Characterization of SMAD3 function in skeletal muscle growth

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NANYANG
TECHNOLOGICAL
UNIVERSITY

**CHARACTERIZATION OF SMAD3 FUNCTION IN
SKELETAL MUSCLE GROWTH**

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SCHOOL OF BIOLOGICAL SCIENCES

2012

**CHARACTERIZATION OF SMAD3 FUNCTION IN
SKELETAL MUSCLE GROWTH**

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School of Biological Sciences

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partial fulfilment of the requirement for the degree of Doctor of
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Abstract

Smad3 is a key intracellular signaling mediator for Myostatin and TGF- β 1, two TGF- β superfamily members and are key regulators of skeletal muscle growth and homeostasis. Despite extensive studies about the functions and signaling of Myostatin/TGF- β 1, little is known about the regulatory role of Smad3 in muscle growth. Thus the main emphasis of this thesis is to investigate the role of Smad3 in post-natal myogenesis.

Firstly, this thesis demonstrated that there is pronounced skeletal muscle atrophy in Smad3-null mice and this is due to increased *Myostatin* expression. Enhanced *Myostatin* signaling promoted sarcomeric protein degradation through the ubiquitin proteasome system in Smad3-null skeletal muscle. Increased *Myostatin* level also resulted in impaired SC proliferation, and reduced self-renewal resulting in reduced SC number in Smad3-null mice. Therefore, treatment of Smad3-null myotubes with Myostatin antagonist significantly increased the expression of sarcomeric proteins. Consistent with this result, double knockout (DKO) mouse (Smad3^{-/-}/Myostatin^{-/-}) had improved muscle weights and *in vitro* myogenic capacity when compared to that of Smad3-null mice. Therefore, up-regulated *Myostatin* expression is partially responsible for the muscle atrophy in Smad3-null mice.

To further validate the impaired SC function found in Smad3-null mice, muscle regeneration induced by notexin injection was analyzed. Smad3-null mice showed impaired *M. tibialis anterior* (TA) muscle regeneration, with a reduced inflammatory response and less activated myoblasts and nascent myofibers formation. Moreover, Smad3-null regenerated muscle had remarkable reduced oxidative enzyme activity, which might be due to impaired mitochondrial biogenesis since the expression of TFAM, the master regulator of mitochondrial biogenesis, was down-regulated.

Furthermore, Smad3 deficiency led to reduced scar tissue formation in the regenerated muscle. Most importantly, similar to the uninjured muscle, Smad3-null muscle had consistently up-regulated *Myostatin* expression during the course of muscle regeneration. Therefore, the inhibitory role of Myostatin on muscle growth may be one major molecular mechanism underlying the impaired muscle regeneration of Smad3-null mice.

To advance our understanding of molecular mechanisms through which Smad3 regulates muscle growth and metabolism, gene expression analysis of Smad3-null muscle was performed. In total, 25 up-regulated and 31 down-regulated genes ($P < 0.05$, fold change ≥ 1.5 or ≤ -1.5) were identified in Smad3-null muscle when compared to Wild-type control muscles. Altered gene expression changes may help to explain the muscle phenotype seen in Smad3-null mice. Up-regulated expression of genes encoding two inflammatory cytokines (*S100a8* and *S100a9*), may help to explain the systemic inflammation observed in Smad3-null mice. Furthermore, down-regulated expression of 5 genes encoding ECM components in Smad3-null muscle suggests an indispensable role of Smad3 in muscle fibrosis. In addition, down-regulated expression of *Pkm2*, and up-regulated expression of *Dlat* suggests a reduced glycolysis and pyruvate oxidation in Smad3-null muscle.

In conclusion, results presented in this thesis highlight the essential role of Smad3 in post-natal myogenesis. Using *in vitro* and *in vivo* myogenesis models, I demonstrated that Smad3 deficiency led to defective post-natal myogenesis, atrophied musculature and impaired muscle regeneration. Up-regulated *Myostatin* levels partially contribute to the post-natal myogenic defects in Smad3-null mice. Moreover, this thesis also sheds light on the regulatory role of Smad3 in skeletal muscle metabolism, as well as ECM synthesis.

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List of Abbreviations

The following abbreviations have been used in this thesis:

°C	degree celsius
3'	3 prime
5'	5 prime
aa	amino acid
Acetyl-CoA	acetyl coenzyme A
ACh	acetylcholine
AChRs	acetylcholine receptors
ActRIIB	activin type IIB receptor
AMPK	AMP-activated protein kinase
ATP	adenosine triphosphate
bFGF	basic fibroblast growth factor
bHLH	basic helix-loop-helix
BMPs	bone morphogenic proteins
bp	base pair
BSA	bovine serum Albumin
C2C12	Murine myoblast cell line
CCGs	clock-controlled genes
Cdk2	Cyclin-dependent kinase 2
Cdks	Cyclin-dependent kinases
CEE	chicken embryo extract
Chad	Chondroadherin
CK	creatine kinase
CKD	chronic kidney diseases
Colla1	Collagen I alpha 1
Colla2	Collagen I alpha 2
ColIIIa1	Collagen III alpha 1
ColIVa1	Collagen IV alpha 1
Comp	Cartilage oligometric matrix protein

Co-Smad	common Smad
CPT	carnitine palmitoyltransferase
Cry1	Cryptochrome
CSA	Cross Sectional Area
c λ	carrageenan λ
DAVID	Database for Annotation, Visualization and Integrated Discovery
DEX	dexamethasone
DGC	Dystrophin-glycoprotein complex
Dia	Diaphragm
DKO	double knockout
Dlat	dihydrolipoamide S-acetyltransferase
DM	differentiation medium
DMD	Duchenne muscular dystrophy
DMEM	Dulbecco's Modified Eagle Medium
ECM	extracellular matrix
EGF	endothelial growth factor
EMCL	extramyocellular lipids
epox	epoxomicin
ERKs	extracellular signal regulated kinases
FA	fatty acid
FACS	fluorescence-activated cell sorting
FAK	focal adhesion kinase
FBS	Fetal Bovine Serum
FC	fold change
FFA	free fatty acids
FLRG	Follistatin-related gene
Fmod	Fibromodulin
FoxO1	Forkhead box protein O1
FoxO3	Forkhead box protein O1
Fstl 1	Follistatin-like 1
g	gram
G ₀	Gap 0 phase of the cell cycle
G ₁	Gap 1 phase of the cell cycle

G ₂	Gap 2 phase of the cell cycle
G-6-P	glucose-6-phosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Gas and Sol	<i>M. gastrocnemius and soleus</i>
GASP-1	Growth and differentiation factor-associated serum protein-1
GDF-8	growth and differentiation factor-8
GH	Growth hormone
GO	Gene Ontology
GP	glycogen phosphorylase
GS	glycogen synthase
H & E	Hematoxylin and Eosin
HGF	Hepatocyte growth factor
HRP	horse-radish peroxidase
HS	Horse Serum
HSL	Hormone sensitive lipase
ICC	immunocytochemistry
IGF-1	Insulin-like growth factor-1
IgG	Immunoglobulin G
IL	Interleukin
IMCL	intramyocellular lipids
IPA	Ingenuity Pathway Analysis
I-Smads	inhibitory Smads
JNK	c-Jun N-terminal kinase
kDa	Kilo Dalton
LAP	latency-associated protein
LCFA	Long-chain fatty acids
LCFA-CoA	long-chain fatty acyl-CoA
LPL	Lipoprotein lipase
LTBPs	Latent TGF- β -binding proteins
M phase	Mitosis phase of the cell cycle
MAD	mothers against decapentaplegic
MAPK	mitogen-activated protein kinases
MGI	Mouse Genomics Informatics database

MH1	Mad homology domain-1
MH2	Mad homology domain-2
MIBC	muscle invasive bladder cancer
min	minutes
miRNA	microRNAs
MQ	Milli Q water
mRNA	messenger RNA
MuRF-1	Muscle specific ring finger-1
MyHC	myosin heavy chain
MyLC	myosin light chain
NF- κ B	Nuclear factor-kappa B
NES	nuclear export sequence
NLS	nuclear localization signal
NGS	normal goat serum
NLS	nuclear localization signal
NMJ	neuromuscular junction
NO	Nitric oxide
OCT	Optimal Cutting Temperature
PAGE	Polyacrylamide gel electrophoresis
PASMCs	primary pulmonary smooth muscle cells
Pax3	Paired box gene 3
Pax7	Paired box gene 7
PCr	phosphocreatine
PCR	Polymerase chain reaction
PCs	proprotein convertases
PDC	pyruvate dehydrogenase complex
PDGF	platelet-derived growth factor
Pdk4	pyruvate dehydrogenase kinase 4
PEG	Polyethylene glycol
PEP	phosphoenolpyruvate
PFA	paraformaldehyde
PGs	proteoglycans
PI3K	phosphatidylinositol 3-kinase

Pkm2	pyruvate kinase isoenzyme M2
pRb	hypo-phosphorylated Rb
pRb ^{PP}	hyper-phosphorylated Rb
pre-miRNAs	miRNA precursors
pri-miRNAs	primary miRNAs
PVP	Polyvinylpyrrolidone
Quad	<i>M. quadriceps</i>
R	Pearson's correlation coefficient
Rb	Retinoblastoma
R-Smads	receptor-regulated Smads
R-SBE	Smad binding element in primary transcripts of miRNA
RT	reverse transcriptase
RT-PCR	quantitative real time PCR
SARA	Smad anchor for receptor activation
SBEs	Smad binding elements
SC	satellite cell
SDH	succinate dehydrogenase
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SHP-2	SH2 domain-containing protein-tyrosine phosphatase
shRNA	short hairpin RNA
SLRPs	small leucine rich repeat proteoglycans
Smad	small mother against decapentaplegic
SP	signal peptide
Spon2	Spondin 2
SR	sarcoplasmic reticulum
T tubules	transverse tubules
TA	<i>M. tibialis anterior</i>
TAE	Tris acetate EDTA
<i>Taq</i>	DNA polymerase from <i>Thermus aquaticus</i>
TBS	Tris buffered saline
TBST	Tris buffered saline tween 20
TCA cycle	tricarboxylic acid cycle

TE	Tris EDTA
TG	Triglyceride
TGF- β	transforming growth factor- β
TNF- α	Tumor necrosis factor-alpha
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
U	Units
UTR	Untranslated region
VLDLs	very-low-density lipoproteins
VLDL-TG	very-low-density lipoproteins-triglyceride
w/v	weight per volume
WAT	white adipose tissue
α -GPD	α -glycerophosphate dehydrogenase
β Me	β -Mercaptoethanol

This Thesis is dedicated to My Dear Family.

Chapter 1 Literature review

1.1 Skeletal muscle

Skeletal muscle contributes to around 50% of total human body mass, which makes it the largest organ (264). Skeletal muscle is one of the three major muscle types, i.e. skeletal, cardiac, and smooth muscle and is composed of myofibers, extracellular matrix (ECM), and an organized network of nerves and blood vessels (Figure 1.1a) that connect individual myofibers into bundles to form muscles. Most skeletal muscles articulate into bones through bundles of collagen fibers known as tendons and as such are responsible for locomotion. Skeletal muscle is also responsible for thermogenesis, energy storage and influencing overall body metabolism (113). Under the microscope, skeletal muscle appears striated (Figure 1.1b), therefore skeletal muscle is also known as striated muscle (30). Skeletal muscle myogenesis starts very early during embryonic development and continues throughout post-natal life. Various growth factors and signaling molecules have been implicated in regulating myogenesis. This section reviews the most relevant literatures pertaining to the structure and function of skeletal muscle, Myostatin (a member of the TGF- β superfamily) and its downstream signaling molecule, Smad3 protein.

1.1.1 Skeletal muscle structure

1.1.1.1 Myofiber

Skeletal muscle consists of bundles of elongated multinucleated muscle cells called myofibers. Each myofiber is mainly composed of organized myofibrils. These myofibrils make up most of the cytoplasm of myofibers (sarcoplasm), while the nuclei are located just under the plasma membrane (sarcolemma). Myofibrils consist of repeated subunits called sarcomeres, with one myofibril consisting of approximately 10,000 sarcomeres (107).

The sarcomere is the smallest structural and functional element of myofibrils. Sarcomeres contain two types of protein filaments, thick filaments (composed primarily of myosin) and thin filaments (composed primarily of actin), as well as the proteins which regulate the interaction between the two filaments (Figure 1.2a). This interaction is ultimately responsible for producing muscle contraction. Myosin is a rod-shaped protein with two globular heads at one end. The rod part polymerises to form the filament backbone (thick filaments). Actin is a globular protein and it polymerises to form long helical filaments (thin filaments). A sarcomere extends from Z disc to Z disc (Figure 1.2a). Due to the differences in density and distribution of the two filaments, the sarcomere shows a banded appearance, with a dark band (A band) and a light band (I band). The A band is located in the center of a sarcomere and the length of the A band is equal to the length of thick filament. The M line is the center line of a sarcomere and it is the site where thick filaments are connected to each other. Under the resting state, the A band includes two zones, the portion on either side of the M line that contains only thick filaments (H zone), and the portion where thick

filaments and thin filaments overlap (zone of overlap). The I band contains only thin filaments (113) (Figure 1.2a).

When skeletal muscle contracts, sarcomeres shorten in length. The H-zone and I band decrease their widths while the width of A band remains unchanged. The sliding filament theory explains the mechanism by which sarcomeres shorten without changing the length of both filaments (Figure 1.2b). Two types of filaments slide past each other. The head region of myosin binds to actin filaments and pulls them towards the center of sarcomere. This results in the shortening of the sarcomere and triggers the overall contraction of myofibers. The head part of myosin also has a catalytic domain (ATPase), responsible for powering myosin/actin sliding, via hydrolysis of adenosine triphosphate (ATP) (282).

a

b

Figure 1.1 Skeletal muscle structure

(a) Skeletal muscle is made up of a group of fascicles, each of which is enclosed by the perimysium. Fascicles are made up of bundles of muscle fibers, each of which is enclosed by the endomysium. Finally the epimysium encloses the entire skeletal muscle. Muscle fibers are mainly composed of myofibrils, which are further made up of repeated units called, sarcomeres. (b) Skeletal muscle appears striated under light microscopy. Figures are modified from Jenkins *et al.* (113).

a

b

Figure 1.2 Sarcomere structure

(a) Sarcomeres contain thin and thick filaments, and is primarily made up of a dark A-band and a light I-band. Figure is modified from Jenkins *et al.* (113). (b) Interaction of thick filaments and thin filaments leads to the shortening of the sarcomere and myofibril contraction (Sliding filament theory). Figure is modified from Wakabayashi *et al.* (282).

1.1.1.2 Extracellular matrix

Extracellular matrix (ECM) plays an important role in skeletal muscle. It is involved in the maintenance and regeneration of skeletal muscle, and strongly influences muscle cell functions. Skeletal muscle ECM includes tendon and three layers of connective tissue: the layer around myofibers (endomysium), the layer around groups of myofibers (perimysium), and the layer which ensheaths the whole skeletal muscle (epimysium) (Figure 1.3). Collagen is the major protein in skeletal muscle ECM. However, a number of proteoglycans (PGs), mainly belonging to a family of small leucine-rich proteoglycans (SLRPs), are also present in the ECM, such as decorin, biglycan, fibromodulin, and lumican. PGs and collagen bind together and they determine the structural organization of skeletal muscle ECM. Perimysium is primarily made up of type I collagen and PG, such as decorin. Epimysium and endomysium are mainly made up of equal amounts of type I and III collagen, as well as other PGs. Muscle basal lamina is primarily made up of type IV collagen (6). PGs are known to associate with growth factors, therefore, skeletal muscle ECM functions as a reservoir of growth factors. Once these growth factors are released by enzymes present in ECM, they initiate cell signaling pathways and strongly regulate myofiber function in an autocrine fashion (110, 197). Skeletal muscle ECM also functions as the Satellite Cell (SC) niche. SCs are muscle stem cells located between the basal lamina and sarcolemma of individual muscle fibers (124, 170).

Figure 1.3 Extracellular matrix (ECM) in skeletal muscle

Skeletal muscle ECM includes tendons and three layers of connective tissue, endomysium, perimysium, and epimysium. Figure is modified from Allison *et al.* (6).

1.1.1.3 Neuromuscular junction

The site where a motor neuron is attached to a group of myofibers is called the neuromuscular junction (NMJ) or neuromuscular synapse (Figure 1.4a). It transmits signals from the motor neurons to myofibers. In addition to myofibers and motor neurons, NMJ include Schwann cells and kranocytes, which cap the nerve terminals. Electrical signals from the motor neurons release the chemical messenger, acetylcholine (ACh). ACh then diffuses across the narrow gap (synaptic cleft) between the motor nerve terminal and myofibers and then trigger myofiber contraction by binding to its receptor (AChR) (Figure 1.4b). AChRs are located on a region of the myofiber, which is called the motor endplate. One motor neuron innervates a group of myofibers and this motor neuron, together with all the myofibers it innervates is called a motor unit (101).

Figure 1.4 Neuromuscular junction (NMJ) in skeletal muscle

(a) The NMJ in skeletal muscle is composed of myofibers and motor neurons. (b) Electrical signals from the motor neurons release the chemical messenger, Acetylcholine (ACh). ACh diffuses across the synaptic cleft and then triggers myofiber contraction by binding to its receptor (AChR) on myofibers. Figures are modified from Jenkins *et al.* (113).

1.1.2 Skeletal muscle function

1.1.2.1 Skeletal muscle as a locomotion organ

Skeletal muscle is responsible for supporting body weight, and performing movements. It converts chemical energy into mechanical motion via myofibril contraction. Stimulation by nerve impulse (neuromuscular signals) causes depolarization of the sarcolemma (reversal of the membrane charge polarity). Like other plasma membranes, sarcolemma has unequal distribution of positive and negative charges across the sarcoplasm. Under the resting state, the extracellular face is more positive while the inside face is more negative. Upon nerve impulse stimulation, chemically regulated ion channels open, which results in a more negative charge outside the sarcolemma and a more positive charge inside the myofibers. This depolarization of sarcolemma is spread deep into the sarcoplasm via transverse tubules (T tubules), which are a deep invagination of the sarcolemma. T tubules allow the depolarization to penetrate deep into the sarcoplasm to activate the sarcoplasmic reticulum (SR). Upon activation, SR opens the calcium ion channels and releases calcium ions. Calcium ions diffuse into the surrounding sarcoplasm and trigger the conformation changes in contractile proteins, which generate directional motion and force (Figure 1.5). When the intracellular calcium ions are pumped back into storage and the calcium ion level goes down to normal, myofibers go back to the resting state (113).

Figure 1.5 Muscle function: locomotion

Stimulated by neuromuscular signals, the sarcolemma undergoes depolarization. Transverse tubules (T tubules) spread this depolarization deep into the sarcoplasm, which activates the sarcoplasmic reticulum (SR). Upon activation, SR releases calcium ions, which further trigger conformation changes in contractile proteins and generate directional motion and force. Figure is modified from Sadava *et al.*(230).

1.1.2.2 Skeletal muscle as an endocrine organ

In addition to locomotion, skeletal muscle is believed to function as an endocrine organ, secreting cytokines to influence the function or metabolism of other tissues and organs (213). Researchers have proposed the term “myokines”, to define the cytokines secreted by myofibers (212).

Skeletal muscle has been shown to release significant amounts of inflammatory cytokines into circulation, especially IL-6, in response to prolonged intense exercise (206). This up-regulation of IL-6 in skeletal muscle is not due to acute inflammatory response caused by muscle damage, but due to muscle contraction *per se*. It is hypothesized that IL-6, as a signal released by contracting skeletal muscle, stimulates liver to release glucose in times of energy depletion (70, 99). IL-6 also activates AMP-activated protein kinase (AMPK) to stimulate lipolysis and fat oxidation (213). In addition to IL-6, other myokines have been identified in recent years. IL-8 is released by contracting skeletal muscle, possibly to stimulate angiogenesis by targeting microvascular endothelial cells (3, 77). IL-15, which is an anabolic factor and functions as a mediator between muscle and fat cross-talk, may also act as a myokine (203, 217). In addition, Follistatin-like 1 (Fstl 1), an extracellular glycoprotein secreted by myofibers, may act as a myokine through coordinating blood vessel recruitment with muscle growth (209). Identification of new myokines will help us better understand skeletal muscle function as an endocrine organ.

1.1.2.3 Energy source and energy metabolism pathways in skeletal muscle

During muscle contraction, ATP is the immediate energy source. However, intracellular ATP storage is depleted very quickly and different metabolic pathways are activated to supply ATP by catabolizing a number of molecules (fuels) (231). Skeletal muscle uses either intramyocellular fuel reserves, such as phosphocreatine (PCr), glycogen, and lipids, or circulating fuels such as glucose or lipids. There are two different metabolic pathways, namely anaerobic and aerobic. Aerobic pathways occur in mitochondria and result in high energy release. Anaerobic pathways occur in the sarcoplasm and are not as efficient as aerobic pathways for energy production.

Skeletal muscle shows high plasticity, with the ability to change its metabolic profile in response to changing functional demands. Different catabolic pathways demonstrate different efficiency and rate of ATP production. The intensity and duration of exercises performed determine the catabolic pathways used in skeletal muscle. Exercises with low intensity usually rely on aerobic pathways, while high intensity exercises rely more on anaerobic mechanisms (116, 287).

1.1.2.3.1 Creatine phosphate as an energy source for skeletal muscle contraction

Creatine phosphate (PCr) is used by myofibers as a way to rapidly replenish ATP. It donates a phosphate group to ADP to form ATP ($\text{PCr} + \text{ADP} \leftrightarrow \text{Cr} + \text{ATP}$). This reaction is catalyzed by creatine kinase (CK) within seconds and it is one of the dominant anaerobic pathways for ATP generation. This is a reversible reaction, such that when ATP demand is low, PCr is replenished (122).

1.1.2.3.2 Glucose as an energy source for skeletal muscle contraction

Glucose is an essential energy substrate in skeletal muscle. Due to its large mass, skeletal muscle is one of the major tissues for glucose disposal, accounting for around 80% of all glucose uptake/disposal. Glucose is stored in the form of glycogen (polysaccharide of glucose) in “glycogen granules”, which are subcellular bead-like structures within myocytes, that also contain enzymes and proteins for glycogen metabolism (86). Glycogen is the most important intramyocellular fuel reserve. Glucose metabolism in skeletal muscle includes glycogen synthesis, during times of energy abundance, and glycogen break down to produce ATP, during muscle contraction. The synthesis and break down processes are coordinated with metabolic demands (87).

