrophy might be impaired in situations characterized by low levels of their ligands, IGFs and insulin.

To investigate the role of *IGF-I*, the main ligand of IGF-IR, hypophysectomized (HYPOX) animals characterized by low circulating and muscle IGF-I were used. This model allowed to demonstrate that the GH-IGF-I axis is not required for the hypertrophy induced by FS (31), loading (41) or testosterone (32). This model is probably the only non-lethal model characterized by a profound and global IGF-I deficiency. Indeed, IGF-I null mice are not viable (15-17) and IGF-I null mice obtained by Cre-Lox technique have a better but still limited viability (42). However, it has to be realized that HYPOX animals are not only deficient in GH/IGF-I but also in other pituitary hormones (in particular sexual hormones) despite the hormonal substitution in thyroxine and glucocorticoids. The lack of testosterone, a major regulator of muscle mass, was circumvented in our study by using female HYPOX rats. Moreover, in contrast to IGF-I null mice, HYPOX animals, are still exposed to IGF-I, albeit at very low concentrations. For this reason, we cannot exclude the possibility that these very low IGF-I concentrations were sufficient enough for the FS-induced muscle hypertrophy. Finally, as GH deficiency does not influence markedly the levels of IGF-II and insulin, the possibility still exists that these two anabolic hormones compensate for the reduced IGF-I. However, considering the major growth retardation observed in HYPOX animals, these two hormones are clearly not sufficient to support normal body and muscle growth.

To investigate the role of *IGF-II*, another ligand of the IGF-IR involved in skeletal muscle development, IGF-II knock out mice were used. Our work demonstrated that IGF-II is not crucial for skeletal muscle hypertrophy induced by FS. However, in rodents, the role of IGF-II during postnatal growth is questionable, in contrast to humans (43). Indeed, IGF-II is predominantly expressed during fetal growth and it its expression in adult animals is hardly detectable in contrast to IGF-I (44). Moreover, the postnatal growth curves are the same in wild-type and IGF-II knock out mice (19). Finally, as the model used to investigate the role of IGF-II was constitutive, an adaptation with a compensation by IGF-I is still possible.

To investigate the role of *insulin*, a model of streptozotocin (STZ)-induced diabetes was used. This model allowed to demonstrate that insulin is required for the hypertrophy caused by FS overexpression or Mstn deficiency (45), but not if caused by overloading (39). One of the problem that we had to face is that insulinopenia is often associated with decreased IGF-I circulating concentrations, making difficult to decide which from insulin or IGF-I is required for this hypertrophy. Indeed, besides GH, insulin is also a major regulator of the IGF-I production by the liver (46). To circumvent this problem, IGF-I was infused in insulinopenic animals to show that even if IGF-I is not mandatory (cf. HYPOX model) for FS-induced hypertrophy, it can compensate for the lack of insulin (STZ-diabetic animals).

A central component of the cascade activated by IR and IGF-IR is the *Akt* kinase. This kinase plays a crucial role in the hypertrophy of myotubes induced by Mstn inhibition (47) and testosterone (10). In contrast, Akt inhibition by PI3K antagonist or Akt-1 genetic loss has no effect on the stimulation of protein synthesis induced by exercise (36). Using a dominant negative (dn) form of Akt transfected in muscle, we extended the *in vitro* data by showing that Akt is required for the hypertrophic effect of FS. This particular result contrasts with those of Goncalves *et al*. Indeed,

this group showed that neither Akt1 nor Akt2 isoforms are required for the hypertrophic response to Mstn inhibition obtained by a soluble form of its receptor ActIIRB (sActIIRB-FC) (48). The discrepancy between this last report and ours might result from differences in the choice of the tool to inhibit Akt-1. In our conditions, Akt inhibition was local and postnatal while, in their conditions, the Akt inhibition was systemic and developmental, allowing some degree of compensation.