Glucose can be catabolized either by aerobic (oxidative phosphorylation) or anaerobic pathways (substrate level phosphorylation). Glucose released from glycogen undergoes glycolysis and is ultimately converted to pyruvate, producing two ATP molecules. These reactions are regulated by glycogen phosphorylase (GP) and do not need oxygen. Pyruvate can be further converted to lactate and hydrogen ions by lactate dehydrogenase in an anaerobic way (123). This is another predominant anaerobic pathway used by skeletal muscle. Compared to aerobic metabolism, the energy produced in this pathway is remarkably low: 3 ATP per glucosyl unit vs. the far greater 38 ATP per glucosyl unit produced during oxidative phosphorylation (287). However, the advantage is that under anaerobic mechanisms there is a high rate of ATP production, which is necessary during high intensity exercise, when the ATP consumption rate is high. Pyruvate can also be used in the aerobic pathway. Once Pyruvate is taken into mitochondria via the pyruvate dehydrogenase complex (PDC), it

is oxidized into acetyl coenzyme A (Acetyl-CoA) and then completely oxidized through the tricarboxylic acid cycle (TCA cycle) (116).

Glucose is taken up into skeletal muscle in response to insulin stimulation and can be stored as glycogen through glycogen synthesis. Extramyocellular glucose is transported into myocytes via the GLUT4 transporter (glucose uptake). GLUT-4 is a low-capacity glucose transporter in skeletal muscle. Upon stimulation, it translocates from intracellular vesicles to the plasma membrane (114, 272). Once glucose is transported in, it is immediately phosphorylated to glucose-6-phosphate (G-6-P) by hexokinase II. G-6-P is either stored as glycogen or enters glycolysis to produce ATP. For glycogen synthesis, G-6-P is converted to UDP-glucose, and through the action of glycogen synthase (GS), a glucose residue from UDP-glucose is added to the growing glycogen chain. The activity of GS controls the rate-limiting step in glycogen synthesis (25, 86).

Insulin levels in plasma also regulate the metabolism of glucose in skeletal muscle. When insulin concentration is high, the majority of glucose is converted to glycogen. However, during the fasting state, around half of the glucose is used for glycogen synthesis and the remaining half is used to produce ATP via glycolysis (1).

1.1.2.3.3 Lipids as an energy source for skeletal muscle contraction

Lipids are another fuel source for ATP production in skeletal muscle in aerobic metabolism. Prolonged, low intensity, exercise enhances lipid utilization. Long-chain fatty acids (LCFA), very-low-density lipoproteins-triglyceride (VLDL-TG) in blood plasma or intramuscular lipids are the lipid source that skeletal muscle utilizes (126).

LCFA is one of the major circulating lipid sources used for ATP production in exercising skeletal muscle. There are two mechanisms for the transport of LCFA from blood plasma into skeletal muscle. Firstly, LCFA passively diffuses into skeletal muscle when the plasma free fatty acid (FFA) level is high (216). Secondly, cell surface proteins, such as CD36 (fatty acid translocase), are thought to mediate the active transport of LCFA (2). After uptake in skeletal muscle, LCFA is activated into long-chain fatty acyl-CoA (LCFA-CoA). LCFA-CoA either enters the β -oxidation pathway to generate ATP or is used for triglyceride (TG) synthesis. For ATP production, LCFA-CoA is transported into mitochondria from the sarcoplasm, and is converted to Acetyl-CoA via the β -oxidation pathway and then completely oxidized through the TCA cycle. The transport of LCFA across the mitochondrial membrane is L-carnitine-dependent and is catalyzed by the carnitine palmitoyltransferase (CPT) enzyme system (20). In addition to being a fuel source for energy production, LCFA-CoA might also be involved in enzyme activation and cell signaling (32, 69).

Triglyceride (TG) is another circulating lipid source. In liver, it is wrapped into very-low-density lipoproteins (VLDLs) before it is secreted into circulation. VLDLs-TG is the major circulating form of TG, and it is this form that permits the delivery of the hydrophobic TG to peripheral tissues. Circulating TG may also contribute to energy production during exercise, depending on the exercise intensity and diet (126). Circulating VLDL-TG is hydrolyzed, releasing FFAs to be taken up into skeletal muscle for oxidation. Lipoprotein lipase (LPL) is the rate-limiting enzyme in the VLDL-TG hydrolysis and it is located at the capillary luminal surface within muscle. LPL activity is an important determinant for VLDL-TG degradation in skeletal muscle (186).

Intramuscular lipids can also be utilized as an energy source during exercise. These include both intramyocellular lipids (IMCL) and extramyocellular lipids (EMCL). IMCL are the local lipids stored within myofibers and EMCL are the lipids from adipocytes, located between or along myofibers. The majority of the intramuscular fat (99%) is EMCL and it may be recruited into myofibers for utilization (126). Hormone sensitive lipase (HSL) is an important lipase responsible for the hydrolysis of intramuscular lipids (76).

1.1.2.3.4 Muscle fiber type classification based on myosin heavy chain (MyHC) isoforms

To cope with the diversity of activities, skeletal muscle contains myofibers with different contractile properties and metabolic profiles. Based on the expression of myosin heavy chain (MyHC) isoforms, rodent muscles consist of four different muscle fiber types, type I/IIA/IIB/IIX. It is noteworthy that human muscles do not express type IIB MyHC. The rate of myofiber contraction is determined by the MyHC isoform expressed in it. Type II fibers (fast-twitch) consume ATP more rapidly and rely mainly on anaerobic metabolism (more on glycolysis) to support explosive contraction. Type II fibers are further classified into type IIA/IIB/IIX depending on metabolic profile and MyHC isoforms. Type I fibers (slow-twitch) are energy conserving fibers, which mainly depend on aerobic metabolism (more on FFA oxidation) to support prolonged low-intensity movements (287).

1.1.3 Myogenesis

1.1.3.1 Embryonic myogenesis

Skeletal muscle of vertebrates originates from paraxial mesoderm during embryogenesis. Mesoderm segments into somites and the dorsal part of the somites gives rise to dermomyotome, which eventually forms the dermis, as well as skeletal muscles of the body and limbs. The hypaxial dermomyotome gives rise to most of the body musculature and limb musculature, whereas the epaxial dermomyotome gives rise to deep back musculature (37).

Muscle progenitor cells delaminate from the epithelium of the dermomyotome and migrate into limb buds, where they proliferate and differentiate to form skeletal muscles (67). Hepatocyte growth factor (HGF) and its tyrosine kinase receptor, c-met, play a critical role in the delamination and migration of muscle progenitor cells because mutation of either gene leads to the absence of skeletal muscle in limbs (24). The paired box gene 3 (*Pax3*), the paired box transcription factor, is believed to act up-stream of c-met and loss of the *Pax3* gene also blocks limb muscle formation (257). Muscle progenitor cells do not express the myogenic determination genes, *MyoD* and *Myf5*, until they migrate into limb buds. *MyoD* and *Myf5*, as well as the other basic helix-loop-helix (bHLH) transcriptional factors of the MyoD family play important roles in embryonic myogenesis. Further studies in mice show that *Myf5* is the first bHLH transcriptional factor to be expressed in embryonic myogenesis and acts genetically up-stream of *MyoD*, along with *Pax3* (257). Mutation of the *MyoD* gene in mice results in delayed myogenesis but with virtually normal musculature (119), similarly mutation of the *Myf5* gene also results in normal muscle formation but with slightly reduced muscle fiber size and muscle mass (119). However, mice with

mutation of both *MyoD* and *Myf5* show complete absence of skeletal muscle (229). These studies suggest a redundancy in the function of *MyoD* and *Myf5* during myogenesis.

1.1.3.2 Post-natal myogenesis

Adult skeletal muscle has powerful regenerative capacity, and in fact can regenerate in response to myotrauma throughout the entire life of the organism. Muscle regeneration, in some respects, recapitulates the process of the embryonic myogenesis (303). It is a multi-step process and mainly includes three phases: degeneration, regeneration and remodelling (88) (Figure 1.6b). In the degenerative phase, the necrotic muscle releases chemotactic factors, which recruit inflammatory cells in the damaged area to promote phagocytosis of fiber debris and activation of Satellite Cells (SCs) (268). In the second phase, SCs are activated, giving rise to myoblasts. Myoblasts then proliferate and differentiate to repair the damaged myofibers or fusing together to form nascent myofibers. In the third phase, nascent myofibers increase in size and undergo maturation. Infiltrated fibroblasts are responsible for the formation of ECM. Excessive accumulation of ECM during muscle regeneration leads to scar tissue (234).

Figure 1.6 Muscle regeneration and Satellite Cells (SCs)

(a) An electron micrograph highlighting a human muscle Satellite Cell (S) and myonucleus (M). SCs are located outside of the sarcolemma, while the myonucleus is located inside the sarcolemma. Figure is modified from Roth *et al.* (228). (b) Post-natal myogenesis is attributed to SCs. In response to muscle injury, SCs are activated. Activated SCs re-enter the cell cycle, proliferate and finally differentiate. They then either fuse together to form nascent myofibers or fuse with damaged fibers. Another population of myoblasts are fusion-incompetent cells, which eventually withdraw from cell cycle and return to quiescent state to replenish the SC population. Figure is modified from McCroskery *et al.* (173)

1.1.3.2.1 Satellite Cell function in muscle regeneration

Muscle regeneration is attributed to SC function. SCs are muscle stem cells that are located between the basal lamina and sarcolemma of individual muscle fibers (124, 170) (Figure 1.6a). SCs exist normally in a quiescent state, but are activated in response to myotrauma. Quiescent SCs have relatively small cytoplasm and condensed chromatin. Activated SCs re-enter the cell cycle, proliferate, differentiate and then either fuse together to form nascent myofibers or fuse with existing damaged fibers. These myoblasts are usually termed fusion-competent cells. Another population of myoblasts, referred to as fusion-incompetent cells, eventually withdraw from the cell cycle and return to a quiescent state to replenish the SC population (Figure 1.6b) (173).

At birth, about 30% of sub-laminar muscle nuclei in mice are SCs, however in adulthood, the number of SCs goes down and is maintained at 5% through SC self-renewal (23). There are currently two mechanisms proposed for SC self-renewal: asymmetric and symmetric SC division. In the asymmetric model, SC division gives rise to two different daughter cells, one quiescent and one fusion-competent (52, 138). In the symmetric model, the same type of daughter cells are produced (28, 137). Most proliferating myoblasts down-regulate *Pax7* expression and differentiate by up-regulating *MyoD* expression. However, a small population of cells maintain *Pax7* expression, while down-regulating *MyoD* expression, and as such become the self-renewed population (205).

The microenvironment (niche) of SCs plays an important role in the regulation of SC function and post-natal myogenesis. The structure of the SC niche influences SC function, including mechanical, chemical or electrical signals released by the niche (139). Blood capillaries within the muscle might also influence SCs since the number

of the capillaries per muscle fiber is correlated to SC number (45). Importantly, disruption of the sarcolemma and basal lamina during muscle injury initiates SC activation (23, 106, 248). Integrin $\alpha 7 \beta 1$ is a laminin receptor in skeletal muscle, which connects the cytoskeleton of SCs and the laminin in the basal lamina (250). M-cadherin is an adhesion receptor that links SCs with adjacent muscle fibers. It is known to initiate calcium-dependent myoblasts fusion and the fusion between myoblasts and myotubes (47). These structural proteins mediate signal transduction for regulation of SC function, such as migration, proliferation and fusion (29, 40, 290).

Moreover, systemic factors in serum have also been reported to profoundly regulate SC activity (50-51). Various growth factors and cytokines are known to influence SC functions. For example, Hepatocyte growth factor (HGF) is able to induce SC activation (262). Insulin-like growth factor-1 (IGF-1) and endothelial growth factor (EGF) enhance SC proliferation (117). Myostatin is a potent negative regulator of skeletal muscle growth. Studies conducted in our lab demonstrate that Myostatin inhibits SC activation, proliferation, differentiation and self-renewal (142, 172, 176). The local production of Nitric oxide (NO), as one of inflammatory mediators, has also been shown to be essential for the SC activation (9).

1.1.3.2.2 Inflammatory response in muscle regeneration

Inflammatory response induced through acute myotrauma includes the sequential infiltration of monocytes into the damaged muscle, which consists mainly of neutrophils and a specific subpopulation of macrophages (238). Figure 1.7 demonstrates the time course of monocyte infiltration. These monocytes play a pivotal role in the regulation of muscle regeneration. Neutrophils dominate the initial

inflammatory response in muscle regeneration. Their number in the damaged area enhances dramatically within hours of muscle injury. Neutrophils release proteases, free radicals, and pro-inflammatory cytokines rapidly. Free radicals target necrotic myofibers to be phagocytosized. Proteases are responsible for the degradation of the debris, while pro-inflammatory cytokines amplify inflammatory response and recruit macrophages to the damaged area (289).

Following neutrophil infiltration, a subpopulation of CD68 expressing macrophages (CD68⁺ or ED1⁺ MPs) from circulation participate. They are infiltrating macrophages and are responsible for rapid removal of debris by phagocytosis. This population of macrophages displays a pro-inflammatory phenotype, and release pro-inflammatory cytokines and nitric oxide. Once the necrosis is over, another subpopulation of CD163 positive macrophages (CD163⁺ or ED2⁺ MPs) dominates. They are resident macrophages and do not phagocytose muscle debris. They can de-activate CD68⁺ macrophages by releasing anti-inflammatory cytokines such as IL-10 (181). IL-10 is a potent anti-inflammatory cytokine which terminates the inflammatory response and enables macrophages to shift phenotypes (196). ED2⁺ macrophages are associated with muscle regeneration (167, 182, 269).

In addition to phagocytosis, macrophages have a proregenerative role. They are essential for the activation, proliferation, and differentiation of SCs. During muscle regeneration, macrophages and SCs interact with each other to regulate muscle regeneration. Specifically, infiltrated macrophages secrete important mitogenic growth factors, which regulate SC function, such as transforming growth factor- β (TGF- β), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) (225). In addition, macrophages also protect SCs from apoptosis through direct contact (44). A previous study from Arnold *et al.* suggests that pro-inflammatory macrophages

promote myoblast proliferation via a TNF- α /IL-6-mediated mechanism and repress differentiation, whereas the anti-inflammatory macrophages stimulate myoblasts differentiation and fusion (13). Suppression of macrophages leads to incomplete regeneration (delayed SC proliferation and differentiation *in vivo*) and enhanced collagen deposition (241). In addition, depletion of circulating monocytes at the time of injury completely blocks muscle regeneration (13). Moreover, inhibition of anti-inflammatory macrophages leads to a small regenerating myofiber size (263) Since inflammatory cells promote both muscle injury and repair, the overall effect of inflammatory response on muscle regeneration depends on the magnitude of the inflammatory response.

Figure 1.7 *Relative time-course of monocyte infiltration and muscle membrane disruption.*

Neutrophils dominate the initial inflammatory response and the number of neutrophils increases dramatically within hours of muscle injury. Following neutrophil infiltration, a subpopulation of pro-inflammatory macrophages ($CD68^{+}$) are recruited. These macrophages release pro-inflammatory cytokines and nitric oxide to promote inflammatory reactions, and are mainly responsible for the phagocytosis of muscle debris. Once the necrotic tissue is cleared, pro-inflammatory macrophages undergo a phenotype switch. Anti-inflammatory macrophages ($CD163^{+}$) rapidly dominate and release anti-inflammatory cytokines to terminate the inflammatory response. Figure is modified from Schiaffino and Partridge (238).

1.2 TGF- β superfamily and Smad proteins

1.2.1 TGF- β superfamily and signaling pathways

The TGF- β superfamily is a large group of extra cellular growth factors, which are involved in autocrine, paracrine and endocrine regulation of diverse biological processes: including embryogenesis, immune and inflammatory response, as well as cellular processes including proliferation, differentiation, migration, and adhesion (130, 252). TGF- β superfamily members signal through a cell surface receptor complex and a group of intracellular signal transducers, called Smad proteins (Figure 1.8a) (134). Smad (small mother against decapentaplegic) proteins are a family of proteins identified to be homologous to the *Drosophila* proteins, encoded by gene, mothers against decapentaplegic (*MAD*) (223). Cell surface receptor complexes consist of two distinct trans-membrane serine/threonine kinases, type I and type II. Ligand binding induces the association of the two receptors, which further leads to the trans-phosphorylation of the C-terminal SSXS motif (a conserved Gly/Ser-rich sequence) of the type I receptor by the type II receptor (59). The activated receptor complex now recruits and phosphorylates the intracellular signaling mediators, namely Smad proteins. Activated Smad proteins then translocate into the nucleus to regulate transcription of target genes in a cell type-specific manner (93).

Smad proteins function as mediators of a short-circuit signaling pathway, transducing signals directly from cell surface receptors to the nucleus. In addition, Smad-independent pathways are also found to be involved in TGF- β family signaling, such as the Erk, JNK and p38 MAPK pathways. TGF- β also activates phosphatidylinositol-3-kinase (PI3K) and Rho-like GTPases, which have been shown to further regulate Smad activation (60, 121).

1.2.2 Structure and function of Smad proteins

To date, eight Smad proteins have been identified in mammals, which can be categorized into 3 subgroups: receptor-regulated Smads (R-Smads), a common Smad (Co-Smad), and inhibitory Smads (I-Smads). Smad proteins are characterized by the presence of a Mad homology domain-1 (MH1) and/or a Mad homology domain-2 (MH2), which are connected by a less-homologous linker region (Figure 1.8b). The MH1 domains of R-Smads and Co-Smads contain nuclear localization signals (NLS). The C-terminal of R-Smads has two serine residues, which can be phosphorylated by the activated TGF- β receptor complexes (79).

Five R-Smad proteins have been found in mammals (Smad1, Smad2, Smad3, Smad5 and Smad8). Smad2 and Smad3 are involved in the signaling of TGF- β -like ligands. Smad1, Smad5 and Smad8 are responsible for bone morphogenic protein (BMP)-like ligand signaling (71). More proteins have been found to mediate the interaction between R-Smads and receptor complexes (33). Smad anchor for receptor activation (SARA) is one of the proteins which is associated with the plasma membrane and recruits R-Smads to the TGF- β receptor complexes (274). In addition to SARA, R-Smads interact with a number of cytoplasmic and membrane bound adaptor proteins, which can also regulate R-Smad activation (33). Non-phosphorylated R-Smads are auto-inhibited through interactions between their own MH1 and MH2 domains in the cytoplasm (91). After activation, R-Smads undergo a conformational change and form heteromeric complexes with the Co-Smad, Smad4. Before activation, the NLS of R-Smads and the Co-Smad are both masked. Upon activation, the NLS is exposed, which triggers the nuclear translocation of R-Smads and the Co-Smad (291). However, results suggest that R-Smads can also translocate into the nucleus in a Smad4-independent manner in some cell lines, such as cancer cell lines (73).

Figure 1.8 TGF- β signaling and Smad proteins

(a) TGF- β canonical signaling pathway. Upon ligand binding, the cell surface receptor complex recruits and phosphorylates Smad3 or Smad2 proteins. After phosphorylation, Smad2/3 forms a heteromeric complex with the co-Smad, Smad4 and the complex translocates to nucleus to regulate expression of TGF- β superfamily target genes, through associating with a variety of co-activators and co-repressors. Figure is modified from ten Dijke and Hill (265). (b) The structure of Smad proteins, the essential intracellular signal mediators in the TGF- β canonical signaling pathway. Smads proteins contain domains of nuclear localization signal (NLS) and nuclear export sequence (NES). Figure is modified from Brown *et al.* (33).

a

b

Once in the nucleus, the R-Smads/Co-Smad complex regulates the transcription of down-stream genes by associating with a variety of co-associated proteins (33). The R-Smads/Co-Smad complex binds to conserved Smad binding elements (SBEs), a 5'AGAC 3' sequence, in the promoters of target genes through the MH1 domain (127). In addition to SBEs, Smad3 and Smad4 are also shown to bind GC-rich motifs (133). Since the interaction of R-Smads with promoters is not strong, R-Smads need additional DNA transcription factors to facilitate transcription of target genes, such as AP1, Ets and basic helix-loop-helix (bHLH) families of transcription factors (166). SBEs exist in high frequency in the mouse genome, thus in addition to enhancing the interaction between R-Smads and promoters, it is hypothesized that transcription factors or co-factors impart selectivity of R-Smads transcription activity (15, 71). Specifically, over fifty proteins have been identified to interact with R-Smads (33). For example, the AP1 family of transcription factors are well-studied proteins which interact with the Smad3/4 complex (108).

Inhibitory Smads (I-Smads), namely Smad6 and Smad7, inhibit TGF- β signaling by competing with R-Smads for receptor interacting or through directly binding to Smad4. Interestingly, I-Smads, which are activated by TGF- β superfamily signaling, can in turn block TGF- β superfamily signaling and thus provide a negative feedback mechanism (202). Smad7 is known to specifically inhibit TGF- β -like ligand signaling, while Smad6 specifically inhibits BMP-like ligands signaling (191). Previously, our lab has demonstrated that Myostatin, a member of the TGF- β superfamily, auto-regulates its own expression through a negative feedback mechanism involving Smad7 (75).

Recent studies add a new dimension to our understanding of the role of Smad proteins in regulating gene expression. Smad proteins regulate gene expression by controlling

the biogenesis of microRNAs (miRNA) (55-56, 271). miRNAs are small RNAs which regulate gene expression at the post-transcriptional level. miRNAs target the 3' untranslated region of specific mRNA, to repress translation or initiate the degradation of the target mRNA in a deadenylation-dependent mechanism (17). The biogenesis of miRNA is tightly regulated at various levels. miRNAs are first transcribed as primary miRNA (pri-miRNAs), which are then processed by the microprocessor complex (including Drosha, the RNase III enzyme and its cofactor DGCR8), to give rise to miRNA precursors (pre-miRNAs). Dicer, an RNase III enzyme, cleaves the pre-miRNA to mature miRNAs (129). In response to TGF- β and BMP4 treatment in primary pulmonary smooth muscle cells (PASMCs), Smad proteins have been shown to stimulate Drosha-mediated processing of pri-miRNAs through binding directly to a conserved 5'CAGAC3'motif (R-SBE) in the pri-miRNAs, which is similar to SBEs in the promoters of Smad target genes. The MH1 domain of Smads is required for this binding, however other regions, such as the MH2 domain, also are essential for miRNA processing (56).

1.2.3 Smad3-null mice

In humans, Smad2 and Smad4 are well-known as tumor suppressors. Both Smad2 and Smad4 mutations have been identified in a number of human cancers, such as pancreas, breast, ovary, head, neck, esophagus, colon, and lung cancers (222). However, to date no Smad3 mutation has been detected in any human cancer (33).

In mouse models, targeted mutagenesis of *Smad* genes was performed to help researchers understand the function of Smad proteins. Knockout of either the Smad2 or Smad4 gene in mice results in embryonic lethality (204, 283). This demonstrates

that both genes play non-redundant roles during embryonic development. However, in contrast, Smad3-null mice are viable and can survive to adulthood, which suggests distinct roles for these three Smad proteins in mouse embryonic development (54, 297, 308). Smad3-null mice provide an *in vivo* model to analyze the function of TGF- β superfamily members / Smad signaling in development and disease progression.

Three different Smad3-null mice strains have been developed thus far, each of which targets different *exons* of the *Smad3* gene (54, 297, 308). Zhu *et al.* developed Smad3-null mice via targeted disruption of *exon 2* of the *Smad3* gene (Smad3^{ex2/ex2} mice). Adult Smad3^{ex2/ex2} mice show distended abdomens and dilated large and small bowels. They gradually develop metastatic epithelial colorectal cancer and usually die between 4 and 6 months. In humans, mutation of TGF- β type II receptor, Smad2, or Smad4 are identified in colorectal cancers. This suggests a relationship between colorectal cancer and blockade of TGF- β signaling (308).

Smad3^{ex1/ex1} mice, which contain a disrupted *exon 1* of the *Smad3* gene, show malformed forelimbs. No major immune system defect was observed in Smad3^{ex1/ex1} mice. However, TGF- β failed to inhibit the proliferation of Smad3^{ex1/ex1} immune cells (splenocytes, activated B and T-cells). These cells are of lymphoid origin and show highest Smad3 expression. TGF- β -mediated inhibition of cytokines such as gamma interferon (IFN- γ), IL-2, IL-13 and IL-15 production in Smad3-null splenocytes is also blocked in Smad3^{ex1/ex1} mice (54).

Yang *et al.* developed Smad3-null mice by targeted disruption of *exon 8* of the *Smad3* gene (297). Smad3^{ex8/ex8} mice express a truncated SMAD3 protein, lacking 89 amino acids from the C-terminus, which normally contains the SSVS consensus phosphorylation site and an L3 loop. These domains are essential for the

phosphorylation of Smad3 by TGF- β receptors (159). Smad3^{ex8/ex8} mice have a major defect in immune function and typically die between 1 and 3 months. Infection (inflammatory lesions) was found in a number of organs, including the nasal mucosa, stomach, pancreas, colon and small intestine. Due to this inflammatory response, Smad3^{ex8/ex8} mice shows increased circulating neutrophils and monocytes. The susceptibility to infection in Smad3^{ex8/ex8} mice was attributed to the impaired regulation of mature leukocyte function by TGF- β . For example, Smad3^{ex8/ex8} thymocytes and peripheral T cells are completely resistant to the proliferation inhibitory effects of TGF- β . Moreover, mutant neutrophils display defective chemotactic response to TGF- β (297). Smad3^{ex8/ex8} mice also have significantly reduced thickness of the smooth muscle layer in the colon and reduced, or in some cases no interstitial cells of Cajal, which leads to colonic dilation in a 40% Smad3^{ex8/ex8} mice (278). Although Smad3^{ex8/ex8} mice do not die of cancer, recent study suggests that they are more susceptible to colon cancer triggered by bacteria. Epithelial cells in the colon of Smad3^{ex8/ex8} mice show increased proliferation and survival, which is due to increased expression of pro-inflammatory cytokines, pro-oncogenic and anti-apoptotic proteins, as well as enhanced activation of NF- κ B due to chronic inflammation (162).

TGF- β /Smad signaling plays a critical role in tissue fibrogenesis. Smad3^{ex8/ex8} mice are protected against skin fibrosis after radiation (74). Loss of Smad3 also protects against fibrosis following over-expression of TGF- β in lungs. In addition to skin and lung, lack of Smad3 also confers resistance to tissue fibrosis in the kidney, liver and intestine (144).

Although all three Smad3-null mice have smaller body size when compared to Wild-type littermates, there is phenotype disparity between Smad3-null mice with different

Smad3 gene exon disruptions. This difference suggests that specific domains of Smad3 protein might be involved in the regulation of different downstream targets (278).

1.3 Myostatin

1.3.1 Myostatin and its function in myogenesis

Myostatin (or growth and differentiation factor-8, GDF-8), a TGF- β superfamily member, is a potent negative regulator of skeletal muscle growth (120, 183). It is highly conserved across species, such as bovine (120), dog, mice, sheep (49) and humans (239). Targeted disruption of the *Myostatin* gene or inhibition of Myostatin expression leads to increased skeletal muscle growth, due to both muscle hyperplasia (increase in muscle fiber number) and hypertrophy (increase in muscle fiber size) (120, 183) (Figure 1.9a). Systemic over-expression of Myostatin in mouse causes muscle atrophy and cachectic-like muscle wasting (309). Myostatin over-expression results in reduced skeletal muscle mass, decreased muscle fiber size and myonuclear number (219).

Myostatin regulates muscle growth by inhibiting myoblasts proliferation (177, 266, 296), differentiation (118, 142, 177, 224), as well as negatively regulating SC activation and self-renewal (172, 176). In addition to SC functionality, Myostatin also regulates protein synthesis and degradation in skeletal muscle (155, 179, 284).

The molecular mechanisms behind Myostatin function in myogenesis are well studied. Studies performed in our laboratory and others have shown that Myostatin inhibits myoblasts proliferation by up-regulating the expression of p21, which is a Cdk2 inhibitor (177, 266). Myostatin also induces cyclin D1 degradation to down-regulate

Cdk4 activity (296). Progression of the cell cycle is regulated by a group of proteins termed cyclins and Cdks (cyclin-dependent kinases). The cyclin-Cdk complex initiates the phosphorylation of the retinoblastoma (Rb) family (tumor suppressor proteins) and abrogates their inhibitory activity on cell cycle progression (175, 247, 266). Inhibition of Cdk2 activity or degradation of cyclin D1 by Myostatin leads to cell cycle arrest in the G1-phase and inhibition of myoblast proliferation (177, 266). These studies suggest that Myostatin may inhibit myoblast proliferation through multiple mechanisms.

Previously, our lab has demonstrated that Myostatin negatively regulates myoblast differentiation by down-regulating *MyoD* expression (142). MyoD is a basic helix-loop-helix transcription factor, which is essential for specification of the myogenic lineage and initiation of myogenic differentiation. The association of Myostatin with its receptor complex induces the phosphorylation and activation of Smad3. The activated Smad3 translocates into nucleus and physically interacts with the bHLH domain of MyoD. The binding of Smad3 and MyoD interferes with MyoD and E protein (E12/E47) interaction, which is required for initiation of *MyoD* expression (142). Myogenin, another myogenic gene essential for myoblast differentiation, is also targeted by Myostatin (118, 224). Much like what is seen for the response to Myostatin, active Notch signaling inhibits myogenesis through down-regulating *MyoD* and *Myogenin* expressions (34). A recent study in human myoblasts, conducted by our lab, suggests that Myostatin activates the Notch signaling pathway to negatively regulate myoblast differentiation (177). Specifically, Myostatin promotes the expression of Notch downstream target genes, including *Hes1*, *Hes5*, and *Hey1*, to inhibit *MyoD* expression, possibly through Smad3/Notch-1 direct interaction.

Moreover, it was shown that blockade of Notch signaling through inhibitor treatment rescues Myostatin-mediated differentiation inhibition (177).

However, a study by Manceau *et al.* suggests a controversial function for Myostatin in the terminal differentiation of muscle progenitors during chick and mouse embryogenesis (164). During embryonic myogenesis, Myostatin activates *P21* and *MyoD* expression to inhibit the proliferation of muscle progenitors, whereas promote cell cycle arrest and terminal differentiation. Through regulating the balance between proliferation and differentiation of embryonic muscle progenitors, Myostatin plays an important role in maintaining the proportion of progenitor population and differentiated myofibers during development. Therefore, over expression of Myostatin in embryos results in remarkable muscle hypotrophy, due to a reduction in the muscle progenitor population during embryogenesis. However, loss of Myostatin in embryos delays the onset of the terminal differentiation program and stimulates continuous proliferation of progenitors, which leads to muscle hyperplasia. The different environment context between *in vivo* resident muscle progenitors and *in vitro* myoblasts may explain this different role of Myostatin in the regulation of myogenic differentiation (164).

In addition to regulating proliferation and differentiation of myoblasts, Myostatin also regulates SC functions. Studies in our lab demonstrate that Myostatin not only inhibits SC activation, through negatively regulating cell cycle progression from the G1 phase to S phase (172), but also inhibits SC self-renewal by signaling through Pax7 (176).

Myostatin is a potent anti-anabolic regulator of skeletal muscle. Studies conducted in our lab revealed that Myostatin promotes ubiquitin-mediated proteolysis to induce muscle wasting. The Akt-forkhead box protein O1 (FoxO1) pathway, but not NF- κ B,

was shown to be important in this regulation (179). Previous data, together with unpublished data from our lab also suggests that Myostatin treatment promotes the degradation of sarcomeric proteins by up-regulating the expression of two important muscle-specific ubiquitin E3 ligases, MuRF1 and Atrogin-1 (179), with Smad3 required for Myostatin-mediated up-regulation of Atrogin-1 (Sudarsanareddy *et al.*¹). On the other hand, Myostatin inhibits muscle protein synthesis by interfering with the Akt protein synthesis pathway, through blocking the phosphorylation of p70 S6 kinase and ribosomal S6 (284). Myostatin inhibits IGF-1-induced Akt activation in C2C12 myoblasts (192), whereas Myostatin-null mice show increased levels and activity of Akt and mTOR/S6K in muscle, which leads to enhanced protein synthesis in skeletal muscle (156).

A study in our lab has found that Myostatin-null mice display accelerated skeletal muscle regeneration and reduced scar tissue formation following injury (173). Fibroblasts are responsible for the formation of scar tissue in muscle regeneration. Myostatin has been shown to directly stimulate fibroblast proliferation (151). Therefore, the reduced fibrosis in Myostatin-null mice during muscle regeneration is not secondary to increased myofiber regeneration, but due to absence of Myostatin stimulatory effects on fibroblast growth (151). Consistent with this result, mRNA levels of several collagen genes were down-regulated in muscle from mice with post-natal inactivation of *Myostatin* (285).

¹ Sudarsanareddy *et al.* manuscript in preparation

Figure 1.9 Myostatin function and synthesis

(a) Mutation of the *Myostatin* gene in (i) bovine (Belgian Blue cattle) (120), (ii) mouse (183), (iii) dog (246), or (iv) human (child at 7 months of age) (239), leads to increased skeletal muscle growth. (b) Biosynthesis and processing of Myostatin protein: Myostatin is synthesized as a pre-proprotein (376 aa) which undergoes two proteolytical cleavages. In the first processing event, N-terminally located signal peptide (24 aa) is firstly cleaved. In the second processing event, RSRR (Arg-Ser-Arg-Arg) site at 264-267 aa is cleaved, which releases the N-terminally located latency-associated protein (LAP) and C-terminally located mature Myostatin. After processing, Myostatin dimer linked by disulfide bond associate with LAP in a non-covalent way as a latent complex. Figure is modified from Lee (147).

Figure 1.10 Regulation of Myostatin expression

Myostatin expression is under strict regulation. Studies conducted in our lab suggest that a couple of proteins have been shown to regulate Myostatin expression. MyoD, a critical myogenic regulatory factor, up-regulates Myostatin expression to promote myoblast withdrawal from cell cycle. Myostatin regulates its own expression through a Smad7-dependent negative feedback loop: Myostatin initiates the expression of inhibitory Smad7 and in turn Smad7 inhibits Myostatin promoter activity. Unpublished data demonstrates another potential negative auto-regulation of Myostatin expression: Myostatin initiates microRNA-27 (miRNA-27) through Smad3-dependent mechanism and miRNA-27 targets Myostatin mRNA and inhibits its expression.

1.3.2 Regulation of Myostatin expression

It is well established that the expression and activity of Myostatin are both under strict regulation to help maintain the balance between muscle growth and muscle wasting.

The expression of Myostatin is limited to several organs and tissues. It is mainly expressed in skeletal muscle, with low levels of *Myostatin* also seen in adipose and cardiac tissues (244). A study conducted in our lab indicates that MyoD, as a transcription factor, directly interacts with E-boxes in Myostatin promoter to regulate its expression (187). Moreover, a separate study from our lab has demonstrated that Myostatin regulates its own transcription through a Smad7-dependent negative feedback loop (75). A recent study suggests that Myostatin expression may be regulated at the post-transcriptional level by microRNA-27a/b (5). In addition, unpublished data from our lab reveals that Myostatin is a target of miRNA-27a/b and that Smad3 may play a role in miRNA-27a/b regulation of Myostatin expression. In support, loss of Smad3 leads to down-regulation of miRNA-27a/b expression, which contributes to higher expression of Myostatin in Smad3-null muscle (Arigela *et al.*²). The relationship between Myostatin, MyoD, Smad2/3, Smad7 and microRNA-27 is presented in Figure 1.10.

Like other TGF- β superfamily members, Myostatin is synthesized as an inactive full length protein (pre-proprotein) which is modified at the post-translational level (Figure 1.9b). After synthesis, a homodimer of the precursor proteins are linked together by disulfide bonds. The precursor protein undergoes two proteolytical cleavage events to generate mature Myostatin. In the first cleavage, an N-terminally located 24 amino acid signal peptide (SP), consisting of a hydrophobic core of amino acids in N-

² Arigela *et al.* manuscript in preparation

terminus, is removed to target the protein for secretion. In the second cleavage, the N-terminal latency-associated protein (LAP or propeptide, 26 kD) and a C-terminal mature protein (12.5 kD) are formed, following proteolytic processing at the RSRR site (Arg-Ser-Arg-Arg). The disulfide-linked C-terminal mature Myostatin is the active ligand. The furin family of proprotein convertases (PCs) is responsible for proteolytic processing of Myostatin (236). This proteolytic processing is believed to occur within cells (the Golgi apparatus) with mature Myostatin subsequently secreted into circulation. In serum, a mature Myostatin homodimer is non-covalently linked with its propeptide (LAP) to remain in a latent form. However, a new study suggests that unlike in serum, in skeletal muscle tissue, Myostatin is predominantly secreted as a Myostatin precursor protein rather than the mature Myostatin form. This precursor can then be cleaved extracellularly by furin proteases (10). Moreover, a study in our lab has revealed that during myogenesis, mature Myostatin negatively regulates furin protease transcription, suggesting the presence of an auto-regulatory loop during the proteolytic processing of mature Myostatin (178).

Myostatin levels are up-regulated during food deprivation, which results in the degradation of muscle tissue to produce amino acids for blood glucose maintenance (4). In addition, both Myostatin mRNA and protein levels are up-regulated in muscle biopsies from aged humans (around 70 years old) (148). Myostatin mRNA and protein levels are also elevated in a rat model of muscle disuse (141), mouse model of muscle denervation (301) and mice with chronic kidney disease. Inflammatory cytokine such as TNF- α also induces Myostatin expression through an NF- κ B-dependent pathway.

1.3.3 Regulation of Myostatin activity

In addition to Myostatin expression, the activity of Myostatin is also strictly regulated. A number of proteins, both in circulation and muscle tissue, have been shown to regulate Myostatin activity by limiting its access to cell surface receptors, in circulation or muscle tissue (146) (Figure 1.11).

Follistatin is a potent Myostatin antagonist and also binds to other TGF- β superfamily members (58, 201). In support, Follistatin-null mice have reduced diaphragm and intercostal muscle, consistent with increased Myostatin activity (169). In addition, the circulating Myostatin latent complex in mice or humans, has been shown to bind to Follistatin-related gene (FLRG), which further suggests that FLRG may be a negative regulator of Myostatin *in vivo* (94). Growth and differentiation factor-associated serum protein-1 (GASP-1) was also found to associate with the Myostatin latent complex in circulation. GASP-1 contains domains found in protease-inhibitory proteins, and in addition to mature Myostatin, it also directly binds to Myostatin propeptide (95).

Myostatin activity is also locally regulated by ECM components. Decorin is a small leucine-rich proteoglycan in the ECM. Studies including one conducted by our lab, demonstrate that decorin sequesters Myostatin into ECM by binding to mature Myostatin protein. As a result of this interaction Myostatin activity is thus inhibited (132, 189). Furthermore, *decorin* gene transfer improves muscle regeneration (150). *In vitro* experiments demonstrate that other ECM proteins, such as fibromodulin, fibronectin and laminin can also bind to Myostatin, suggesting additional ECM components which may regulate Myostatin activity (190). Latent TGF- β -binding proteins (LTBPs), are a group of proteins required for efficient folding and secretion of TGF- β superfamily members (221). Currently, research on the interaction between

LTBPs and Myostatin has remained controversial. Although a study has shown that LTBP-3, which is predominantly expressed in skeletal muscle, associates with Myostatin precursor protein to regulate its activity locally (10), a study conducted in our lab indicates that LTBPs do not interact with Myostatin as assessed through yeast two hybrid and co-immunoprecipitation techniques (178).

Modulating Myostatin level or Myostatin signaling has proved to be a potentially promising treatment for muscular diseases or muscle-related disorders. Antibody-mediated Myostatin inhibition attenuates muscle loss with aging (Sarcopenia) in mouse models (199). In addition, aged Myostatin-null mice showed improved cardiac function and metabolism (serum insulin and glucose levels) when compared to Wild-type mice (193). Furthermore, blocking Myostatin signaling by down-regulating the Myostatin receptor, ActRIIB, or through neutralizing Myostatin using a specific monoclonal antibody improves muscle function in *mdx* mice, the mouse model of Duchenne muscular dystrophy (DMD) (27, 65). Inhibition of Myostatin signaling by systemic injection of its propeptide also improves both bone and muscle healing in mice (90). Cachexia is a complication associated with chronic illnesses, such as cancer, and is characterized by the progressive loss of both muscle and fat mass, regardless of nutritional intake (12). Inhibition of Myostatin and related ligands by systemic administration of the Activin receptor extracellular domain potently decreases muscle wasting in cachexia (19). Moreover, inhibition of Myostatin by injecting an anti-Myostatin peptibody decreases muscle loss and circulating inflammatory cytokines in a mouse model of chronic kidney diseases (CKD) (302). Over-expression of a Myostatin inhibitor, exclusively in mice liver, profoundly improves muscle mass and function in *mdx* mice, which suggests that an inhibitor secreted by liver is enough to inhibit Myostatin function throughout the whole body (66).

Figure 1.11 Regulation of Myostatin activity

Myostatin activity is under strict regulation in both tissue and circulation. A number of proteins have been identified to be involved in the regulation of Myostatin activity. Follistatin-related gene (FLRG) and Growth and differentiation factor-associated serum protein-1/2 (GASP-1/2) have been shown to inhibit Myostatin activity in circulation. Extracellular matrix (ECM) components, such as decorin, associate with Myostatin, by which sequester Myostatin in ECM and inhibit its activity. Follistatin, an autocrine glycoprotein, is a potent Myostatin inhibitor and overexpression of follistatin in mouse leads to increased muscle mass.

1.3.4 Myostatin signaling pathways

1.3.4.1 Canonical signaling

Like other TGF- β superfamily members, Myostatin predominantly signals through Smad proteins, mainly Smad2/3 and Smad4. Studies show that Myostatin suppresses the expression of MyoD, Myogenin and MEF2 through Smad3, rather than Smad2 (142, 157-158), and Myostatin also activates Smad7 expression in a Smad3-dependent manner (75). The molecular mechanism by which Myostatin inhibits MyoD expression is well studied. Myostatin binds to its heteromeric cell surface receptor complex, consisting of activin type IIB receptor (ActRIIB), and TGF- β type I receptor (ALK4 or ALK5). This association induces the phosphorylation of Smad3. The now activated Smad3 then associates with Smad4, to allow for translocation into the nucleus to physically interact with the bHLH domain of MyoD. The association of Smad3 and MyoD interferes with MyoD and E protein (E12/E47) interaction on the *MyoD* promoter. The association of MyoD and E is required for MyoD regulation of its own expression (142, 157-158).

1.3.4.2 Non-canonical signaling

In addition to Smad-dependent signaling, other signaling pathways, including JNK, p38, ERK, and Akt signaling are also involved in Myostatin signaling (7, 104, 115, 214, 296).

Akt signaling plays a pivotal role in the regulation of skeletal muscle growth by controlling both protein degradation and protein synthesis (200). Myostatin regulates muscle protein metabolism through Akt signaling. In addition, studies in our lab have

demonstrated that Myostatin induces muscle wasting by down regulating Akt phosphorylation (179). Both *in vivo* and *in vitro* studies show that over-expression of Myostatin in mouse skeletal muscle decreases Akt phosphorylation (7). Furthermore, treatment of human myoblasts with Myostatin decreases the levels of phosphorylated Akt, as well as the phosphorylation of its downstream target p70S6 kinase. However, both Smad2 and Smad3 are required for this inhibition, as blockade of Smad2 or Smad3 by siRNA reduces Myostatin-mediated inhibition of myoblast differentiation (273).

Studies also suggest that Myostatin inhibits myogenesis by decreasing Akt signaling activity (273). Myostatin down-regulates cyclin D1 mRNA level, via Akt/Gsk3 β signaling pathway to inhibit proliferation (115). In addition, Myostatin inhibits myoblasts differentiation by interfering with Akt/TORC1/p70S6K signaling. Interestingly, blockade of the Akt/mTOR signaling pathway increases Smad2 phosphorylation induced by Myostatin, which suggests that blocking of Akt signaling amplifies Myostatin canonical signaling (273). In mouse C2C12 myoblasts, Myostatin induces cyclin D1 degradation through inhibiting Akt phosphorylation on Ser 473 and GSK-3 β phosphorylation on Ser 9, however, this inhibition appears to be ActRIIB-dependent, but not Smad3-dependent (296).

MAPK signaling pathways are key modulators of extracellular and growth factor signals to the nucleus. MAPK consists of three subfamilies, ERKs (extracellular signal-regulated kinases), JNKs (c-Jun N-terminal kinases) and p38 kinases. Myostatin has been previously shown to inhibit myoblast proliferation by activating the p38 MAPK pathway (214). In addition, the Erk1/2 MAPK pathway is also involved in Myostatin-mediated inhibition of muscle cell growth and differentiation (294). Moreover, Myostatin induces fibroblast proliferation by sequentially stimulating Smad

(specifically smad2 and smad3), p38 MAPK (mitogen-activated protein kinases) and Akt pathways (151). Thus, it is well established that Myostatin signals through multiple signaling pathways to elicit biological function, and there exists cross-talk between these signaling pathways to finely regulate Myostatin function.

1.3.4.3 Myostatin signaling is cell dependent

Growing evidence suggests that Myostatin regulation of cellular responses is cell-dependent. Myostatin inhibits myoblast proliferation by increasing p21 expression (266) or by decreasing Akt phosphorylation to down-regulate cyclin D1 expression (115, 296). On the other hand, Myostatin promotes muscle fibroblast proliferation through increasing both Akt and p38 phosphorylation (151).

1.4 Aims and objectives

TGF- β and Myostatin are well established as important regulators of skeletal muscle growth. Both growth factors have been shown to signal through a Smad2/3-dependent signaling pathway. However, the specific role of Smad3 in skeletal muscle growth is not clearly known. Therefore, the overall aim of this thesis is to systematically investigate the specific requirement for Smad3 in post-natal muscle growth using a Smad3-null mouse as model with the following aims:

- Characterization of the role of Smad3 in normal skeletal muscle growth and Satellite Cell biology
- Investigation of the role of Smad3 in the regulation of skeletal muscle regeneration
- Genome-wide transcript analysis of Smad3-null skeletal muscle to elucidate the underlying molecular mechanism for Smad3 function in muscle growth

Chapters 2 Materials and Methods

2.1 Materials

2.1.1 Oligonucleotides

All oligonucleotides used in this thesis were synthesized by Sigma (Singapore). Stock solutions (100 μ M) were stored at -20°C. Working concentrations of the primers were 2.5 μ M for quantitative real time PCR (RT-PCR) and 10 μ M for genotyping PCR and cloning PCR. Information about the oligonucleotides is listed in the Table 2.1.