Increases in muscle mass are often correlated with enhanced *mTOR* activity and several evidences suggest that signaling through mTOR is necessary for these changes to occur. To investigate the role of mTOR, a pharmacological specific inhibitor, rapamycin, was used. This drug allowed to demonstrate that mTOR is required for the muscle hypertrophy induced by BMP administration (7) or by loading (35). Moreover, the contraction-induced increase in muscle protein synthesis in humans is also blocked by rapamycin (34). Interestingly, the relatively modest role of mTOR in the muscle hypertrophy induced by Mstn inhibition as we and other groups (49,50) observed contrasts with what has been observed the other models of muscle hypertrophy. However, as rapamycin can exert nonspecific, mTOR independent actions, it is more prudent to mention that a rapamycin-sensitive mechanism is necessary for the hypertrophic effects of these stimuli. In fact, rapamycin inhibits only partially mTORC1 activity and a large fraction of mTORC1 substrates is insensitive to rapamycin (51). The use of novel highly selective compounds that block the kinase activity of mTORC1 and mTORC2 will allow to decipher more completely the role of mTOR in the FS-induced skeletal muscle hypertrophy (52).

S6K which is activated by nutrients and insulin/IGFs is essential for the control of muscle size by Akt and mTOR. To investigate its role, S6K null mice were used. These mice present a reduction of body weight associated with serious organ growth defect especially in nutrient and insulin/IGFs target tissues such as skeletal

muscle with reduced muscle fiber size (30). Here, we demonstrated that S6K is not mandatory in FS-induced muscle hypertrophy. These results are in line with those of Mc Mullen *et al.* showing that deletion of S6K1/2 have no impact on the development of physiological, pathological or IGF-IR-PI3K-induced cardiac hypertrophy (53). As the mice model used to investigate the role of S6K was constitutive and global, we cannot excluded that the activation of a compensatory pathway in S6K1/2 KO mice may have masked the role of S6Ks in this model of muscle hypertrophy (54). In fact, the activity of Akt, a crucial mediator of FS-induced skeletal muscle hypertrophy, is upregulated in S6K1/2 KO mice (54). In contrast to FS-induced hypertrophy, myotube hypertrophy induced by IGF-I is severely attenuated by rapamycin (3) or S6K1/2 deletion (30). This divergence suggests that, although the stimulation of the IGF-IR/Akt signaling is mandatory for the FS-induced hypertrophy, mTOR and S6K activities seem less crucial for the hypertrophy induced by FS, supporting the role of other pathways in this hypertrophy model.

Functional benefits of Mstn inhibition

Although the muscle hypertrophy caused by FS is undisputable, we did not demonstrate that this mass gain is translated to the production of muscle force. Indeed, the TA muscle which was used in most of our experiments is not suitable for force assessment, in contrast to EDL or soleus muscles. However, the muscle function issue is particularly important to consider in the clinical settings.

The effect of Mstn inhibition on muscle force is controversial. Indeed, an increased muscle force, as assessed by grip strength *in vivo* or by maximum tetanic force *ex vivo*, was reported in Mstn KO mice by some authors (55-57), but not all (58-61). In general, muscle force, expressed in mN, is proportional to the muscle cross-sectional area (CSA in mm^2), but a gain of muscle mass does not always result

in parallel enhancement in force (62). Indeed, when normalized to the muscle CSA, the force production (or specific force expressed in mN/mm^2), a marker of muscle contractile efficiency, is reduced in Mstn KO mice compared to wild-type mice (57,58,60,61). Moreover, by using single muscle fiber preparation, it was shown that Mstn inhibition does not increase the force production of individual muscle fibers but reduces the specific maximum isometric force (63). In contrast, muscle hypertrophy caused by muscle-specific overexpression of IGF-I, albeit less marked than after Mstn deletion, is accompagnied by increased maximum tetanic force (64). The reason of this discrepancy is not clear. It might be related to the increase in the myonuclear domain observed in Mstn KO mice and not in mTr-IGF-I (65). Alternatively, it could be related due to changes in pennation angle caused by massive hypertrophy as the one observed in Mstn KO mice. Indeed, endurance exercise and food restriction, which cause a reduction in muscle size, improve the muscle force of Mstn KO mice (66,67).

Besides muscle force, fatigability and exercise tolerance are also crucial to assess if one considers the benefit of FS on physical performance. Genetic loss of Mstn results in an increase in the proportion of fast-twitch glycolytic fibers (IIb). Moreover, muscles of these mice are characterized by a decrease in capillary density and in mitochondria number (58,61,68). Overall, this suggests that loss of Mstn is associated with loss of oxidative characteristics of skeletal muscle. It is not therefore unexpected that Mstn KO mice present a lower endurance capacity and higher fatigability (59,61,68,69). Indeed, Mstn KO mice ran less time and a shorter distance than wild-type mice. In conclusion, Mstn deficient mice are less suited to endurance running and better suited to short sprint.

Altogether, these results suggest that Mstn inhibition in healthy mice causes an increase in muscle mass without parallel increase in force production. However,

this not necessarily means that inhibition of Mstn cannot be beneficial in treating muscle wasting diseases, as illustrated in *mdx* mice (see infra).

Unsolved questions and scientific perspectives

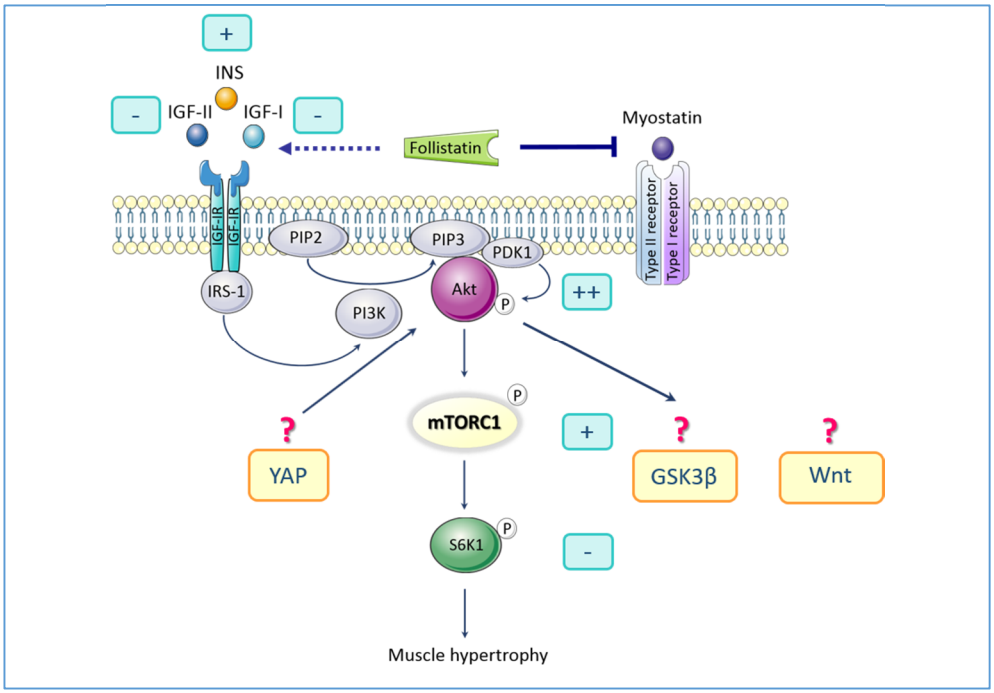
Although our data indicate that the muscle hypertrophy caused by Mstn inhibition requires the IGF-R/Akt/mTOR pathway, they also reveal that other pathways are probably involved. Indeed, Akt inhibition, and even more mTOR inhibition, do not blunt completely the FS-induced muscle hypertrophy, suggesting (20,49,50) that muscle growth is partially mTOR independent in this model of muscle hypertrophy. It is therefore likely that other proteins upstream or downstream of the Akt signaling might contribute to the muscle hypertrophy induced by Mstn inhibition.

One potential candidate upstream of Akt is the *Yes-associated protein* (YAP), a nuclear transcription cofactor of the Hippo signaling, known to regulate cellular growth. YAP is known to activate the PI3K/Akt pathway in cardiomyocytes (70). Interestingly, a relation between Mstn and the Hippo/YAP pathways has already been suggested. Indeed, Mstn inhibition results in the activation of the Hippo/YAP pathway in skeletal muscle (71). In opposite, overexpression of YAP in skeletal muscle results in the inhibition of Smad2/3 activity (72), the transcription factors mediating most of the Mstn actions. Moreover, activation of the Hippo/YAP pathway in skeletal muscle stimulates myogenesis. Indeed, *in vitro* activation of the Hippo/YAP pathway blocks satellite cells proliferation thereby enhancing their differentiation into myotubes (73). Furthermore, *in vivo* overexpression of Yap in skeletal muscle by electrotransfer is sufficient to promote muscle hypertrophy (72,74). The muscle hypertrophy induced by YAP is characterized by increased protein synthesis (74) and does not require mTORC1 activation (72). Together,

these results suggest that YAP might contribute to the hypertrophic effect of Mstn inhibition via the activation of Akt and independently of mTOR.