Table 2.1 Oligonucleotides

Primer	Sequence (5'-3')	Usage
Smad3-p1	5'CCACTTCATTGCCATATG CCCTG3'	Genotyping
Smad3-p2	5'CCCGAACAGTTGGATTACACA 3'	Genotyping
Smad3-p3	5'CCAGACTGCCTTGGGAAAAGC3'	Genotyping
Myostatin-P1	5'GATGTGCTCTCACTTCCTTG3'	Genotyping
Myostatin-P2	5'CAGCCATGGTAGTAGACCG 3'	Genotyping
Myostatin-P3	5'TCTATCGCCTTCTTGACGAG3'	Genotyping
Atrogin-1-F	5'GCGTGTTCTCTGGCAACATA3'	RT-PCR
Atrogin-1-R	5'GAGAAGAGGTGCAGGGACTG3'	RT-PCR
Caveolin 3-F	5'GGATCTGGAAGCTCGGATCAT3'	RT-PCR
Caveolin 3-R	5'TCCGCAATCACGTCTTCAAAAT3'	RT-PCR
Coll α 1-F	5'AGACATGCTCAGCTTTGTGGATAC3'	RT-PCR
Coll α 1-R	5'CGTACTGATCCCGATTGCAAAT3'	RT-PCR
Coll α 2-F	5'AGACATGCTCAGCTTTGTGGATAC3'	RT-PCR
Coll α 2-R	5'CGTACTGATCCCGATTGCAAAT3'	RT-PCR
Col3 α 1-F	5'ACGTAGATGAATTGGGATGCAG3'	RT-PCR

Col3 α 1-R	5'GGGTTGGGGCAGTCTAGTG3'	RT-PCR
Col4 α 1-F	5'CTGGCACAAAAGGGACGAG3'	RT-PCR
Col4 α 1-R	5'ACGTGGCCGAGAATTTACACC3'	RT-PCR
Dlat-F	5'TCCCTCCGCATCAGAAGGTT3'	RT-PCR
Dlat-R	5'CCAACTGGAACATCTCTGGTC3'	RT-PCR
eMyHC-F	5'TGGAGAACGACAAGCAACAG3'	RT-PCR
eMyHC-R	5'GGCCCTCTCTGCCTCTATCT3'	RT-PCR
Fmod-F	5'AGCAGTCCACCTACTACGACC3'	RT-PCR
Fmod-R	5'CAGTCGCATTCTTGGGGACA3'	RT-PCR
Gadd45g-F	5'GGGAAAGCACTGCACGAACT3'	RT-PCR
Gadd45g-R	5'AGCACGCAAAAGGTCACATTG3'	RT-PCR
GAPDH-F	5'ACAACTTTGGCATTGTGGAA3'	RT-PCR
GAPDH-R	5'GATGCAGGGATGATGTTCTG3'	RT-PCR
IGF-1-F	5'TGCTCTTCAGTTCGTGTG3'	RT-PCR
IGF-1-R	5'ACATCTCCAGTCTCCTCAG3'	RT-PCR
Mup1-F	5'CCCAGAGAGTATATAAGGACAAGCAAAGG3'	RT-PCR
Mup1-R	5'AGTATGCCATTCCCCATTAATCTTTTCTAC3'	RT-PCR
Mup2-F	5'ATTAATGGGGAATGGCATACTA3'	RT-PCR
Mup2-R	5'ATTAATGGGGAATGGCATACTA3'	RT-PCR
MuRF1-F	5'CATGTCTGGAGGTCGTTTCCG3'	RT-PCR
MuRF1-R	5'GCTGAGGTTCTGTCTGCGG3'	RT-PCR
MyHC I-F	5'GCTGGCACTGTGGACTACAA3'	RT-PCR
MyHC I-R	5'CTTTCTTTGCCTTGCCTTTG3'	RT-PCR
MyHC IIB-F	5'CAAGTCATCGGTGTTTGTGG3'	RT-PCR
MyHC IIB-R	5'GGCCATGTCCTCAATCTTGT3'	RT-PCR
MyHC IIX-F	5'CACGCTGGATGCTGAGATTA3'	RT-PCR
MyHC IIX-R	5'AGGTGCAGCTGAGTGTCTT3'	RT-PCR
MyoD-F	5'GACAGGGAGGAGGGGTAGAG3'	RT-PCR
MyoD-R	5'TGCTGTCTCAAAGGAGCAGA3'	RT-PCR
Myostatin-F	5'AGTGGATCTAAATGAGGGCAGT3'	RT-PCR
Myostatin-R	5'GTTTCCAGGCGCAGCTTAC3'	RT-PCR
NRF-1-F	5'GTAGCGCAGCCGCTCTGAGG3'	RT-PCR
NRF-1-R	5'TCCCCCAGCCTGGTTTCCCC3'	RT-PCR

P57-F	5'CGAGGAGCAGGACGAGAATC3'	RT-PCR
P57-R	5'GAAGAAGTCGTTTCGCATTGGC3'	RT-PCR
Pdk4-F	5'AGGGAGGTCGAGCTGTTCTC3'	RT-PCR
Pdk4-R	5'GGAGTGTTCACTAAGCGGTCA3'	RT-PCR
Pkm2-F	5'GCCGCCTGGACATTGACTC3'	RT-PCR
Pkm2-R	5'CCATGAGAGAAATTCAGCCGAG3'	RT-PCR
S100A8-F	5'AAATCACCATGCCCTCTACAAG3'	RT-PCR
S100A8-R	5'CCCACT TTTATCACCATCGCAA3'	RT-PCR
S100A9-F	5'ATACTCTAGGAAGGAAGGACACC3'	RT-PCR
S100A9-R	5'TCCATGATGTCATTTATGAGGGC3'	RT-PCR
SHP-2-F	5'ATGACATGGCGGAGATGGTTT3'	RT-PCR
SHP-2-R	5'GGGTTACTCTTACTGGGCCTT3'	RT-PCR
Smad3-F	5'GCACAGCCACCATGAATTAC3'	RT-PCR
Smad3-R	5'GGAGGTAGAACTGGCGTCTC3'	RT-PCR
TFAM-F	5'GGCGCTGTTCCGGGGAATGT3'	RT-PCR
TFAM-R	5'TCTGCCGGGCCTCCTTCTCC3'	RT-PCR
β_1 D integrin-F	5'CATCCCAATTGTAGCAGGCG3'	RT-PCR
β_1 D integrin-R	5'GAGACCAGCTTTACGTCCATAG3'	RT-PCR
ActRIIB-F	5'GAGGATCCGTCCGGGCGAGGGGAGGCT3'	Cloning
ActRIIB-R	5'GGATCCTCACGTGAGCAGGGTGGGGGC3'	Cloning
shSmad3-1-top	5'GATCCACTTTCTACTGCCACTTGGTTCAAGA GACCAAGTGGCAGTAGAAAGTTTA3'	shRNA
shSmad3-1-bottom	5'AGCTTAACTTTCTACTGCCACTTGGTCTCTT GAACCAAGTGGCAGTAGAAAGTG3'	shRNA
shSmad3-2-top	5'GATCCGTTCTCCAGAGTTAAAAGCTTCAAGA GAGCTTTTAACTCTGGAGAACTTA3'	shRNA
shSmad3-2-bottom	5'AGCTTAAGTTCTCCAGAGTTAAAAGCTCTCT TGAAGCTTTTAACTCTGGAGAACG3'	shRNA

2.1.2 Primary antibodies

Information about the primary antibodies used in this thesis is listed in the Table 2.2.

Table 2.2 Primary antibodies

Antibodies	Source	Catalog No.	Dilution	Usage
Akt1/2/3	Santa Cruz	SC-8312	1:3000	Western blot
Cdk2	Santa Cruz	SC-6248	1:400	Western blot
cyclin-E	Santa Cruz	SC-481	1:400	Western blot
FoxO1	Santa Cruz	SC-11350	1:1000	Western blot
Myf-5	Santa Cruz	SC-302	1:400	Western blot
MyoD	Santa Cruz	SC-304	1:400	Western blot
Myogenin	Santa Cruz	SC-576	1:400	Western blot
MyHC	DSHB	MF20-a	1:2000	Western blot
MyLC	DSHB	T14	1:5000	Western blot
p21	BD Biosciences	556430	1:400	Western blot
p-FoxO1	Santa Cruz	SC-101681	1:5000	Western blot
p-Akt (Thr 308)	Santa Cruz	SC-16646-R	1:3000	Western blot
p-Akt (Ser 473)	Santa Cruz	SC-7985-R	1:3000	Western blot
Retinoblastoma	BD Biosciences	554136	1:100	Western blot
Tubulin	Sigma	T9026	1:10,000	Western blot
Ubiquitin	Santa Cruz	SC-8017	1:1000	Western blot
Pax7	DSHB	PAX7	1:100	Immunofluorescence
MyoD	Santa Cruz	SC-304	1:100	Immunocytochemistry
MyoD	BD Biosciences	554130	1:25	Immunohistochemistry
Mac-1	BD pharmingen	550282	1:100	Immunohistochemistry
Embryonic MyHC	DSHB	F1.652-C	1:100	Immunohistochemistry
Type I MyHC	DSHB	A4.840-a	1:25	Immunohistochemistry
Type IIA MyHC	DSHB	2F7-C	1:25	Immunohistochemistry
Type IIB MyHC	DSHB	10F5-C	1:25	Immunohistochemistry
Type IIX MyHC	DSHB	6H1-C	1:25	Immunohistochemistry

2.1.3 Solutions

Table 2.3 TAE buffer

Tris-acetate	40 mM
EDTA (pH 8.0)	2 mM

Table 2.4 TE buffer

Tris-HCl	10 mM
EDTA (pH 8.0)	1 mM

Table 2.5 HCl (5 M)

Concentrated HCl	42 ml
Top up with Milli-Q water to	100 ml

Table 2.6 KOH (1 M)

KOH	5.61g
Top up with Milli-Q water to	100 ml

Table 2.7 Scott's tap water

Sodium Bicarbonate	2 g
Magnesium Sulphate	20 g
Thymol	1 Crystal
Top up with Milli-Q water to	1000 ml

Table 2.8 Van Giesen solution

1% (g:ml) aqueous acid fuchsin	10 ml
Saturated picric acid	90 ml
Concentrated HCl	0.25 ml

Table 2.9 Protein lysis buffer

1 M Tris-HCl pH 7.5	0.5 ml
5 M NaCl	0.5 ml
0.5 M EDTA	0.1 ml
IGEPAL [®] CA-630	10 μ l
Complete protease inhibitor	½ tablet
Top up with Milli-Q water to	10 ml

Table 2.10 RIPA buffer

Aprotinin	0.4 ml
700 mM NaVO ₄	30 μ l
500 mM NaF	1.4 ml
0.5 M EDTA	140 μ l
50 x protease inhibitor stock	400 μ l
Top up with Milli-Q water to	14 ml
100 mM PMSF (just before using)	140 μ l

Table 2.11 Stacking protein gel (4%)

30% Acrylamide	1 ml
Tris-HCl pH 6.8	750 μ l
10% SDS	60 μ l
10% APS	60 μ l

TEMED	10 μ l
Milli-Q water	4.1 ml

Table 2.12 Resolving protein gel (10%, for proteins of low molecular weights)

30% Acrylamide	6.7 ml
Tris-HCl pH 8.8	7.5 ml
10% SDS	200 μ l
10% APS	200 μ l
TEMED	10 μ l
Milli-Q water	5.4 ml

Table 2.13 Resolving protein gel (6%, for proteins of high molecular weights)

30% Acrylamide	4 ml
Tris-HCl pH 8.8	7.5 ml
10% SDS	200 μ l
10% APS	200 μ l
TEMED	20 μ l
Milli-Q water	8.1 ml

Table 2.14 Tris-glycine SDS running buffer

Tris	30 g
Glycine	18.8 g
10% SDS	10 ml
Top up with Milli-Q water to	1000 ml
Store at room temperature	

Table 2.15 Western blot transfer buffer

Tris	15.1 g
Glycine	75 g
Methanol	1000 ml
Top up with Milli-Q water to	5000 ml
Store at 4°C	

Table 2.16 TBS-T

1 M Tris-HCl pH 7.5	50 ml
5 M NaCl	30 ml
TWEEN-20	1 ml
Top up with Milli-Q water to	1000 ml

Table 2.17 PVP blocker

Polyvinylpyrrolidone (PVP)	1 %
Polyethylene glycol (PEG)	1 %
Bovine serum Albumin (BSA)	0.3 %
top up with TBS-T to	100 ml
Sterile filter before use, store at 4°C	

Table 2.18 MOPS buffer (10 x)

MOPS	41.9 g
1 M Sodium acetate	50 ml
0.5 M EDTA	10 ml
pH to 7	
Top up with Milli-Q water to	500 ml
Sterile filter and store at 4 °C, protected from light	

Table 2.19 RNA loading dye (2 x)

MOPS (10 x)	10%
deionised formaldehyde	20%
deionised formamide	50%
bromophenol blue	0.02%
glycerol	5%
EDTA (pH 8.0)	1 mM
ethidium bromide	40 µg/ml

Table 2.20 DNA loading dye (10 x)

ficoll	15%
bromophenol blue	0.25%
SDS	1%
EDTA (pH 8.0)	1 mM

Table 2.21 Carrageen λ (0.35%)

carrageen λ	0.175 g
Top up with PBS	50 ml

Table 2.22 Stock buffer for hydroxyproline assay

citric monohydrate	50 g
glacial acetic acid	12 ml
sodium acetate trihydrate	120 g
NaOH	34 g
pH to 6.0	
Top up with Milli-Q water to	1000 ml

Table 2.23 Chloramine T solution

chloramine T	2.64 g
Milli-Q water	11.6 ml
Stock buffer for hydroxyproline assay	29 ml
methyl cellosolve	17.4 ml

Table 2.24 Phenolphthalein solution (1%)

phenolphthalein	1 g
95% ethanol	100 ml

Table 2.25 Sodium borate buffer (0.1 M, pH 8.7)

sodium tetraborate	38.2 g
pH to 8.7	
Top up with Milli-Q water to	1000 ml

Table 2.26 Sodium thiosulfate (3.6 M)

sodium thiosulfate	89.3 g
Top up with Milli-Q water to	100 ml

Table 2.27 Ehrlich's Reagent

Concentrated sulfuric acid	37.4 ml
alcohol	400 ml
p-dimethylaminobenzaldehyde	120 g

2.1.4 Enzymes

Information of enzymes used in this thesis is listed in Table 2.28. Enzymes were used according to the manufacturer's instruction.

Table 2.28 Enzymes

Eyzmes	Source
Proteinase K	Promega
RNase H	Bio-Rad (iScript™ cDNA Synthesis Kit)
Reverse transcriptase	Bio-Rad (iScript™ cDNA Synthesis Kit)
<i>Taq</i> DNA Polymerase	Ferments
T4 DNA ligase	Invitrogen

2.2 Methods

2.2.1 Genotype analysis

Genotype analysis was performed according to Ge *et al.*(81). A 0.2×0.2 cm² section of each ear was digested in 300 µl of tissue lysis buffer containing 10 mM Tris-HCl (pH 8.0), 100 mM EDTA, 0.5% SDS and 100 µg/ml Proteinase K (Promega) for 2 h at 56°C and centrifuged at 13,000 rpm at room temperature for 10 min. The supernatant was transferred to a new tube and 500 µl isopropanol and 1 ml ice-cold 70% ethanol was added. The mixture was centrifuged at 13,000 rpm at room temperature for 10 min. The supernatant was decanted and DNA pellet was washed with 2 ml of 70% ethanol. After air drying, the pellet was re-suspended in 100 µl of 10 mM Tris-HCl (pH 8.0) for 5 min at 65°C. PCR was performed to identify Wild-type, heterozygous and null mice using the oligonucleotides as listed in Table 2.1 Reaction conditions were as follows: 3 min at 94°C and 40 amplification cycles (20 s at 94°C, 20 s at 60°C, and 20 s at 72°C).

PCR products were separated in 1.5% agarose gel (UltraPure™, Invitrogen) in 1 x TAE running buffer.

Details of the other experiment methods used in this thesis are included in Chapter 3-5.

Chapter 3 Smad3 signaling is required for satellite cell function and myogenic differentiation of myoblasts

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Smad3 signaling is required for satellite cell function and myogenic differentiation of myoblasts

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Abstract

TGF- β and Myostatin are the two most important regulators of muscle growth. Both growth factors have been shown to signal through a Smad3 dependent pathway. However to date, the role of Smad3 in muscle growth and differentiation is not investigated. Here we demonstrate that Smad3-null mice have decreased muscle mass and pronounced skeletal muscle atrophy. Consistent with this we also find increased protein ubiquitination and elevated levels of the Ubiquitin E3 ligase MuRF1 in muscle tissue isolated from Smad3-null mice. Loss of Smad3 also led to defective Satellite Cell (SC) functionality. Smad3-null SCs showed reduced propensity for self-renewal, which may lead to a progressive loss of SC number. Indeed, we did find decreased SC number in skeletal muscle from Smad3-null mice showing signs of severe muscle wasting. Further *in vitro* analysis of primary myoblast cultures identified that Smad3-

null myoblasts have impaired proliferation, differentiation and fusion, resulting in the formation of atrophied myotubes. A search for the molecular mechanism confirmed that loss of Smad3 results in increased Myostatin expression in Smad3-null muscle and myoblasts. Given that Myostatin is a negative regulator, we hypothesize that increased Myostatin levels are responsible for the atrophic phenotype in Smad3-null mice. Consistent with this theory, inactivation of *Myostatin* in Smad3-null mice rescues the atrophy phenotype.

3.1 Introduction

The ability of skeletal muscle to regenerate is due to availability of adult muscle stem cells, termed Satellite Cells (SCs) (22). SCs are quiescent cells that are located between the basal lamina and sarcolemma. Upon injury to skeletal muscle, SCs are activated to enter the cell cycle and as a result rapidly proliferate to give rise to myoblasts, which then differentiate and fuse to give rise to multinucleated nascent muscle fibers (89). At the same time, some of the myoblasts re-enter the quiescent phase to self renew the SC population. Among several growth factors, Myostatin (GDF8) has been shown to be a profound regulator of skeletal muscle during prenatal and postnatal growth. Disruption of the *Myostatin* gene or inhibition of Myostatin expression in mice leads to increased skeletal muscle growth, which is due to both muscle hyperplasia (increase in muscle fiber number) and hypertrophy (increase in muscle fiber size) (120, 184). In contrast, over-expression of Myostatin in mice results in reduced skeletal muscle mass, decreased muscle fiber size and myonuclear number (219), moreover systemic over-expression of Myostatin in adult mice leads to the

development of cachectic-like muscle wasting (309). Myostatin functions by inhibiting myoblast proliferation and differentiation (266) as well as negatively regulating SC activation and SC self-renewal (172, 176). In addition to Myostatin, TGF- β has also been shown to inhibit myogenesis (157-158). Mechanistically the Smad2/3 pathway plays a significant role in Myostatin and TGF- β signaling and inhibition of myogenesis (157-158). Specifically, Smad3 is required for the inhibition of myoblast differentiation by Myostatin (142) and for the activation of fibroblast proliferation by Myostatin (151). The Smad3-dependent pathway begins when activated Myostatin receptors (Activin type IIB receptor/ALK5) form heterotetrameric complexes, which then recruit, phosphorylate and activate the receptor-regulated SMADS (R-Smads). The now activated Smad3, complexes with Smad4 and then translocates to the nucleus to activate/repress gene expression necessary for muscle growth and differentiation. Although Smad3 is a key signaling molecule/transcription factor, its role in SC biology is not studied in greater detail. Here we make use of Smad3-null mice and show that Smad3 is essential for normal skeletal muscle growth, as the absence of Smad3 promotes dramatic skeletal muscle atrophy due to defective SC functionality, impaired *in vitro* myogenesis and enhanced skeletal muscle protein degradation. Interestingly, loss of Smad3 results in the up-regulation of Myostatin expression. Furthermore the elevated level of Myostatin plays a key role in the development of the skeletal muscle atrophy observed in Smad3-null mice.

3.2 Materials and Methods

3.2.1 Animal care

Heterozygote Smad3-null mice were kindly gifted by Prof. Walter Wahli (University of Lausanne, Lausanne, Switzerland), which were bred and genotyped according to the previously published protocol (297). Heterozygote Myostatin-null mice were a gift from Prof. Se-Jin Lee (The Johns Hopkins University, Baltimore, MD) and were genotyped according to previously published reports (152, 183). Mice were maintained on standard chow diet at a constant temperature (20°C) under a 12 h/12 h artificial light/dark cycle with unlimited access to water. All experiments were performed according to the approved protocols of the institute animal ethics committee (IACUC), Singapore.

3.2.2 Cell culture

C2C12 myoblasts were cultured in proliferation medium, which consisted of Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen). For differentiation, C2C12 cells were plated at a density of 25,000cells/cm². After an overnight attachment period, C2C12 cells were induced to differentiate in low serum media (DMEM with 2% Horse Serum [HS, Invitrogen]). Primary myoblasts were cultured from hind-limb muscles using a modified method of Partridge TA (210). Briefly, hind-limb Muscles were excised, minced and then digested in 0.2% collagenase type 1A for 90 min. Fibroblasts were removed by pre-plating the cells on uncoated plates for 3 h. Adhered myoblasts were cultured on 10% Matrigel (BD Biosciences) coated plates and were maintained in

proliferation medium, which consists of DMEM with 20% FBS, 10% HS, and 1% Chicken Embryo Extract (CEE) at 37 °C/5% CO₂. Primary myoblast differentiation was induced as described above. For the neutralization of Myostatin in primary culture, the isolated primary myoblasts were seeded at the density of 25,000 cells/cm² in growth media on 10% Matrigel-coated 6-well plates. After overnight attachment, primary myoblasts were induced to differentiate with (0 h + 3 µg/ml soluble ActRIIB) or without soluble ActRIIB (0 h + 0 µg/ml soluble ActRIIB) for 48 h. For the treatment of proteasome inhibitor, primary myotubes (48 h) were incubated for 10 h with epoxomicin (48 h + 100 nM epox) (Sigma, US) or without epoxomicin (48 h + 0 nM epox).

3.2.3 Proliferation assay

Myoblast proliferation was assessed as described previously (266). Briefly, primary myoblasts or C2C12 cells were seeded on 96 well plates at a density of 1,000 cells per well in proliferation medium. Myoblasts were subsequently grown and fixed at regular 12 h intervals. Proliferation was assessed using the methylene blue photometric end-point assay as previously described (266).

3.2.4 Determination of SC number on single muscle fibers

Single muscle fibers from *M. gastrocnemius* muscle were isolated by the method of Rosenblatt *et al.* (227). Isolated muscle fibers were placed in four-well Lab-Tek chamber slides (Nunc), fixed in 4% paraformaldehyde for 10 min and rinsed in PBS. The fibers were then permeabilized in 0.5% TritonX-100 in PBS and blocked with 10% normal goat serum (NGS) and 0.35% carrageenan λ (c λ) in PBS for 1 h. Fibers were

incubated with mouse anti-Pax7 antibody (1:100; Developmental Studies Hybridoma Bank; DSHB) in 5% NGS and 0.35% c λ in PBS overnight, followed by incubation with the biotinylated sheep anti-mouse IgG (1:300; GE Healthcare Life Sciences) in 5% NGS and 0.35% c λ in PBS for 1 h. After 3 x TBS-T washes, fibers were incubated with the streptavidin conjugated Alexa Fluor 488 (1:400; Molecular Probes) in 5% NGS and 0.35% c λ in PBS for 1 h, counterstained with DAPI (1:1000; Molecular Probes) and mounted with ProLong Gold antifade reagent (Invitrogen). Immunofluorescence images were captured using the Carl Zeiss LSM 510 META laser scanning confocal microscope and LSM 510 META software, with a Zeiss Plan-Neofluar 10 $\times$ /0.30 objective (Carl Zeiss MicroImaging, Inc.).

3.2.5 Pax7/MyoD immunocytochemistry

Primary myoblasts were fixed with 70% ethanol: formaldehyde: acetic acid (20:2:1) for 30 sec and rinsed three times with PBS. Cells were permeabilized in 0.1% TritonX-100 in PBS and washed with PBS. Cells were blocked with 5% normal goat serum (NGS), 5% normal sheep serum (NSS) and 0.35% carrageenan λ (c λ) in PBS and incubated with mouse anti-Pax7 antibody (1:100; Developmental Studies Hybridoma Bank; DSHB) and rabbit anti-MyoD antibody (1:100; Santa Cruz) in 5% NGS and 0.35% c λ in PBS overnight at 4 °C. After washing with TBS-T for three times, myoblasts were incubated for 1h with biotinylated sheep anti-mouse IgG (1:300; GE Healthcare Life Sciences) in PBS containing 5% NGS and 0.35% c λ and then for 1h in streptavidin conjugated Alexa Fluor 488 (1:400; Molecular Probes) and Alexa Fluor 546 conjugated goat anti-rabbit IgG (IgG) (1:300; Molecular Probes) in 5% NGS, 5% NSS and 0.35% c λ in PBS. After washing in TBS-T twice, myoblasts were stained with DAPI (1:1000; Molecular Probes) and mounted with ProLong Gold

antifade reagent (Invitrogen). Immunofluorescence images were captured using the Carl Zeiss LSM 510 META laser scanning confocal microscope and LSM 510 META software, with a Zeiss Plan-Neofluar 10×, and 20× objectives (Carl Zeiss MicroImaging, Inc.). For single muscle fibers, isolated muscle fibers were cultured in four-well Lab-Tek Permanox chamber slides (Nunc) coated with 10% Matrigel (BD Biosciences). After 144h of culturing, primary myoblasts which migrated from single muscle fibers were fixed and immunostained with Pax7 and MyoD antibodies as described above.

3.2.6 Hematoxylin and Eosin (H & E) staining

H & E staining on cell cultures was performed according to McFarlane *et al.* (176). Primary cultures were grown on Thermanox coverslips with 10% Matrigel coating. Myotubes were fixed at 24 h intervals and stained with Gill's hematoxylin followed by 1% eosin (Merck). For H & E staining on skeletal muscle sections, *M. tibialis anterior* (TA) muscles were covered with OCT (Optimal Cutting Temperature) compound and then frozen in isopentane cooled with liquid nitrogen. 10 µm transverse sections were cut and mounted on slides for H & E staining. Images were captured using the Leica CTR 6500 microscope, equipped with the Leica DFC 310 FX camera and Image Pro Plus software (Media Cybernetics, Bethesda, MD).

3.2.7 Western blot

Protein extracts from Myoblasts were made by lysing myoblasts in protein lysis buffer (50 mM Tris, pH 7.6, 250 mM NaCl, 5 mM EDTA, 0.1% P-40, complete protease inhibitor cocktail from Roche Molecular Biochemicals). Skeletal muscle protein

lysates were prepared by homogenizing muscles in RIPA buffer with EDTA, PMSF, aprotinin, NaF and NaVO₄. Equal amount of proteins were resolved by SDS-PAGE (4-12% gradient, Invitrogen) and transferred to a nitrocellulose membrane. The membranes were blocked in 5% milk in TBS-T, and then incubated with primary and secondary antibodies in 5% milk in TBS-T. Immunoreactivity was detected using Western Lightning™ Chemiluminescence Reagent Plus (PerkinElmer). Band density was analyzed using the Quantity One imaging software (Bio-Rad). For Retinoblastoma phosphorylation analysis proteins were separated by 6% SDS-PAGE. Information of the antibody sources and dilutions used in this chapter are listed in Table 2.2.

3.2.8 RNA preparation and quantitative real time PCR analysis

Total RNA was isolated using TRIzol reagent (Invitrogen) and RNA integrity was monitored by electrophoresis. cDNA was synthesized according to iScript cDNA Synthesis kit (Bio-Rad). Quantitative real time PCR reactions were carried out in triplicate using SsoFast EvaGreen supermix (Bio-Rad) and the CFX96 Real Time System (Bio-Rad). Gene expression levels were normalized against that of *Gapdh*. Fold change of gene expression was calculated using the delta-delta Ct method. The sequences of primers used in this chapter are listed in Table 2.1.

3.2.9 Establishment of stable shSmad3 and control C2C12 cell lines

Two different sets of short hairpin RNA (shRNA) sequences were designed to specifically target the mouse *Smad3* gene using the online software, RNAi finder and RNAi converter, available at the Applied Biosystems website (http://www.ambion.com/techlib/misc/siRNA_finder.html, and

http://www.ambion.com/techlib/misc/psilencer_converter.html): 5'-GAT CCA CTT TCT ACT GCC ACT TGG TTC AAG AGA CCA AGT GGC AGT AGA AAG TTT A-3' (sense) and 5'-AGC TTA AAC TTT CTA CTG CCA CTT GGT CTC TTG AAC CAA GTG GCA GTA GAA AGT G-3' (anti-sense) for shSmad3-1 and 5'-GAT CCG TTC TCC AGA GTT AAA AGC TTC AAG AGA GCT TTT AAC TCT GGA GAA CTT A -3' (sense) and 5'- AGC TTA AGT TCT CCA GAG TTA AAA GCT CTC TTG AAG CTT TTA ACT CTG GAG AAC G -3' (anti-sense) for shSmad3-2. The oligo's were annealed and ligated into the pSilencer vector according to protocol recommended by the manufacturer (Applied Biosystem, USA). Sequence verified constructs were selected and transfected into C2C12 myoblasts using Lipofectamine 2000 (Invitrogen). The stable cell lines were selected by continued puromycin treatment for a period of 20 days. A negative control pSilencer vector that expresses a hairpin siRNA with limited homology to any known sequences in the human, mouse, and rat genomes databases was used. Negative control cells were maintained simultaneously, under the same conditions as described above.

3.2.10 Flow cytometry

Control and shSmad3 C2C12 myoblasts were cultured in proliferation medium and harvested. 1-3 million cells were counted and centrifuged at 1000xg for 5 min. After washing with cold PBS, cells were transferred to 10 ml glass tubes and washed again. Supernatant was carefully aspirated and 1 ml of ice-cold 70% ethanol was slowly added while vortexing at low speed. Fixed cells were stored at -20 °C until staining. Cells were centrifuged at 1000xg for 5 min and 70% ethanol was carefully aspirated. While vortexing at low speed, 1 ml of staining mixture (0.1 mg/ml Propidium iodide and 0.05 mg/mL Ribonuclease) was added slowly. After 45 min of incubation at 4°C,

cells were washed twice with cold PBS. 1 ml of cold PBS was added and cells were transferred to FACS tubes and examined for propidium iodide fluorescence using a flow cytometer (FACSCalibur, Becton-Dickinson) and analyzed using ModFit software (Becton-Dickinson).

3.2.11 Purification of the recombinant extracellular domain of mouse ActRIIB

The extracellular domain of mouse ActRIIB cDNA was amplified by PCR and cloned in the pET-16b protein expression system (Novagen) according to the manufacturer's protocol (Novagen). The construct was transformed into BL21 bacterial strain. The soluble ActRIIB protein was expressed and purified according to Sharma *et al.* (244). The biological activity of the recombinant soluble ActRIIB was measured by myoblast proliferation assay.

3.2.12 Statistical analysis

Statistical significance was performed using one-way ANOVA and the two-tailed Student's T test and results were considered significant at $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***). Results were expressed as mean $\pm$ SEM (standard error of mean).

3.3 Results

3.3.1 *Smad3*-null mice demonstrate pronounced skeletal muscle atrophy

Smad3-null mice had an overall reduction in body size, however, there was no significant decrease in the muscle weights when calculated as a percentage of body weight (Figure 3.1a-c) (Table 3.1). To evaluate muscle fiber histology, *M. tibialis anterior* (TA) muscles were sectioned and stained with Hematoxylin and Eosin (H & E) (Figure 3.1d). As shown in Figure 3.1e, no significant difference in muscle fiber number was noted between Wild-type and *Smad3*-null mice. However *Smad3*-null mice had a greatly reduced muscle fiber Cross Sectional Area (CSA) when compared to Wild-type mice (Figure 3.2a). Specifically, in Wild-type mice ~63% of muscle fibers had an area of $\geq 2000 \mu\text{m}^2$, however in the *Smad3*-null mice, only ~8% were found to be of similar diameter.

Genotype	Sex	Age	TA	Gas and Sol	Quad
Wild-type	F	5weeks	0.1429 ± 0.0044	0.3947 ± 0.0118	0.5488 ± 0.0172
<i>Smad3</i> ^{-/-}	F	5weeks	0.1633 ± 0.0133	0.3903 ± 0.0063	0.6209 ± 0.0658
<i>Mstn</i> ^{-/-}	F	5weeks	0.2108 ± 0.0039	0.6415 ± 0.0062	0.9188 ± 0.0159
<i>Smad3</i> ^{-/-} , <i>Mstn</i> ^{-/-}	F	5weeks	0.2661 ± 0.0204	0.5883 ± 0.0157	0.8076 ± 0.0470

Table 3.1 *Proportion of muscle weight to body weight (%)*.

Data represents skeletal muscle weights expressed as a percentage of total body weight and is given as mean ± SEM (n=3). *Mstn*^{-/-} denotes Myostatin-null mice. TA denotes *M. tibialis anterior*. Gas and Sol denotes *M. gastrocnemius and soleus*. Quad denotes *M. quadriceps*.

Figure 3.1 Analysis of $Smad3$ -null muscles.

(a) Representative images of body size and skeletal muscle mass differences between Wild-type ($Smad3^{+/+}$), $Smad3$ Heterozygous null ($Smad3^{+/-}$) and $Smad3$ -null ($Smad3^{-/-}$) mice. (b) Quantification of the change in individual skeletal muscle weights between $Smad3$ -null and Wild-type control mice ($n=3$). Error bars represent $\pm$ SEM ($n=3$). (c) Individual muscle weights of $Smad3$ -null and Wild-type mice expressed as a percentage of total body weight. Error bars represent $\pm$ SEM ($n=3$). (d) Representative images of Hematoxylin and Eosin (H & E) stained Wild-type and $Smad3$ -null TA muscle. Scale bar represents 50 μ m. (e) Total muscle fiber number in TA muscles from Wild-type and $Smad3$ -null mice ($P>0.05$) ($n=3$). Error bars represent $\pm$ SEM ($n=3$). For all relevant figure panels, statistical differences are indicated, $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***).

Figure 3.2 Characterization of Skeletal muscle atrophy in *Smad3*-null mice

(a) Frequency distribution of *M. tibialis anterior* (TA) skeletal muscle fiber Cross Sectional Area (CSA) between Wild-type and *Smad3*-null mice. (b-c) *Smad3*-null primary myoblasts and sh*Smad3* C2C12 cells were grown under proliferating conditions for a period of 60 h or 72 h respectively, with proliferation monitored using the methylene blue assay. Error bars represent $\pm$ SEM (n=2). (d) Western blot analysis of Cdk2, cyclin-E, and Retinoblastoma in primary myoblasts (left) and sh*Smad3* C2C12 cells (right). Ponceau S staining and analysis of Tubulin expression were performed to ensure equal loading of samples. The hyper-phosphorylated (pRb^{PP}) and hypo-phosphorylated (pRb) forms of Rb are indicated. (e) Western blot analysis of Myf5, p21, MyoD, Myogenin, MyHC and Tubulin expression at 0 h, 24 h, 48 h, 72 h and 96 h of differentiation in Wild-type and *Smad3*-null primary myotubes. Tubulin expression was analyzed to ensure equal loading of samples. (f) Fusion index of Wild-type and *Smad3*-null myoblasts at 24 h, 48 h, 72 h and 96 h differentiation. Error bars represent $\pm$ SEM (n=3). (g) Total average myonuclei number in Wild-type and *Smad3*-null myotubes at 24 h, 48 h, 72 h and 96 h differentiation. Error bars represent $\pm$ SEM (n=3). (h-j) Relative mRNA expression levels of *caveolin 3*, β_1D *integrin* and *SHP-2* in Wild-type and *Smad3*-null primary myotubes as measured by quantitative real time PCR. Graphs display fold change $\pm$ SEM, normalized to *Gapdh* (n=3). For all relevant figure panels, statistical differences are indicated, P<0.05 (*), P<0.01 (**) and P<0.001 (***).

3.3.2 *Smad3*-null primary myoblasts have a reduced rate of proliferation

One of the reasons for postnatal muscular atrophy is the impaired myogenic nature of SCs. In order to analyze myogenic potential, proliferation rate of myoblasts derived from *Smad3*-null SCs and Wild type SCs were compared *in vitro*. Data showed that *Smad3*-null primary myoblasts had a significantly reduced proliferation rate when compared to Wild-type primary myoblasts (Figure 3.2b). In addition, we generated a *Smad3* knock down C2C12 cell line (sh*Smad3*) (Figure 3.3), and analysis of proliferation demonstrated that shRNA-mediated knock down of *Smad3* also results in a reduction in the proliferation rate (Figure 3.2c). Consistent with reduced proliferation, fluorescence-activated cell sorting (FACS) revealed that shRNA-mediated knock down of *Smad3* results in a reduction in the percentage of cells in S phase of the cell cycle, concomitant with increased percentages of cells in both G₁ and G₂/M phases of the cell cycle (Table 3.2). Cyclin dependent kinase 2 (Cdk2), cyclin-E and Retinoblastoma protein have all been shown to be critical in regulating the G₁/S transition in myoblasts (247). Therefore we next analyzed the levels of these proteins via western blot analysis. As shown in Figure 3.2d, down-regulation or inactivation of *Smad3* leads to reduced expression of both Cdk2 and cyclin-E (Figure 3.2d). Retinoblastoma (Rb) is the major substrate of G₁ Cdks, moreover, Cdk-mediated phosphorylation of Rb relieves repression on E2F/DP1, resulting in continued cell cycle progression (266). Subsequent western blot analysis revealed a reduction in the levels of hyper-phosphorylated Rb (pRb^{PP}) in both *Smad3*-null primary myoblasts and sh*Smad3* C2C12 cells, concomitant with an increase in hypo-phosphorylated Rb (pRb) (Figure 3.2d).

Genotype	Cells in G ₁	Cells in S	Cells in G ₂ /M
shsmad3	70.32 ± 0.58 %	18.54 ± 0.20 %	11.14 ± 0.38 %
Control	67.00 ± 0.79 %	22.15 ± 0.71 %	10.87 ± 0.08 %

Table 3.2 Cell cycle distribution of *shSmad3* C2C12 myoblasts and control.

Percentage of cells from 10,000 counts in G₁, S, or G₂/M phases of the cell cycle as analyzed by FACS. Data is given as mean ± SEM (n=2). Statistical differences were determined by two-tailed Student's T test and $P_S < 0.01$, $P_{G1} < 0.05$.

Figure 3.3 Relative *Smad3* mRNA expression levels in *shSmad3* and *shControl* C2C12 myoblasts.

Relative *Smad3* mRNA expression levels in *shSmad3* and *shControl* C2C12 myoblasts, as measured by quantitative real time PCR. Graph displays fold change ± SEM, normalized to *Gapdh* (n=3). Statistical differences are indicated, $P < 0.001$ (***).

3.3.3 Smad3-null primary myoblasts have impaired myogenic differentiation

In addition to proliferation rate, we also measured the differentiation ability of Smad3-null myoblasts. The results demonstrate that Smad3-null myoblasts differentiated to form smaller myotubes with reduced branching, as compared to Wild-type myoblasts (Figure 3.4). Consistent with histology, gene expression results showed that the critical myogenic genes, Myf5, p21, MyoD, Myogenin, and MyHC are down-regulated in Smad3-null myotubes, especially in early (0 h) and late phases of differentiation (72 h and 96 h) when compared to Wild-type cultures (Figure 3.2e and Figure 3.5).

This defective differentiation can also result from inefficient fusion of myoblasts and myotubes. Therefore we determined fusion index of Smad3-null myotubes. Our analysis showed that the Smad3-null myotubes had decreased fusion index and reduced average myonuclei number in myotubes (Figure 3.2f-g). This suggests that Smad3-null myoblasts and myotubes have fusion defects during differentiation. We also analyzed the expression levels of some of the genes that have been shown to be involved in fusion during myogenesis. Caveolin-3 is a scaffolding protein primarily associated with caveolae membrane domains in skeletal muscle tissue (78). β_1 D integrin is a muscle-specific membrane receptor, which appears to play a critical role in myoblast fusion (240, 304). SHP-2 (SH2 domain-containing protein-tyrosine phosphatase) positively regulates myogenesis through activation of RhoA (135), and resulting phosphorylation of focal adhesion kinase (FAK), essential for myoblasts fusion (57). Results from real time PCR analysis showed that these fusion-related genes were down-regulated in Smad3-null myotubes at late time points (48 h and 72 h) (Figure 3.2h-j), which further supports the fusion defects observed in the Smad3-null myoblasts.

Figure 3.4 Representative images of differentiating primary myoblasts from Wild-type and Smad3-null mice.

Representative images of differentiating primary myoblasts from Wild-type and Smad3-null mice at 0 h, 24 h, 48 h and 72 h of differentiation; cells were stained with Hematoxylin and Eosin (H & E). Scale bar represents 50 μ m.

Figure 3.5 Densitometric analysis of myogenic gene expression in primary myotubes.

Densitometric analysis of myogenic gene (Myf5, p21, MyoD, Myogenin and MyHC) expression in primary myotubes isolated from Wild-type and Smad3-null mice, normalized to Tubulin expression.

3.3.4 The role of Smad3 signaling in regulation of SC function

As described earlier, Smad3-null mice show signs of muscular atrophy, which we hypothesized, may be attributed to impaired SC function namely SC self-renewal. We used two independent experimental systems, namely, tissue dissociated SC and single muscle fiber culture to quantify self-renewed SC. The self-renewal ability of Smad3-null myoblasts was quantified through *in vitro* immunostaining analysis using antibodies directed against MyoD and Pax7 (Figure 3.6a). Cells that are positive for MyoD only (MyoD⁺/Pax7⁻) were considered fusion competent and cells positive for Pax7 only (MyoD⁻/Pax7⁺) were quantified as a measure of the fusion incompetent, self-renewing lineage (quiescent SCs). The results indicate that there was a significant decrease in the percentage of the MyoD⁻/Pax7⁺ myoblasts (from 19 % $\pm$ 0.95 % to 9 % $\pm$ 0.63 %), with a concomitant increase in the percentage of the MyoD⁺/Pax7⁻ myoblast population (from 27 $\pm$ 0.84 % to 40 $\pm$ 2.87 %) in Smad3-null myoblasts when compared to Wild-type (Figure 3.6b) in tissue dissociated SC experimental system. Similarly in single muscle fiber cultures, we observed a significant reduction in self-renewed population of SC (Figure 3.6c-d). We next quantified SC numbers through immunostaining with Pax7 antibodies (Figure 3.7). Our results show that Smad3-null mice had significantly reduced SC number when compared to Wild-type controls (Figure 3.6e-f). Therefore, Smad3 appears to play a role in the regulation of SC function. Specifically, loss of Smad3 results in reduced SC self-renewal capacity, which may lead to a progressive decrease in the number of SCs.

Figure 3.6 Satellite Cell (SC) number and self-renewal in Smad3-null mice.

(a) Representative images of MyoD and Pax7 immunostaining on tissue dissociated SCs. (b) The graph represents the proportions of MyoD⁺/Pax7⁺, MyoD⁻/Pax7⁺ and MyoD⁺/Pax7⁻ cells expressed as a percentage of total cell number in tissue dissociated SCs. (c) Representative images of MyoD and Pax7 immunostaining on SCs migrated from single muscle fibers. (d) The graph represents the proportions of MyoD⁺/Pax7⁺, MyoD⁻/Pax7⁺ and MyoD⁺/Pax7⁻ cells expressed as a percentage of total cell number in single muscle fiber cultures. For representative images, MyoD⁻/Pax7⁺ (Yellow arrowheads), MyoD⁺/Pax7⁻ (red arrows) and MyoD⁺/Pax7⁺ (white asterisks) populations are indicated. Scale bar represents 50 μ m. (e) The graph represents the number of SCs, per 100 myonuclei (%), in single muscle fibers isolated from Smad3-null and Wild-type mice. (f) The graph represents the number of SCs, per single muscle fiber. Error bars represent $\pm$ SEM (n=3). For all relevant figure panels, statistical differences are indicated, P<0.01 (**) and P<0.001 (***).

Figure 3.7 Representative images of Pax7 ICC on single muscle fibers.

Representative images of Pax7 ICC on single muscle fibers (The arrows indicate the location of SCs). Scale bar represents 50 μm .

3.3.5 Altered levels of Myostatin and IGF-1 are observed in *Smad3*-null skeletal muscle tissue and primary myoblast cultures

In order to analyze the molecular basis for the observed atrophy, we performed real time PCR analysis to measure the expression of candidate genes that regulate muscular atrophy, such as Myostatin and IGF-1. The expression of Myostatin was up-regulated in *Smad3*-null *M. quadriceps* (Quad) muscle (Figure 3.8a). In contrast, the expression of IGF-1 was significantly down-regulated in Quad muscles from *Smad3*-null mice when compared to Wild-type controls (Figure 3.8b). In addition we analyzed the expression of both Myostatin and IGF-1 in primary myoblast cultures isolated from both *Smad3*-null and Wild-type mice. Consistent with the results outlined above, we find increased expression of Myostatin concomitant with reduced expression of IGF-1 in *Smad3*-null primary myotube cultures when compared to Wild-type mice-derived cultures (Figure 3.8c-d).