One candidate downstream of Akt and potentially implicated in the muscle hypertrophy induced by Mstn inhibition is *GSK-3β*. In fact, it is well established both *in vitro* and *in vivo* that GSK-3β kinase and some of its targets play a crucial role for skeletal muscle growth (3,75). Moreover, Mstn treatment in C2C12 cells activates GSK-3β through an inhibition of the PI3K/Akt pathway (76). Further studies will have to decipher the role of this pathway in the muscle hypertrophy caused by Mstn inhibition.

Wnt proteins might represent another potential mediator of the muscle hypertrophy induced by Mstn inhibition. Muscle transcriptional analysis of the Mstn deficient mice showed an upregulation of the expression of several Wnt proteins and in particular of Wnt4 (77). Overexpression of Wnt4 induces myotube hypertrophy by repressing Mstn signaling (78). Moreover, Wnt7α overexpression increases skeletal muscle mass by increasing muscle fiber size and the number of satellite cells (79). This hypertrophy results from the activation of the PI3K/Akt/mTOR pathway independently of the IGF receptor activation. Altogether, these results suggest that Wnt signaling represents a downstream target of Mstn which might be implicated in the hypertrophic effect of Mstn inhibition.



Recently, two research groups identify BMP signaling as the dominant signaling controlling muscle mass (7,80). BMP signaling seems to be dominant over Mstn signaling and is required for the hypertrophic action of FS. Therefore, the BMP signaling could present a new opportunity to develop therapeutics to limit muscle mass loss. However, the administration of BMP signaling agonists should be less muscle specific than Mstn inhibition. Indeed, the BMP family is large and most of its members are ubiquitously expressed. Moreover, BMPs have essential role in bone and cartilage formation. Therefore, at first glance, manipulation of BMPs to enhance muscle mass and function seem less attractive than inhibiting Mstn. On the other hand, the fact that BMPs control muscle and bone development may constitute an advantage to treat conditions in which both tissues are affected (aging) (81). In conclusion, the possible development of therapies based on the BMPs will clearly need a better understanding of their mechanisms of action.

2. Clinical perspectives

The interest for a better understanding of the intracellular mediators involved in the muscle hypertrophic effect of Mstn inhibition is encouraged by several promising clinical perspectives. Although our work did evaluate the action of FS in healthy animals, Mstn inhibition has already been tested in several pathological conditions not only in animals, but also in humans.

Clinical applications of Mstn inhibition

The progressive skeletal muscle loss and weakness are major clinical features of inherited myopathies and common in acquired muscle atrophies associated with aging, sepsis, glucocorticoids and cancer (82). These conditions have major impact on mobility, whole-body metabolism, disease resistance and quality of life. Thus the development of strategies to block or attenuate this muscle mass loss is likely to have major therapeutic benefits for a diverse set of clinical conditions.

The facts that Mstn mainly targets skeletal muscle to limit muscle size has raised the possibility that inhibition of Mstn pathway may be a promising way to counteract muscle atrophy. Moreover, Mstn functions extracellularly and is therefore accessible to pharmacological agents.

Tools to inhibit Mstn

FS overexpression into the muscle, as we used, is not clearly acceptable for treating patients. As demonstrated, systemic administration of FS is able also to induce some muscle hypertrophy, but needs to be repeated very regularly due to the short half-life of FS protein (83). Fortunately, many other approaches to inhibit the Mstn pathway are available and are under preclinical or clinical investigation. These approaches act extracellularly to block Mstn binding to its ActIIRB/ALK4/5

receptor complex. The use of Mstn propeptide or neutralizing monoclonal antibodies against Mstn represents a very specific way to inhibit Mstn signaling. Another avenue consists of the use of a soluble form of its receptor, sActIIRB-Fc. Like FS (84), this approach does not specifically block Mstn as ActIIRB-Fc (85,86) and FS bind other growth factors in addition of Mstn, which is not necessarily a disadvantage as some of these ligands, Activin A in particular, may also negatively control muscle mass (87).