Figure 3.8 *Myostatin* and *IGF-1* expression in *Smad3*-null muscle and primary myotubes.

Relative mRNA expression levels of *Myostatin* (a) and *Igf-1* (b) as measured by quantitative real time PCR in Quad muscle tissue from Wild-type and *Smad3*-null mice. Relative mRNA expression levels of *Myostatin* (c) and *Igf-1* (d) as measured by quantitative real time PCR in primary myotube cultures from Wild-type and *Smad3*-null mice. Graphs display fold change $\pm$ SEM, normalized to *Gapdh* (n=3). For all figure panels, statistical differences are indicated, P<0.01 (**) and P<0.001 (***).

3.3.6 Increased ubiquitination of proteins in Smad3-null skeletal muscle

Since Smad3-null mice have increased levels of Myostatin and decreased levels of IGF-1, we next investigated if there was an increase in ubiquitination of intracellular proteins in Smad3-null muscles. Indeed proteins of Quad muscle in the molecular weight range of 22-60 kDa appear to be highly ubiquitinated as compared to Wild-type muscles (Figure 3.9a), which is consistent with the increased muscle atrophy observed. However, despite the increase in Myostatin, there was no change in the expression and phosphorylation status FoxO1 (Figure 3.9a). In addition, both mRNA expression and protein level of MuRF1 were up-regulated, while no change in the expression of Atrogin1 was observed, in the muscle tissue from Smad3-null mice when compared to controls (Figure 3.9a-b). MuRF1 has been previously demonstrated to interact with and promote the degradation of MyHC protein in myotubes treated with dexamethasone (DEX) (48). Consistent with this, we find decreased expression of MyHC at the protein level in Smad3-null mice, with no appreciable change in mRNA expression of MyHC detected between Wild-type and Smad3-null muscle tissue (Figure 3.9a-b). Muscle mass is maintained through a balance of protein synthesis and protein degradation. Therefore we next analyzed the expression of Akt, a key signaling intermediate involved in protein synthesis. Subsequent western blot analysis demonstrated that the expression and the level of active phosphorylated Akt remained unaltered between Smad3-null and Wild-type muscle tissue (Figure 3.9a).

Figure 3.9 Ubiquitination of intracellular proteins in *Smad3*-null skeletal muscle.

(a) Western blot analysis of MuRF1, Ubiquitin, MyHC, FoxO1, p-FoxO1, Atrogin1, Akt, p-Akt (Thr 308) and p-Akt (Ser 473) expression in Quad muscle from Wild-type and *Smad3*-null mice. Ponceau S staining was performed to ensure equal loading of samples. (b) Quantitative real time PCR analysis of MuRF1, MyHC I, MyHC IIB, and MyHC IIX expression in muscle of Wild-type and *Smad3*-null mice. Graphs display fold change $\pm$ SEM, normalized to *Gapdh* (n=3). (c) Treatment of proteasome inhibitor epoxomicin in Wild-type and *Smad3*-null primary myotubes. Differentiated Wild-type and *Smad3*-null primary myotubes (48 h) were incubated with epoxomicin (48 h + 100 nM epox) or without epoxomicin (48 h + 0 nM epox) for 10 h before sample collection. The MyHC expression was measured by Western Blot analysis. Tubulin levels were measured to ensure equal loading of samples. (d) The expression of MyHC in Wild-type and *Smad3*-null primary myotubes treated with or without epoxomicin, normalized to Tubulin expression. For all the relevant figure panels, statistical differences are indicated, $P < 0.001$ (***)

3.3.7 Treatment with proteasome inhibitor reduces MyHC degradation in *Smad3*-null primary myotubes

Smad3-null skeletal muscle demonstrated elevated protein ubiquitination and MuRF1 expression level, which suggested the involvement of ubiquitin proteasome system in *Smad3*-null muscle atrophy. One proteasome inhibitor, epoxomicin (epox), was used to determine whether the inhibition of the ubiquitin proteasome system will improve the loss of MyHC protein in *Smad3* myotubes. *Smad3*-null primary myotubes showed significantly reduced MyHC expression as compared to Wild-type primary myotubes. However, 10 h treatment with epox partially improved the MyHC expression in *Smad3*-null myotubes (Figure 3.9c-d). This partial rescue of MyHC expression in *Smad3*-null myotubes with the treatment of epox suggested that proteasome system might be involved in myotube atrophy in *Smad3*-null mice.

3.3.8 Inactivation of *Myostatin* partially alleviates the muscle atrophy and impaired myogenesis observed in *Smad3*-null mice

In order to genetically prove that increased *Myostatin* is responsible for the observed muscular atrophy in *Smad3*-null mice, we crossed the *Smad3*-null mice into a *Myostatin*-null genetic background to generate a Double knockout mouse model (DKO; *Smad3*^{-/-}/*Myostatin*^{-/-}). Inactivation of mature *Myostatin* expression and expression of an inactive *Smad3* allele is shown in Figure 3.10.

DKO mice have reduced body size and decreased musculature when compared to *Myostatin*-null mice (Figure 3.11a). Skeletal muscle weights were similar between DKO mice and Wild-type mice, however, when muscle weights were normalized to

total body weight, we found increased skeletal muscle mass when compared to that of Wild-type mice (Table 3.1) (Figure 3.12a-b). Histological analysis of muscle sections (Figure 3.11b), indicated that muscle Cross Sectional Area (CSA) of DKO was similar to that of Wild-type (Figure 3.12c). However, quantification of muscle fiber number revealed an increase in fiber number in muscle sections from DKO mice when compared to Wild-type controls, which suggests that the increased muscle mass observed in the DKO mice may be due to muscle fiber hyperplasia (Figure 3.12d), which is consistent with the phenotype observed in *Myostatin*-null mice. Despite increased muscle fiber number, DKO mice had decreased average muscle fiber area when compared to Wild-type controls, which is similar to that observed in the *Smad3*-null mice (Figure 3.12e). Therefore, inactivation of *Myostatin* in *Smad3*-null mice rescues, at least in part, the atrophic phenotype observed in the *Smad3*-null mice, resulting in an increase in muscle mass back to levels comparable to Wild-type mice. Furthermore, it appears that the improvement in muscle mass results from an increase in muscle fiber number (hyperplasia), rather than an increase in individual muscle fiber area (hypertrophy).

We next isolated primary myoblasts from DKO mice and analyzed their myogenic potential *in vitro*. DKO myoblasts had a significantly increased proliferation rate when compared to Wild-type myoblasts (Figure 3.12f). Analysis of myogenic differentiation also revealed an enhanced fusion index and an increase in average myonuclei number in the primary myotubes from DKO mice when compared to *Smad3*-null controls (Figure 3.11c and Figure 3.12g-h). These data therefore suggest that myoblasts isolated from DKO mice demonstrate improved myogenesis when compared to myoblasts isolated from *Smad3*-null mice, which is consistent with the enhanced proliferation and differentiation observed in *Myostatin*-null mice. Therefore loss of

Myostatin appears to partially rescue the defective myoblast proliferation and differentiation observed in Smad3-null primary myoblast cultures.

Figure 3.10 *The genotypes of the Wild-type, Smad3-null, and DKO mice were confirmed by RT-PCR analysis.*

(a) RT-PCR analysis of Smad3 expression was conducted in muscles from the Wild-type, Smad3-null and DKO mice using primers of Smad3-*exon* 6 and Smad3-*exon* 9. These primers amplified a 431 bp fragment of Wild-type transcript and a 284 bp fragment of mutant transcript. No Wild-type transcript was found in RNA from Smad3-null and DKO mice. **(b)** RT-PCR analysis of Myostatin expression was conducted in muscles from the Wild-type and DKO mice using primers which target the sequence encoding mature Myostatin peptide. These primers amplified a 131 bp fragment in Wild-type mice, but not in Myostatin-null mice.

Figure 3.11 Improved myogenic potential of Double knockout (DKO) myoblasts.

(a) Representative images of body size and skeletal muscle mass differences between DKO and Myostatin-null mice. (b) Representative images of Hematoxylin and Eosin (H & E) stained TA muscle from Smad3-null, Wild-type, DKO and Myostatin-null mice. Scale bar represents 100 μ m. (c) Representative images of differentiating cells from Wild-type, Smad3-null, Myostatin-null and DKO primary myoblast cultures at 0 h, 24 h, 48 h and 72 h of differentiation; cells were stained with Hematoxylin and Eosin (H & E). Scale bar represents 100 μ m.

Figure 3.12 Reduced atrophy in Double knockout (DKO) mice (*Smad3*^{-/-}, *Mstn*^{-/-}).

(a) Quantification of the change in individual skeletal muscle weights between DKO and Wild-type mice. TA denotes *M. tibialis anterior*. Gas and Sol denotes *M. gastrocnemius* and *soleus*. Quad denotes *M. quadriceps*. Error bars represent $\pm$ SEM (n=3). (b) Individual muscle weights from DKO and Wild-type mice expressed as a percentage of total body weight. Error bars represent $\pm$ SEM (n=3). (c) Comparison of the average TA muscle Cross Sectional Area (μm^2) between *Smad3*-null, Wild-type, DKO and Myostatin-null mice. Difference between *Smad3*-null and DKO mice is not significant ($P>0.05$). Error bars represent $\pm$ SEM (n=3). (d) Quantification of the total muscle fiber number in TA muscles from *Smad3*-null, Wild-type, DKO and Myostatin-null mice. Error bars represent $\pm$ SEM (n=3). (e) Frequency distribution of individual muscle fiber Cross Sectional Area in TA muscles from *Smad3*-null, Wild-type and DKO mice, expressed as a percentage of total fiber number. Muscle fiber areas were grouped based on size: $<1000 \mu\text{m}^2$, $1000\text{--}2500 \mu\text{m}^2$ and $>2500 \mu\text{m}^2$. (f) Wild-type, Myostatin-null and DKO primary myoblasts were grown under proliferating conditions for a period of 60 h, with proliferation monitored using the methylene blue assay. Error bars represent $\pm$ SEM (n=2). (g) The fusion index of Wild-type, *Smad3*-null, Myostatin-null and DKO myoblasts at 24 h, 48 h, 72 h and 96 h of differentiation. Error bars represent $\pm$ SEM (n=3). (h) Average myonuclei number in Wild-type, *Smad3*-null, Myostatin-null and DKO myotubes at 24 h, 48 h, 72 h and 96 h of differentiation. Error bars represent $\pm$ SEM (n=3). (i) Neutralization of Myostatin in Wild-type and *Smad3*-null primary culture by soluble ActRIIB treatment. Wild-type and *Smad3*-null primary myoblasts were seeded at a density of 25,000 cells/cm². Following a 12 h attachment period, cells were switched to differentiation media with (0h + 3 $\mu\text{g/ml}$ soluble ActRIIB) or without soluble ActRIIB (0h +0 $\mu\text{g/ml}$ soluble ActRIIB) for 48 h. The MyHC and MyLC expression were measured by Western Blot analysis. Tubulin levels were measured to ensure equal loading of samples. (j) The expression of MyHC and MyLC in Wild-type and *Smad3*-null primary culture treated with or without soluble ActRIIB, normalized to Tubulin expression. For all relevant figure panels, statistical differences are indicated, $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***).

3.3.9 Neutralization of Myostatin results in increased MyHC protein in Smad3 null myotubes.

Loss of Myostatin partially compensates muscle atrophy in Smad3-null mice. In order to confirm the postnatal role of Myostatin in Smad3-null muscle atrophy, we neutralized endogenous Myostatin, by treating Wild-type and Smad3-null primary culture with the soluble extracellular domain of activin type IIB receptor (ActRIIB), also known as soluble ActRIIB. After 48 h treatment, MyHC and MyLC, the important myogenic markers during differentiation, were measured by western blot. The results suggested that neutralizing endogenous Myostatin in primary culture significantly rescued MyHC proteins levels in Smad3-null primary myotubes at 48 h differentiation (Figure 3.12i-j). The expression level of another marker, MyLC, was also improved considerably (Figure 3.12i-j). This result further confirms the role of Myostatin in muscle atrophy of Smad3-null mice.

3.4 Discussion

Among the TGF- β super-family members, TGF- β and Myostatin rely on canonical Smad signaling, specifically Smad2/3, to elicit biological function during myogenesis (75, 142, 158). Since Myostatin is a negative regulator of myogenesis, one might expect that loss of Smad3 would enhance myogenesis, due to interference with the Myostatin signaling pathway. However contrary to this, we now show that lack of Smad3 results in dramatic muscular atrophy. Impaired SC function combined with increased proteasomal mediated protein degradation appears to be the molecular mechanism behind the observed atrophy in Smad3-null muscles.

Smad3 signaling is required for proper function of Satellite cells.

SCs are adult muscle precursor cells and impaired function and/or number of SCs leads to reduced myogenesis and muscular atrophy (267). In Smad3-null mice there is a significant reduction in the SC number (Figure 3.6e-f) and data presented here suggest that this could be due to reduced self-renewal of SCs (Figure 3.6b and 3.6d). SC self-renewal refers to a process whereby a pool of myoblasts (derived from activated SCs), which express high levels of Pax7, withdraw from proliferation to replenish the SC population (205, 299). Therefore, proliferation rate plays an important role in determining the pool of self-renewed SCs (172) during postnatal myogenesis. Cell cycle progression and proliferation of myoblasts are in turn regulated at G₁/S and G₂/M checkpoints and any perturbations at the check points leads to altered proliferation of myoblasts, which of course has consequences on SC self-

renewal. Our data here clearly demonstrates that in Smad3 inactivated myoblasts there is increased cell cycle arrest in G₁ and G₂/M phases of the cell cycle (Table 3.2). Furthermore, there is significant reduction in cyclin-E and Cdk2 expression, which in turn leads to hypo-phosphorylation of retinoblastoma (Figure 3.2d) and cell cycle arrest, resulting in the reduced proliferation rate observed in of Smad3-null myoblasts (Figure 3.2b-c). This, we suspect would lead to a diminished pool of myoblasts and reduced self-renewal of SCs.

Lack of Smad3 severely affects myogenesis

Differentiated Smad3-null myotubes showed an atrophic phenotype (Figure 3.4), with both reduced fusion index and reduced average myonuclei number (Figure 3.2f-g). The fusion defects were further confirmed by reduced expression of three major fusion-related genes, *caveolin3*, β_1D *integrin* and *SHP-2* (Figure 3.2h-j). Caveolin3 and β_1D integrin are membrane-associated proteins, which play important roles in myoblast fusion. SHP-2 is a known upstream modulator of NFATc2 pathway, which is important for fusion between nascent myotubes and myoblasts (112). Therefore results presented here suggest that Smad3 is essential for proper temporal expression of differentiation genes.

Muscle Atrophy seen in Smad3-null muscles could be due to increased Myostatin expression

Both neonatal (data not shown) and 6 week old postnatal Smad3 null mice show severe muscle fiber atrophy (Figure3.2a). Skeletal muscle atrophy could be due to

reduced protein synthesis and/or increased protein catabolism due to targeted ubiquitination of muscle-specific proteins. Two muscle-specific E3 ligases, Atrogin1 and MuRF1, are up-regulated in skeletal muscle tissue in response to numerous atrophic signals (26, 83, 145). Western blot analysis confirmed that there is increased ubiquitination of intracellular proteins in skeletal muscle tissue from Smad3-null mice as compared to control mice (Figure 3.9a). Furthermore, real time PCR and western blot analysis confirmed that MuRF1 was up-regulated in Smad3-null muscles (Figure 3.9a-b). Previous studies showed that up-regulation of MuRF1 could be due to either FoxO1 or Forkhead box protein O3 (FoxO3) transcription factors during skeletal muscle atrophy (232). In this study, we did not detect any changes in the expression of FoxO1 at both total and phosphorylation level (Figure 3.9a). Therefore, we speculate that up-regulation of MuRF1 in Smad3-null mice might be due to an increase in FoxO3 levels or activity. Alternatively, this could also be due to an increase in NF-kappa B transcription factors which can be activated by inflammatory cytokines (242, 297). Consistent with increased MuRF1, MyHC, a known target of MuRF1 (48), was down-regulated (Figure 3.9a-b). Thus these results clearly suggest that the muscular atrophy seen in Smad3-null mice is much akin to cachectic-like muscle wasting. Although there was no significant change in terms of Akt phosphorylation, the impact of reduced protein synthesis cannot be ruled out at this stage since increased Myostatin can also block protein synthesis by down regulating elongation factors (Sudarsanareddy *et al.* ³).

Our search for mechanism revealed increased expression of Myostatin in Smad3-null mice (Figure 3.8a and 3.8c). Myostatin is a secreted growth factor, which has been previously shown to be a potent negative regulator of myogenesis. Loss of Myostatin

³ Sudarsanareddy *et al.* manuscript in preparation

results in increased muscle growth due to increased SC number, and activation (172). Furthermore, myoblasts derived from Myostatin-null SCs have increased proliferation and enhanced differentiation (142-143). In contrast increased levels of Myostatin have been shown to block myoblast proliferation, arresting cells at the G₁ to S transition, further resulting in reduced differentiation (266). Furthermore, increased Myostatin levels have also been shown to block SC activation and self-renewal (172, 176), and also result in the development of severe cachectic-like muscle wasting, through activating components of the ubiquitin-proteasome pathway (179). The data presented in this chapter, as well as previously published reports, strongly support the notion that the increased expression of Myostatin observed in Smad3-null mice may be primarily responsible for the impaired SC function and increased protein catabolism.

Consistent with this hypothesis, myoblasts from DKO mice showed significantly increased proliferation when compared to Smad3-null primary myoblasts and moreover had a higher rate of proliferation than that of Wild-type primary myoblasts (Figure 3.12f). Defects in fusion, during differentiation, were also partially recovered (Figure 3.12g-h and Figure 3.11c). Both fusion index and average myonuclei number increased in DKO myotubes as compared to Smad3-null myotubes. Furthermore, loss of Myostatin, at least in part, rescued the skeletal muscle atrophy phenotype observed in Smad3-null mice, as demonstrated through the increased musculature observed in DKO mice when compared to Smad3-null mice (Table 3.1). Most certainly, the improved muscle mass and enhanced myogenesis in DKO mice provides evidence to support the role of Myostatin in the progression of the atrophic phenotype in Smad3-null mice.

Myostatin is widely known to signal through canonical Smad signaling (mainly Smad3), to inhibit myoblast proliferation, differentiation, SC activation and self-

renewal (75, 142, 307). However, other signaling pathways including Smad2 are also shown to be involved in Myostatin signaling (104, 115, 151, 176, 214, 296). In this study, higher Myostatin level was proven to be responsible for the muscle atrophy in Smad3-null mice. This further suggests that Myostatin could signal independent of Smad3 either via Smad2 or other signaling pathways, including p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), phosphatidylinositol 3-kinase (PI3K) as shown previously (104, 115, 176, 214, 296). In the present chapter, we have provided evidence to support the critical role of Smad3 in regulation of postnatal myogenesis and SC function in mice. In support, absence of Smad3 leads to impaired SC self-renewal, which is important for maintenance of the steady state SC population during postnatal myogenesis. In addition, primary myoblasts derived from Smad3-null SCs also have defective proliferation and differentiation. Therefore we propose that the dramatic atrophy observed in the absence of Smad3 may in part be due to impaired SC function. Smad3-null muscle also undergoes enhanced protein degradation through up-regulation of MuRF1 and the ubiquitin-proteasome system. Furthermore, we also find increased Myostatin expression and decreased IGF-1 expression in Smad3-null muscle and primary cultures. Since Myostatin acts as a potent negative regulator in muscle growth, it is quite possible that elevated levels of Myostatin may contribute to the muscle atrophy in Smad3-null mice. In addition, inactivation of *Myostatin* in Smad3-null mice (DKO) partially rescues the loss of muscle mass, as well as improves the myogenic potential of SC-derived myoblasts *in vitro*. However inactivation of *Myostatin* is unable to fully compensate for the muscle wasting observed in the Smad3-null mice, which suggests the involvement of additional factors, as yet to be determined, which contribute to the progression of the atrophic phenotype in Smad3-null mice.

Chapter 4 Lack of Smad3 signaling leads to impaired skeletal muscle regeneration

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Lack of Smad3 signaling leads to impaired skeletal muscle regeneration

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Abstract

Smad3 is a key intracellular signaling mediator for both TGF- β and Myostatin, two major regulators of skeletal muscle growth. Our previous study revealed pronounced muscle atrophy along with impaired Satellite Cell (SC) functionality in Smad3-null muscles. In this study, we validated the role of Smad3 signaling in skeletal muscle regeneration. Here, we show that Smad3-null mice had incomplete recovery of muscle weight and myofiber size after muscle injury. Histological/Immunohistochemical analysis suggested reduced inflammatory response and less number of activated myoblasts during the early stages of muscle regeneration in the TA muscle of Smad3-

null mice. Nascent myofibers formed after muscle injury were also reduced in number. Moreover, Smad3-null regenerated muscle had decreased oxidative enzyme activity and impaired mitochondrial biogenesis, evident by the down-regulation of gene encoding the master regulator of mitochondrial biogenesis (*TFAM*). However, a reduced fibrotic tissue formation was seen in regenerated Smad3-null muscle. In conclusion, Smad3 deficiency led to impaired muscle regeneration, which indicates the essential role of Smad3 in post-natal myogenesis. Given the negative role of Myostatin in the regulation of muscle regeneration, increased level of *Myostatin* in Smad3-null muscle might contribute to the regeneration defects.

4.1 Introduction

Smad3 belongs to a family of nine proteins named Smad proteins and functions as a key intracellular signaling mediator/transcription factor for TGF- β superfamily members (33, 223). The TGF- β superfamily is a large group of extra cellular growth factors modulating diverse biological processes and tissue homeostasis (131, 252). Mutagenesis of the *Smad3* gene in mouse provides an *in vivo* model for the analysis of Smad3 function. To date, three different Smad3-null mice have been developed, by targeted disruption of *exon1*, *exon 2*, and *exon 8* respectively (54, 297, 308). Smad3-null (*Smad3^{ex8/ex8}*) mice, which was used in this study, express a truncated SMAD3 protein lacking the SSVS consensus phosphorylation site and an L3 loop (297), which are essential domains required for the activation of Smad3 by cell surface receptors (159). Although Smad3-null mice are viable and can survive to adulthood, they usually die between 1 and 8 months due to inflammation in a variety of organs, including the nasal mucosa, stomach, pancreas, colon and small intestine (297). Smad3

deficiency leads to impaired TGF- β regulation of T cell activation. This major immune deficiency increases the susceptibility of Smad3-null mice to bacterial infections and results in the chronic inflammation (297). Due to chronic inflammation, Smad3-null mice are more susceptible to inflammation-associated cancer (162-163).

Although extensive studies have established the importance of Smad3 as a downstream mediator of TGF- β superfamily members, relatively very little is known about the specific functions of Smad3 in skeletal muscle growth and in fact, results to date are controversial (81, 233). A recent report by Sartori *et al.* used RNAi techniques to knock down *Smad3* expression in adult muscles. In this study, muscle hypertrophy was observed in Smad3 knocked down mice, 2 weeks after transfection (233), indicating a negative role for Smad3 in the regulation of post-natal muscle growth. However, in this study, a trace amount of Smad3 protein was still found in muscle fibers (233). A previously published study from our lab has used Smad3-null mice, in which the expression of *Smad3* gene was completely abolished, as a model to investigate the role of Smad3 in muscle growth and Satellite Cell (SC) biology (81). Contrary to Sartori *et al.*'s results, our previous data suggested that Smad3 is essential for normal skeletal muscle growth and that Smad3-null mice have profound muscle atrophy (81). Molecularly we found that muscle atrophy in Smad3-null mice was due to up-regulated *Myostatin* expression (81). Myostatin, a TGF- β superfamily member, is a potent negative regulator of skeletal muscle growth (120, 183), which controls muscle growth by inhibiting myoblast proliferation (177, 266, 296), differentiation (118, 142, 177, 224), as well as SC activation and self-renewal (172, 176). In addition to SC function, Myostatin also inhibits protein synthesis together with promoting degradation in skeletal muscle (156, 179, 284). Our previous data has demonstrated that enhanced Myostatin signaling promotes sarcomeric protein degradation in Smad3-

null muscle through the ubiquitin proteasome system, and also strongly inhibits SC functionality of Smad3-null mice (81); which together contribute to the muscle atrophy observed in Smad3-null mice.

To further characterize the role of Smad3 in the regulation of post-natal myogenesis, here we have induced muscle injury in Smad3-null mice through intra-musculature notexin injection and assessed subsequent muscle regeneration histologically, molecularly, and metabolically. Given the increased *Myostatin* expression and impaired SC functionality in Smad3-null muscle, we hypothesized that Smad3-null muscle would regenerate poorly. Consistent with our hypothesis, thirty days after notexin administration, Smad3-null mice showed impaired muscle regeneration, evident by dramatic loss of muscle weight, profound decline in the size of the regenerated myofibers, and reduced centrally located myonuclei number. An impaired inflammatory response, less activated myoblasts and reduced newly formed myofibers were found in Smad3-null regenerating TA muscle. Moreover, the regenerated TA muscle from Smad3-null mice showed significantly decreased oxidative enzyme activity, due to defects in mitochondrial biogenesis. However, loss of Smad3 led to reduced formation of scar tissue after muscle regeneration.

4.2 Materials and Methods

4.2.1 Animals

Heterozygote Smad3-null mice were kindly gifted by Prof. Walter Wahli (University of Lausanne, Lausanne, Switzerland), and were bred and genotyped according to the previously published protocol (297). Mice were maintained on standard chow diet at a constant temperature (20°C) under a 12 h/12 h artificial light/dark cycle with unlimited access to water. All experiments were performed according to the approved protocols of the Institute Animal Care and Use Committee (IACUC), Singapore.

4.2.2 Muscle injury

6-week-old male mice were anesthetized by intraperitoneal injection of a mixture of 10 mg/ml ketamine and 1 mg/ml xylazine at 0.1 ml/10g body weight. Fifteen micro liters of Notexin (10 µg/ml in 0.9% NaCl; Latoxan) was injected into the *M. tibialis anterior* (TA) muscle of the left leg, using a 28-gauge syringe (Hamilton Co., Whittier, California, USA). The right TA was used as an uninjured control. TA muscles were harvested and weighed on days 1, 2, 3, 7, 30 after the injury.

4.2.3 Histological assessment of muscle regeneration

The dissected TA muscles were embedded in OCT (Optimal Cutting Temperature) compound and then frozen in isopentane cooled with liquid nitrogen. Serial cross sections (8 µm) were cut from the mid-belly of the muscle and mounted on slides for histological, immunochemical, and metabolic staining. Hematoxylin and Eosin (H & E)

staining and Van Giesen's staining were performed according to the manufacturer's instructions (Merck). Images were captured using the Leica CTR 6500 microscope, equipped with the Leica DFC 310 FX camera and Image Pro Plus software (Media Cybernetics, Bethesda, MD). H & E staining was used for Cross Sectional Area (CSA) measurement and Van Giesen's stain was used for fibrotic tissue quantification. Sections which showed the largest lesion area were used for quantification. 5 images (magnification, 10×) around the lesion area from each sample were captured and used for quantification, according to Zeng *et al.* (300). For mean CSA of the regenerated myofibers, the CSA of the centrally nucleated myofibers within the field were measured. For mean myofiber CSA of contralateral uninjured control, the CSA from 1000 myofibers from randomly selected fields were measured. The area of interstitial fibrosis was quantitatively assessed on Van Giesen's stained images and was measured according to Luz *et al.* (161) and calculated as the ratio of the fibrotic area to the total muscle cross sectional area (CSA) (%).

4.2.4 Immunohistochemistry

Immunohistochemical staining on muscle sections was performed as described previously (173). In brief, frozen muscle sections (8 µm) were blocked in 0.2% TritonX-100, 0.2% BSA and 10% normal sheep serum (NSS) in PBS for 1 h at room temperature and then incubated with primary antibodies diluted in blocker overnight at 4°C. After fixation with 10% buffered formalin for 5 min, the sections were incubated with secondary antibodies and/or tertiary antibodies in 0.2% BSA and 5% NSS in PBS-T for 1 h. After washing with PBS, the sections were counter stained with DAPI (1:1000; Molecular Probes) and mounted with ProLong Gold antifade reagent

(Invitrogen). The following antibodies were used in this study for immunohistochemistry: mouse anti-MyoD (1:25; BD pharmingen), mouse anti-Mac-1 (1:100; BD pharmingen), mouse anti-embryonic MyHC (1:100; Developmental Studies Hybridoma Bank F1.652), sheep anti-mouse Immunoglobulin biotinylated secondary antibody (1:300; GE Healthcare Life Sciences), streptavidin conjugated Alexa Fluor 488 (1:400; Molecular Probes) and goat anti-mouse Alexa Fluor 594 (1:1000, GE Healthcare Life Sciences). Embryonic-MyHC positive myofibers were morphometrically assessed in stained sections. Mac-1 and MyoD positive cells were quantified in the stained sections. Information of the primary antibodies used in this chapter is listed in Table 2.2.

4.2.5 Succinate dehydrogenase (SDH), cytochrome c oxidase (COX) and α -glycerophosphate dehydrogenase (α -GPD) staining on muscle section

SDH, COX and α -GPD activities were measured using a colorimetric assay, as described by Masuda *et al.* (41, 168) with slight modification. In brief, for SDH staining, sections were air-dried for 30 min and then incubated in pre-warmed PBS buffer containing 50 mM sodium succinate and 0.6 mM Nitro Blue Tetrazolium (NBT) (Sigma N5514) for 30 min at 37°C. For COX staining, the sections were incubated in pre-warmed 5 mM phosphate buffer (pH 7.4) containing 0.1% of 3,3'-diaminobenzidine (DAB) (Sigma D5637), 0.1% cytochrom c (Sigma C2506), and 0.02% catalase (Sigma C40) for 50 min at 37°C. For α -GPD staining, the sections were incubated in pre-warmed 0.2 M Tris-HCl buffer (pH 7.4) containing 9.3 mM rac-glycerol 1-phosphate disodium salt hexahydrate (Sigma G6014), and 0.24 mM NBT for 45 min at 37°C after air-drying. The reaction was terminated by rinsing the

sections with distilled water thoroughly. After dehydration and clearance by xylene, the sections were mounted using DPX (Sigma). To quantify the enzyme activities in individual regenerated muscle fibers, regenerated muscle fibers were first identified by the presence of centrally located myonuclei from H & E stained images. Around 1000 regenerated fibers were measured for the mean grey value by Image J software (National Institute of Health, USA) and the enzyme activities were expressed as the mean optical density (OD) value of all pixels within a fiber (111). For uninjured muscle, the mean OD value for the whole TA cross sections was measured.

4.2.6 RNA preparation and quantitative real time PCR analysis

Isolation of total RNA from TA muscle was performed using TRIzol reagent (Invitrogen) according to manufacturer's instruction and RNA integrity was monitored by electrophoresis. Subsequent cDNA synthesis was conducted according to iScript cDNA Synthesis kit (Bio-Rad). SsoFast EvaGreen supermix (Bio-Rad) and the CFX96 Real Time System (Bio-Rad) were used for quantitative real time PCR reactions. Gene expression fold change was calculated using the delta-delta Ct method, normalized against *Gapdh* gene. The sequences of primers used in this chapter are listed in Table 2.1.

4.2.7 Western blot

Protein extracts from TA muscles were made in protein lysis buffer (50 mM Tris, pH 7.6, 250 mM NaCl, 5 mM EDTA, 0.1% P-40, complete protease inhibitor cocktail from Roche Molecular Biochemicals). 20µg of proteins were resolved by SDS-PAGE (4-12% gradient, Invitrogen) and transferred to a nitrocellulose membrane. Following

blocking with 5% milk in TBS-T, the membrane was incubated with anti-TFAM primary antibody (1:400, Santa Cruz Biotechnologies) and secondary antibody, goat anti-rabbit IgG conjugated with horseradish peroxidase (1:5000, Bio-Rad) in 5% milk in TBS-T. Immunoreactivity was detected using Western Lightning™ Chemiluminescence Reagent Plus (PerkinElmer). Band density was analyzed using the Quantity One imaging software (Bio-Rad).

4.2.8 Collagen concentration analysis

TA muscle samples were acid-hydrolyzed in 5M HCl (1ml 5M HCl / 10 mg of wet muscle weight) at 130°C for 12 h. Two aliquots for each sample (50 µl per aliquot) were taken for the hydroxyproline assay, using the methods adapted from Switzer and Summer (256). Collagen concentration (µg/mg) of the TA muscle was calculated from the hydroxyproline content assuming that collagen contains 13.4% hydroproline by weight (35).

4.2.9 Statistical Analysis

Statistical difference between groups was performed using unpaired two-tailed Student's T test and results were considered significant at $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***). Results were expressed as mean $\pm$ SEM (standard error of mean).

4.3 Results

4.3.1 Smad3-null injured TA muscle showed reduced muscle weight after regeneration

TA muscle of the left hindlimb of Smad3-null and Wild-type mice was injected with notexin and allowed to regenerate for 30 days. TA muscle from the right hindlimb was used as a contralateral uninjured control. TA muscle weights were measured 1, 2, 3, 7, and 30 days after notexin injection, along with body weights. The weight difference between the injured TA and uninjured control was calculated. Relative muscle weight change was calculated as the muscle weight change (between uninjured and injured muscle) normalized to 100 grams of body weight. Figure 4.1a demonstrates the relative muscle weight change from day 1 to day 30 after injury in both genotypes. During the early phase of regeneration, both genotypes showed a similar pattern of muscle weight change (Figure 4.1a). An increase in the relative weight of the injured TA muscle from day 1 to day 2 was found in both genotypes, which is attributed to the acute inflammatory response (53). On day 7, injured TA muscle weights were reduced when compared to uninjured controls in both genotypes.

However, significant differences between the two genotypes were found on day 3 and day 30. On day 3, enhanced muscle weight was still observed in the Wild-type injured muscle ($0.08 \pm 0.03\%$), whereas the Smad3-null muscle weight decreased ($-0.01 \pm 0.002\%$), when compared with contralateral controls. This suggests less edema and inflammation response in Smad3-null mice on day 3. On day 30, Wild-type TA muscle showed an increase in net weight ($15.07 \pm 1.48\text{mg}$), as compared to respective contralateral controls (Figure 4.1b). In contrast, the regenerated TA muscles from

Smad3-null mice were lighter in weight ($-5.80 \pm 2.40\text{mg}$), when compared to their respective uninjured controls (Figure 4.1b). When muscle weights were normalized to body weight, a similar pattern was observed (Figure 4.1c). The muscle weight loss in Smad3-null regenerated muscle was indicative of impaired muscle regeneration.

Figure 4.1 Decreased muscle weights of regenerated TA muscle of Smad3-null mice at 30 days after injury.

(a) Relative muscle weight change was calculated as the muscle weight change (the weight difference between the injured TA and uninjured TA muscles), normalized to 100 grams of body weight, at 1, 2, 3, 7, and 30 days after notexin injection in Wild-type and Smad3-null mice. Result shown in the black dashed box on the graph was displayed in detail in the third panel of the Figure. (b) Muscle weight change (mg) of TA muscle 30 days after notexin injection. (c) Relative muscle weight change of TA muscle 30 days after notexin injection. Values represent mean $\pm$ SEM. Statistical differences are indicated, $P < 0.05$ (*), $P < 0.01$ (**); $n = 3$ in each group.

4.3.2 Histological analysis during the early phase of Smad3-null mice muscle regeneration

As demonstrated in Figure 4.2, notexin treatment induced complete and extensive muscle degeneration in both genotypes 1 day after notexin injection. Infiltration of mononuclear cells, and edema were observed at day 1. On day 2 and day 3, the inflammatory response extended and necrotic myofibers, which were hyper-stained, were clearly seen. Necrotic myofibers phagocytosed by macrophages were also seen from day 1 to day 3 (Figure 4.2). Newly formed myofibers, characterized by centrally located nuclei and small fiber size, were identified on day 7 in TA muscles from both genotypes. Lower numbers of mononuclear cells were seen in Smad3-null muscle, 1 day after notexin injection (Figure 4.2). On day 2, Wild-type injured muscles were characterized by increased edema compared with muscles on day 1. However, Smad3-null injured muscle on day 2 had less edema as compared to Wild-type muscle on day 2.

Figure 4.2 *Histological analysis during the early phase of TA muscle regeneration in Wild-type and Smad3-null mice.*

TA muscle sections at 1, 2, 3, and 7 days after notexin injection were stained by Hematoxylin and Eosin (H & E) to examine muscle morphology during the early phases of muscle regeneration. Infiltration of inflammatory cells (I), edema (E), necrotic fibers (N) and newly formed myofibers (asterisks) are marked on the images. Scale bar represents 50 μ m.

4.3.3 Reduced inflammatory response in Smad3-null mice

During the initial phase of muscle regeneration, large numbers of inflammatory cells, mainly neutrophils and macrophages, are recruited from the circulation to the damaged muscle (238). Histological analysis of early muscle regeneration found less mononuclear cell infiltration in injured Smad3-null TA muscle (Figure 4.2). Immunohistochemical analysis was then performed to examine the inflammatory response, using anti-Mac-1 antibody to identify macrophages and neutrophils at day 2 and 3 after injury (Figure 4.3). Mac-1 (or CD11b, CD18) positive cells from 20 fields within the lesion area, during early muscle injury (day 2 and 3), were counted. Results show that Smad3-null muscle had significantly reduced inflammatory cells (Figure 4.3), suggesting that loss of Smad3 leads to decreased recruitment of inflammatory cells during muscle regeneration.

Figure 4.3 *Smad3* deficiency leads to dramatically reduced inflammatory response in injured TA muscle.

Anti-Mac-1 antibody was used to identify infiltrating macrophages and neutrophils in muscle sections. Scale bars represent 50 μ m. White asterisks represent the Mac-1 positive cells. Quantification of Mac-1 positive cells from injured TA muscle at 2 and 3 days after injury was conducted. Mac-1 positive cells were expressed as percentage of the total nuclei (DAPI positive). Error bars represent SEM. Statistical difference is indicated, $P < 0.001$ (***) ; $n = 3$ in each group.

4.3.4 Profound reduction in regenerated myofiber size in Smad3-null TA muscle after injury

Morphological analysis, using Hematoxylin and Eosin (H & E) staining, was conducted to quantitatively evaluate muscle regeneration 30 days after injury (Figure 4.4a). Regenerated myofibers are characterized by the presence of centrally located myonuclei (21). One of the obvious morphological differences between the two genotypes, after 30 days of injury, was that the regenerated fibers in Smad3-null muscle were significantly smaller than that of Wild-type (Figure 4.4a). The fiber size of the regenerated myofibers was quantified and represented by the mean Cross Sectional Area (CSA). The mean CSA of regenerated myofibers was about $98 \pm 4.5\%$ of its contralateral counterpart in Wild-type mice, at day 30 post-injury (Figure 4.4b). This demonstrated that Wild-type regenerated myofibers were comparable in size to that of uninjured controls. However, in Smad3-null mice, the mean CSA of regenerated fibers was only $44 \pm 8.0\%$ of its uninjured control (Figure 4.4b). The other observation we made was that in Wild-type regenerated muscle, multiple (2-4) centrally located myonuclei were often observed in each myofibers. However in contrast, the majority of regenerated myofibers in Smad3-null muscle had only one central myonucleus (Figure 4.4a). Therefore, we measured the number of central myonuclei. As shown in Figure 4.4c, $39 \pm 2.6\%$ of regenerated fibers in Wild-type muscle had multiple central myonuclei (2-4), while only $6 \pm 1.9\%$ of the regenerated fibers in Smad3-null muscle had more than 2 central myonuclei.

Figure 4.4 Decreased regenerated myofiber size in Smad3-null TA muscle 30 days after injury.

(a) Representative images of Hematoxylin and Eosin (H & E) staining of TA muscle cross-sections, 30 days post-injury (up) with uninjured controls (down) from Wild-type and Smad3-null mice. Scale bar represents 50 μm . (b) The size of the myofibers in the TA muscle was quantified as the mean Cross Sectional Area (CSA). The percentage of CSA recovery 30 days after injury was calculated as the percentage of the regenerated myofiber CSA, compared to the contralateral uninjured myofiber CSA. (c) Number of centrally formed myonuclei within regenerated myofibers in Wild-type and Smad3-null mice after 30 days of injury. Graph displays the percentage of myofibers with 1, 2, 3, or 4 centrally formed myonuclei. Values represent mean $\pm$ SEM. Statistical differences are indicated, $P < 0.01$ (**), $P < 0.001$ (***); $n = 3$ in each group.

4.3.5 Reduced number of myoblasts were found in Smad3-null regenerating muscle

Skeletal muscle regeneration is dependent on Satellite Cell (SC) function (124, 170). In response to muscle injury, quiescent SCs withdraw from the quiescent state (G_0) and re-enter the cell cycle (173). SCs are activated within 2 h after muscle damage and migrate to the damaged area to aid in repair. The proliferation of myoblasts peaks at 2-3 days post injury, after which myoblasts withdraw from the cell cycle again for differentiation. Differentiated myotubes form nascent myofibers and these myofibers undergo maturation through increasing myofiber size (248). Since MyoD is one of the markers of activated myoblasts as well as myogenic differentiation, we measured the mRNA transcript level of *MyoD* during the course of muscle regeneration (1, 2, 3, 7, and 30 days after notexin injection) by quantitative real time PCR. Gene expression analysis suggested that *MyoD* expression increased to a peak level at day 3 after injury and then progressively declined until day 7 in injured TA muscle from both genotypes. *MyoD* expression returned to the original level by day 30 in injured TA muscle of both genotypes. However, significantly lower *MyoD* expression was found in Smad3-null muscle at day 1, 3, 7, and 30 days after injury when compared to that in Wild-type muscle (Figure 4.5a). In addition to the gene expression analysis, we also performed MyoD immunohistochemical analysis on damaged TA muscle sections on day 3, when *MyoD* expression was at its peak, to identify the activated SCs responding to muscle injury. Consistent with the gene expression analysis, significantly reduced MyoD positive cells were found in Smad3-null muscle (Figure 4.5b and 4.5c), indicating less myoblasts were present in Smad3-null TA muscle after injury.

Figure 4.5 Injured TA muscle from *Smad3*-null mice showed reduced myoblast activation.

(a) Relative mRNA expression of *MyoD* was measured by quantitative real time PCR in the controls and injured muscles on 1, 2, 3, 7, and 30 days after notexin injection. Graph displays fold change $\pm$ SEM, normalized to *Gapdh*. (b) Anti-MyoD antibody was used to identify activated myoblasts in the muscle sections. Scale bars represent 50 μ m. White asterisks represent the MyoD positive myoblasts. (c) Quantification of MyoD positive cells from the TA muscles at 3 days after injury. MyoD positive cells were expressed as a percentage of the total nuclei (DAPI positive). Error bars represent SEM. Statistical differences are indicated, $P < 0.05$ (*), $P < 0.01$ (**); $n = 3$ in each group.

4.3.6 Less embryonic myosin heavy chain (eMyHC) positive nascent myofibers were found in *Smad3*-null regenerating muscle

Expression of embryonic myosin heavy chain (eMyHC) is a hallmark of muscle regeneration. Nascent myofibers express immature MyHC isoforms, such as eMyHC and neonatal MyHC (neoMyHC), rather than adult MyHC (type I/IIA/IIB/IIX) (288). To examine the formation of newly formed myofibers, eMyHC staining was performed on muscle sections at day 7 post injury. At day 7, we observed a large number of eMyHC expressing, centrally nucleated myofibers in Wild-type TA muscle (Figure 4.6a). However, only a few eMyHC positive myofibers were found in *Smad3*-null regenerating muscle (Figure 4.6a). In addition to immunohistochemical staining, we also analyzed *eMyHC* mRNA level in the regenerating muscles from both genotypes by quantitative real time PCR. As Figure 4.6b shows, there was a sharp increase in the *eMyHC* expression for both genotypes on day 3 and then the expression gradually returned to control level on day 30 for both genotypes. However, the peak level of *eMyHC* mRNA (day 3) and the level at day 7 in *Smad3*-null injured TA muscle were significantly lower when compared to that of Wild-type (Figure 4.6b).

Figure 4.6 Injured TA muscle from *Smad3*-null mice had less nascent myofiber formation.

(a) Muscle sections from injured TA muscle at 7 days after injury were stained with a specific antibody against embryonic myosin heavy chain (eMyHC). Scale bars represent 500 μm (left) and 50 μm (middle, higher magnification image). White asterisks represent the positive eMyHC myofibers. Non-specific staining is indicated by the negative control (right). (b) Relative mRNA expression of *eMyHC* was measured by quantitative real time PCR in the control and injured muscles on day 1, 2, 3, 7, and 30 after notexin injection. Graph displays fold change $\pm$ SEM, normalized to *Gapdh*. Statistical differences are indicated, $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***); $n = 3$ in each group.

4.3.7 Metabolic properties of *Smad3*-null regenerated muscle

The metabolic properties of skeletal muscle determine muscle function, as well as metabolic substrate utilization. Here we studied the metabolic phenotypes of TA muscle at day 30 post injury in both genotypes. Three important metabolic enzymes were analyzed in this study, succinate dehydrogenase (SDH) and cytochrome c oxidase (COX), markers of aerobic (oxidative) capacity, and α -glycerophosphate dehydrogenase (α -GPD) for glycolytic potential (41, 168). The activities of the enzymes in the regenerated myofibers were demonstrated by histochemical reactions and quantified by image analysis. We observed uneven staining for both SDH and COX activities in regenerated TA muscles (30 days) of both genotypes (Figure 4.7a, c). However, a significant difference was found between the regenerated muscles from the two genotypes (Figure 4.7a, c). Wild-type regenerated fibers showed strong SDH and COX stainings in the cytoplasm, whereas significantly less intense staining was observed in the regenerated myofibers in *Smad3*-null muscle (Figure 4.7a, c), which suggested lower level of both oxidative enzyme activities. Quantitative analysis demonstrated that SDH activity in *Smad3*-null regenerated myofibers was about 30% lower than that in Wild-type myofibers (Figure 4.7d) and COX activity was about 15% lower in *Smad3*-null regenerated myofibers (Figure 4.7g). Even though slightly weaker staining for α -GPD activity was observed in *Smad3*-null mice, the difference between the two genotypes was not significant ($P > 0.05$) (Figure 4.7b and 4.7e). GPD/SDH ratio is an index of metabolic substrate utilization (85). Figure 4.7f demonstrated that GPD-to-SDH (glycolytic-to-oxidative) ratio was lower in *Smad3*-null muscle at day 30 post injury, when compared to Wild-type muscle. This change in ratio in *Smad3*-null regenerated myofibers suggested enhanced reliance on glycolytic metabolism.