Myostatin inhibition and muscle dystrophy

Several works have demonstrated the beneficial effect of Mstn inhibition in the *mdx* mice, an animal model of Duchenne muscular dystrophy (DMD) (88,89). There is increasing evidence that Mstn inhibition can improve muscle function in *mdx* mice through an increase in muscle mass and total force without consistent improvement of the weakness of dystrophic muscle as illustrated by conflicting results regarding the specific muscle force (90-92). Moreover, the effects of Mstn inhibition on the muscle fibrosis, a feature of the DMD, is controversial (90,93,94). In contrast, the mitigate results obtained in other models of dystrophy (limb-girdle muscular dystrophy, amyotrophic lateral sclerosis or dysferlinopathy) (95-98) suggest that the beneficial effects of Mstn blockade are limited in severely affected or late-stage disease. Mstn inhibition might therefore benefit muscular dystrophy when instituted early or in relatively mild disease. Results of a clinical study with ACE-031, a human ActIIRB-Fc, in DMD boys showed an increase in lean body mass associated with increased physical performance but has to be interrupted due to bleeding (99). Moreover, the administration of an antibody against Mstn (MYO-029) enhanced skeletal muscle mass but without improving strength and muscle function in Becker, limb-girdle and fascioscapulohumeral muscular dystrophy (100).

Further clinical trials will be necessary to investigate the efficiency of a treatment based on Mstn inhibition in inherited human myopathies.

Myostatin inhibition and acquired myopathies

Besides muscular dystrophy, other clinical conditions characterized by muscle atrophy such as cancer, glucocorticoid or aging, may benefit from Mstn inhibition. Cancer cachexia is a muscle wasting syndrome observed in many patients with advanced cancer which is associated with reduced functional performance and decreased survival. In this context, Mstn inhibition represents a promising way to prevent muscle mass and function loss associated with cancer in tumor-bearing mice (101-105). Interestingly, preservation of muscle mass by Mstn inhibition in cachectic mice was associated in some models with prolonged survival (101,105). The use of Mstn antibody (LY2495655) or ActIIRB antibody (BYM-338) are currently under clinical investigation in cancer patients (99). Catabolic conditions are often associated with elevated glucocorticoid production. Moreover, glucocorticoids are in part responsible for the muscle mass loss in the cachexia, starvation or sepsis conditions (106). Blockade of Mstn could therefore limit the muscle wasting observed in these situations. Interestingly, previous work in our laboratory has shown that Mstn deficient mice are protected against muscle atrophy induced by glucocorticoids (107). The work of *Lach-Trifilieff et al.* has recently confirmed these data by showing that Mstn inhibition by an antibody against ActIIRB (BYM388) protects muscle from glucocorticoid-induced atrophy and weakness (108). The potential benefit of Mstn inhibition has also been tested in aging, a situation in which the muscle mass and strength condition the quality of life. Mstn inhibition by specific antibody or Mstn propeptide seems a promising way to limit age related muscle loss and ameliorate physical performance and muscle fatigue associated with aging (103,109-111). In humans, administration of a single dose of soluble

ActIIRB increased total body lean mass in healthy postmenopausal women (112). Moreover, clinical investigation evaluating the treatment with ActIIRB antibody (BYM-338) in aging is actually in progress (82).

Inhibited Mstn signaling in order to enhance muscle mass seems a promising strategy for muscular disorders. Although these strategies ensures a widespread effect on musculature, it may also interfere with ActIIRB signaling in other tissues than skeletal muscle. One possible side effect is the inactivation of ActIIRB signaling in the heart. Indeed, a recent study showed that acute Mstn inhibition in the adult heart of mice leads to cardiac hypertrophy resulting in heart failure and increased lethality (113). In contrast, Mstn inhibition in the heart represents a promising therapy to prevent skeletal muscle mass associated with heart failure (114). Moreover, the use of Act-IIRB-Fc reverses the loss of cardiac muscle associated in cancer-associated cachexia (101). Further clinical investigations are needed to evaluate the risking adverse effects of Mstn blockade.

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