Oxidative metabolic pathways occur in the mitochondria of myofibers and mitochondrial biogenesis is an important event for muscle regeneration (280). We hypothesized that lower oxidative enzyme activities in Smad3-null regenerated TA muscle might be due to impaired mitochondrial biogenesis. Therefore, we analyzed the mRNA expression of two master regulators of mitochondrial biogenesis, mitochondrial transcription factor A (*Tfam*) and nuclear respiratory factor1 (*NRF1*), in regenerated TA muscles from both genotypes (276). Figure 4.7h-i shows that the expression of both genes was down-regulated in Smad3-null TA muscle after 30 days of muscle regeneration. Western blot analysis confirmed the reduced expression of TFAM in Smad3-null regenerated muscle (Figure 4.7j). However, we did not observe a significant difference in the protein level of NRF1 in the regenerated TA muscles from the two genotypes (data not shown).

Figure 4.7 *Smad3*-null regenerated myofibers showed decreased SDH and COX enzyme activities and down-regulated expression of genes relating to mitochondrial biogenesis

(a) succinate dehydrogenase (SDH) staining, (b) α -glycerophosphate dehydrogenase (α -GPD) staining, and (c) cytochrome c oxidase (COX) of Wild-type and *Smad3*-null TA muscle at day 30 after injury. Black dashed boxes on the left images indicate the regions in the magnified image on the right side. Scale bar (left) represents 500 μ m and scale bar (right) represents 50 μ m in higher magnification image. Quantitative analysis of SDH activity (d), α -GPD activity (e), GPD/SDH ratios (f), and COX activity (g) in regenerated muscle 30 days post-injury. Values represent mean $\pm$ SEM. (h-i) Relative mRNA expression of *NRF1* and *Tfam* were measured by quantitative real time PCR in regenerated TA muscle from Wild-type and *Smad3*-null mice (30 days after notexin injection). Graphs display fold change $\pm$ SEM, normalized to *Gapdh*. (j) Western blot analysis of TFAM protein in the regenerated TA muscle from both genotypes (30 days after notexin injection). Ponceau S staining was performed to ensure equal loading of samples. Graph displays the densitometric analysis of TFAM levels, normalized to Ponceau S staining. For all the panels, statistical differences are indicated, $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) ; $n = 3$ in each group.

4.3.8 Decreased scar tissue in *Smad3*-null regenerated muscle

Previous studies show that remodelling of extracellular matrix (ECM) during muscle regeneration begins at about 14 days post injury (234). Excessive growth of ECM and deposition of collagen gives rise to scar tissue formation within the injured muscle tissue, which impairs the full recovery of muscle function. Van Giesen's stain was used to examine fibrotic tissue within the regenerated muscle at day 30 after notexin injection (Figure 4.8a). Quantification of collagen, by morphometric analysis, suggested that *Smad3*-null mice had significantly reduced interstitial fibrotic tissue in regenerated muscle when compared to Wild-type mice (7 ± 0.06 % compared with 20 ± 1.79 %) (Figure 4.8b). Since collagen is the most abundant structural component in skeletal muscle ECM (258), we also measured the total collagen content in regenerated TA muscle ($\mu\text{g/ml}$) via hydroxyproline assay. For Wild-type mice, a remarkable increase in collagen deposition was found in the regenerated TA muscle at day 30 after notexin injection, when compared to uninjured TA muscle (Figure 4.8c). However, collagen accumulation in *Smad3*-null TA muscle after regeneration was significantly lower when compared to Wild-type regenerated muscle (Figure 4.8c). Fibrillar collagen type I and III, and non-fibrillar collagen type IV are the predominant collagen isoforms in skeletal muscle (16). Therefore, the mRNA levels for *collagen Ia1*, *collagen IIIa1* and *collagen IVa1* were also measured by quantitative real time PCR. The results indicated that *Smad3*-null regenerated TA muscle had significantly decreased expression of *collagen Ia1*, *collagen IIIa1* and *collagen IVa1* (Figure 4.8d).

Figure 4.8 *Decreased scar tissue formation in Smad3-null muscle after injury.*

(a) Representative images of Van Giesen's staining of TA muscle cross-sections, 30 days post-injury, from Wild-type and Smad3-null mice. Scale bar represents 50 μ m. (b) Quantification of interstitial fibrotic tissue by morphometric analysis. The percentage of fibrosis in each sample was calculated as the ratio of the fibrotic area to the total muscle cross sectional area (CSA) (%). Values represent mean $\pm$ SEM. (c) The total collagen content of uninjured controls and regenerated TA muscles 30 days after notexin injection as quantified by hydroxyproline assay. Values represent mean $\pm$ SEM. (d) Relative mRNA expression of *Collagen Ia1*, *Collagen IIIa1* and *Collagen IVa1* were measured by quantitative real time PCR in regenerated TA muscle from Wild-type and Smad3-null mice (30 days after notexin injection). Graphs display fold change $\pm$ SEM, normalized to *Gapdh*. For all the panels, statistical differences are indicated, $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***); $n = 3$ in each group.

4.3.9 Consistent up-regulation of *Myostatin* expression in *Smad3*-null TA muscle during the course of muscle regeneration

Myostatin plays a negative regulatory role in muscle regeneration, and thus loss of *Myostatin* results in improved muscle regeneration (173). Therefore, the endogenous expression of *Myostatin* during the course of muscle regeneration was determined by quantitative real time PCR (Figure 4.9). The time course of *Myostatin* expression is illustrated in Figure 4.9. These data demonstrate that during the regeneration process, the expression of *Myostatin* in *Smad3*-null TA muscle was consistently higher when compared to that in Wild-type muscle (Figure 4.9). In Wild-type injured TA muscle, *Myostatin* mRNA level steadily increased after injury and peaked at day 2, then declined until day 7 before returning to uninjured control level (Figure 4.9). However, in *Smad3*-null injured TA muscle, *Myostatin* expression dropped sharply at day 1 from uninjured control level and increased afterwards until day 3. Much like the expression in Wild-type muscle, *Myostatin* level in *Smad3*-null muscle then decreased until day 7 and finally returned to the uninjured control level at day 30 (Figure 4.9).

Figure 4.9 *Relative mRNA expression of Myostatin during the process of muscle regeneration*

Relative mRNA expression of *Myostatin* was measured by quantitative real time PCR in the control and injured muscles at 1, 2, 3, 7, and 30 days after notexin injection. Graph displays fold change $\pm$ SEM, normalized to *Gapdh*. Statistical differences are indicated, $P < 0.01$ (**), and $P < 0.001$ (***); $n = 3$ in each group.

4.4 Discussion

In this study, we used notexin-induced muscle injury to analyze the regeneration capacity of Smad3-null muscle, with the overall aim to further understand the role of Smad3 signaling in the regulation of post-natal myogenesis. Our results suggest that Smad3 is essential for normal muscle regeneration, since Smad3 deficiency resulted in defective muscle repair.

4.4.1 The role of Smad3 in adult muscle regeneration

During the early stage of muscle regeneration, significantly reduced monocyte infiltration was observed in Smad3-null injured muscle (Figure 4.2 and 4.3). This decreased inflammatory response in Smad3-null injured muscle could result from the impaired chemotaxis of Smad3-null monocytes. It is well-known that TGF- β is a potent chemoattractant for neutrophils (218), moreover, Smad3-null monocytes have an intrinsic impaired response towards TGF- β -induced chemotaxis (297). Reduced inflammatory response has been reported in skin excisional wounds (14) and cutaneous wounds of Smad3-null mice (74). Reduced neutrophil infiltration was also found in the infarcted myocardium of Smad3-null mice during the early stage of inflammatory response (38). Pro-inflammatory cytokines released by neutrophils recruit macrophages. A subpopulation of pro-inflammatory macrophages (CD68⁺) release pro-inflammatory cytokines to promote inflammatory response and are also responsible for the phagocytosis of muscle debris. Once the necrotic tissue is cleared, pro-inflammatory macrophages undergo a phenotype switch to become anti-

inflammatory macrophages (CD163⁺) which secrete anti-inflammatory cytokines such as IL-10 to terminate the inflammatory response. This population of macrophages are believed to promote muscle regeneration (196). Therefore, timely activation and repression of the inflammatory response is required and critically important for muscle regeneration. A previous study has shown that suppression of macrophages at the time of injury leads to incomplete regeneration (241). Blocking of anti-inflammatory macrophages leads to small regenerated myofiber size (263). Therefore, the inhibited inflammatory response found in Smad3-null skeletal muscle may contribute to the impaired muscle regeneration.

The weight and the size of regenerated myofibers in Smad3-null TA muscle were remarkably reduced, as compared to uninjured control (Figure 4.1 and 4.4). These defects clearly indicate reduced myogenesis, which could be due to impaired SC activity. In muscle regeneration, SCs play a pivotal role (248). In response to muscle injury, SCs are activated and migrate to the site of injury, in response to chemo-attractants secreted by macrophages, to furnish muscle precursor cells, which then proliferate and differentiate to give rise to nascent muscle fibers (23, 106, 248). Using MyoD as a marker for activated myoblasts, we observed significantly reduced myoblast populations in the damaged area of Smad3-null injured TA muscle (Figure 4.5). Our previous study has revealed that reduced SC number in Smad3-null muscle fibers is due to reduced SC self-renewal capacity. Moreover, *in vitro* experiments also revealed decreased proliferation ability and defective myoblast fusion in Smad3-null primary myoblasts (81). Normally, SCs present in muscle fibers after activation provide sufficient myoblasts within the injured area for myogenesis. However, loss of Smad3 led to a reduction in the activated SC population recruited to the injured area in Smad3-null muscle, contributing to the impaired muscle regeneration. Furthermore,

the majority of regenerated myofibers in Smad3-null mice only have one centralized myonucleus, indicating impaired myoblast fusion (Figure 4.4). In support, previously published primary culture experiments showed that Smad3-null myotubes are smaller, with reduced fusion index and contain less number of myonuclei per myotube. In addition, several fusion-related genes, *β 1D Integrin*, *SHP-2*, and *Caveolin-3* were also down-regulated (81). This intrinsic fusion impairment of Smad3-null myoblasts might explain the smaller size and reduced central myonuclei number in Smad3-null regenerated myofibers, which may contribute to incomplete muscle recovery.

We analyzed the metabolic properties of regenerated muscle from both genotypes through histochemical staining of SDH, COX and α -GPD activity. SDH and COX activity are indicators of mitochondrial oxidative capacity (41, 97) and tricarboxylic acid (TCA) cycle activity in myofibers. α -GPD in both cytosol and mitochondria reflects the activity of the glycerol-phosphate shuttle and is considered to be a key enzyme in muscle fiber glycolysis (207). Our results demonstrated that Smad3-null TA muscle had significantly decreased levels of SDH and COX activity, which suggested an incomplete recovery of oxidative capacity (Figure 4.7). Since difference in the muscle fiber type profile of regenerated muscle might be responsible for the difference in the muscle metabolic profile, we compared the muscle fiber type composition between Wild-type and Smad3-null regenerated muscles. Subsequent immunohistochemical staining with antibodies against adult MyHC isoforms revealed no significant difference between the two genotypes (data not shown). Therefore the remarkable decline in the oxidative enzyme activity of Smad3-null regenerated muscle was not due to the loss of slow twitch fibers or increased fast fibers. We suggest that impaired mitochondrial biogenesis in Smad3-null muscle during regeneration, evident by reduced expression of the master regulator of mitochondrial biogenesis, *TFAM*, at

both the transcriptional and translational levels (Figure 4.7), may be responsible for the metabolic differences. TFAM is a key transcription factor responsible for both the replication and transcription of mitochondrial DNA (237), thus its down-regulation in *Smad3*-null regenerated muscle indicates defective mitochondrial biogenesis. Previous studies suggest an essential role for mitochondria in muscle regeneration, where mitochondria are required for myoblast proliferation and differentiation in addition to its primary function in energy metabolism (226, 243). *In vivo* muscle regeneration studies have indicated that expression of mitochondrial biogenesis-related genes are synchronized with the expression of myogenic genes, such as *MyoD* and *Myogenin* (64, 279). As such, blocking of mitochondrial protein synthesis during muscle regeneration leads to small myofibers (279). In addition, unpublished data from our lab found that genes pertinent to mitochondrial biogenesis were up-regulated at both transcriptional and translational levels in *Myostatin*-null skeletal muscle when compared to Wild-type (Rashid *et al.* ⁴). Moreover, lack of *Myostatin* also enhanced mitochondrial function and activity (Sudarsanareddy *et al.* ⁵). The negative impact of *Myostatin* on mitochondrial biogenesis and function might result in defective mitochondrial biogenesis and reduced oxidative enzyme activity in *Smad3*-null regenerated muscle. Therefore, impaired mitochondrial biogenesis could be one of the contributing factors behind the muscle regeneration defects in *Smad3*-null mice. Moreover, since the largest subunits of SDH are all encoded by the nuclear genome while the three largest subunits of COX are encoded by mitochondrial DNA (mtDNA) (41). Significant reduce in both SDH and COX enzyme activity in *Smad3*-null regenerated myofibers suggests that *Myostatin* might have negative impact on mitochondrial proteins encoded by both genome and mitochondrial DNA.

⁴ Rashid *et al.* manuscript in preparation

⁵ Sudarsanareddy *et al.* manuscript in preparation

4.4.2 Role of Myostatin in the impaired muscle healing in *Smad3*-null mice

Previously published work from our lab revealed that *Myostatin* expression was up-regulated in *Smad3*-null muscle and that this was partially responsible for the profound muscle atrophy observed in *Smad3*-null mice (81). In this study, we observed consistent up-regulation of *Myostatin* mRNA expression in *Smad3*-null injured TA muscle during the process of muscle regeneration (Figure 4.9). Myostatin is a powerful regulator of both muscle growth and regeneration (120, 173, 183), and inactivation of Myostatin significantly enhances muscle regeneration after acute injury (173, 281). Our previous studies demonstrated that Myostatin inhibits SC activation, proliferation and chemotaxis (172-173), therefore, increased *Myostatin* expression in *Smad3*-null regenerating TA muscle might inhibit activation of SCs as well as proliferation of myoblasts. Myostatin is also known to down regulate *MyoD* expression, which is consistent with the lower *MyoD* levels seen in *Smad3*-null muscle when compared to the controls (Figure 4.5). Thus, the inhibitory effect of Myostatin might be responsible for poor regeneration of *Smad3*-null muscle.

4.4.3 The role of *Smad3* in scar tissue formation during muscle regeneration

Connective tissue is an integral part of skeletal muscle and it includes tendons and three sheaths called endomysium, perimysium, and epimysium (258). Remodelling of connective tissue is an essential step in muscle regeneration and fibroblasts are the major mediator of fibrosis. Following muscle degeneration, mesenchymal fibroblasts are activated to become myofibroblasts. They proliferate, differentiate, and express high level of α -smooth muscle actin (α -SMA), which is required for wound closure. Myofibroblasts are also responsible for the synthesis of other ECM components. The

balance between fibrosis and myofiber regeneration determines the functional recovery of skeletal muscle (72). Excessive accumulation of fibrotic tissue during muscle regeneration leads to scar tissue formation, which results in attenuated muscle strength and functional recovery. In this study, we found increased deposition of interstitial fibrous tissue in Wild-type TA muscle at day 30 of regeneration. However, this deposition was significantly attenuated in Smad3-null regenerated muscle (Figure 4.8). Furthermore, gene expression analysis also suggested that genes encoding the major collagen isoforms in skeletal muscle (*Collagen I*, *Collagen III* and *Collagen IV*) were down-regulated (Figure 4.8). These results demonstrate that Smad3 plays an essential role in the accumulation of fibrous tissue during muscle regeneration. Our study is consistent with a large number of studies showing that Smad3 signaling is required for tissue fibrosis. TGF- β 1 is the most potent pro-fibrotic cytokine described to date. It stimulates fibroblast function and promotes ECM protein synthesis (43, 261). Enhanced TGF- β 1 signaling is involved in fibrosis in liver, renal, and lung diseases (80, 109, 140, 235). Growing evidence suggests that TGF- β 1 up-regulates ECM related gene expression through a Smad3-dependent pathway (298). *In vitro*, Smad3^{ex8/ex8} fibroblasts have decreased proliferation and pro-fibrotic response stimulated by TGF- β (140). Microarray data from Smad3-null fibroblasts suggests that Smad3 is required to initiate numerous ECM related gene expression upon TGF- β stimulation (295). Smad3 directly associates with the promoters of ECM related genes, such as Collagen I α 1 and CTGF, to initiate gene expression. Recent studies demonstrate that Smad3 is also involved in myocardial fibrosis by controlling the biogenesis of microRNAs (miRNA) (61). Moreover, loss of Smad3 protects against tissue fibrosis in a number of diseases involving kidney, liver, intestine and skin (74, 144).

In summary, Smad3 plays a critical role in muscle regeneration, from the inflammatory response to myogenesis and finally in fibrosis. Based on the results from this study, we put forward a model which suggests the role of Smad3 in the regulation of SC function, myoblasts fusion, inflammatory response, and ECM remodelling during muscle regeneration (Figure 4.10).

Figure 4.10 Proposed model for the role of Smad3 in muscle regeneration.

Smad3-null mice show defective muscle regeneration. Smad3 deficiency leads to reduced inflammatory response, impaired SC function and defects in myoblast fusion, possibly due to consistent up-regulation of *Myostatin* expression during the course of muscle regeneration. However, loss of functional Smad3 protects regenerating muscle from excessive extracellular matrix (ECM) deposition.

Chapter 5 Gene expression profiling of skeletal muscle of Smad3-null mice

Gene expression profiling of skeletal muscle of Smad3-null mice

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Abstract

Smad3 is a key intracellular signaling mediator for TGF- β superfamily members. Two members of the TGF- β superfamily, TGF- β and Myostatin, are major regulators of skeletal muscle growth and metabolism and have been shown to signal via Smad3. Previous results showed that Smad3-null mice have atrophied musculature and are resistant to diet-induced obesity. In this study, we performed a microarray analysis to identify the molecular mechanisms through which Smad3 regulates muscle growth and metabolism. Smad3-null skeletal muscle showed 29 up-regulated and 36 down-regulated transcripts when compared to Wild-type muscle. Pathway analysis indicated that loss of Smad3 results in down-regulation of genes involved in extracellular matrix

(ECM) and glucose metabolism. The expression of 5 genes encoding ECM components, namely *Colla2*, *Fmod*, *Comp*, *Spon2* and *Chad*, were significantly down-regulated in skeletal muscle due to Smad3 deficiency. Loss of Smad3 also lead to down-regulated expression of genes encoding for key enzymes involved in glycolysis (*Pkm2*) and pyruvate oxidation (*Dlat*), and up-regulated expression of gene encoding for the key inhibitor of pyruvate oxidation (*Pdk4*). Taken together these gene expression changes could lead to inhibited glycolysis and pyruvate oxidation, as well as a possible catabolic fuel shift from glucose oxidation to lipid oxidation in skeletal muscle. Moreover, Smad3 deficiency resulted in the up-regulated expression of two inflammatory cytokines in skeletal muscle, namely *SI00A8* and *SI00A9*, which could be the molecular reason behind the systemic inflammation found in Smad3-null mice. Interestingly, expression of genes critical for circadian rhythm regulation (*Clock*, *Per1*, and *Per2*) was also dysregulated in Smad3-null skeletal muscle. These results therefore underscore the diverse role played by Smad3 signaling in muscle composition and metabolism, inflammation, and circadian rhythms.

5.1 Introduction

The TGF- β superfamily is a large group of extra cellular growth factors which regulate several biological processes, such as embryogenesis, inflammatory response, cell proliferation, differentiation, migration and adhesion (130). Smad3 is one of the essential cellular mediators for TGF- β superfamily signaling pathways, transducing signals directly from cell surface receptors to the nucleus (191). Upon ligand binding, the activated cell surface receptor complex recruits and phosphorylates the two serine

residues in the C-terminus of Smad3 protein. After phosphorylation, Smad3 undergoes a conformational change and forms a heteromeric complex with the co-Smad, Smad4. An exposed nuclear localization signal (NLS) domain in Smad3/Smad4 then triggers the translocation of the complex to the nucleus. Together with a variety of co-activators and co-repressors, Smad3/Smad4 regulate expression of TGF- β superfamily target genes (33).

It is well-accepted that Smad3 is involved in signal transduction for Myostatin, a TGF- β superfamily member, that functions as an important negative regulator of muscle growth (120, 183). However, few studies have been conducted to unveil the specific role of Smad3 in the regulation of skeletal muscle growth. A previous study from our lab investigated the function of Smad3 in post-natal myogenesis by utilizing a Smad3 knockout mouse model. The results indicated that Smad3 deficiency resulted in atrophied skeletal muscle, due to increased muscle protein degradation and impaired satellite cell (SC) functionality (81). Interestingly, enhanced Myostatin expression in muscle and primary cultures partially contributed to the atrophied musculature observed in Smad3-null mice (81). Myostatin has been shown to inhibit myoblast proliferation (266, 296), differentiation (118, 142, 224), as well as satellite cell (SC) activation and self-renewal (172, 176). Our data showed that inactivation of Myostatin signaling either through *Myostatin* gene knock-out or through treatment with a Myostatin antagonist, partially rescued the muscle atrophy observed in Smad3-null mice (81). Furthermore, unpublished data from our lab indicated that skeletal muscle from the Smad3-null mice failed to regenerate efficiently after notexin-induced injury (see Chapter 4).

Although our studies suggest a critical role of Smad3 in post-natal myogenesis, the molecular mechanisms by which Smad3 regulates muscle homeostasis deserve further

study. Analysis of molecular changes associated with the loss of Smad3 function in skeletal muscle will provide further understanding about the regulatory role of Smad3 in skeletal muscle growth, as well as the molecular basis of muscular atrophy observed in Smad3-null mice. Microarray technique enables us to simultaneously assess the expression profiles of a large number of genes. Therefore the main objective of the present study was to detect the changes in global gene expression patterns in skeletal muscle due to Smad3 deficiency through microarray analysis. Functional network analysis revealed that pathways related to extracellular matrix (ECM), inflammation, metabolism, and circadian rhythm were dysregulated in Smad3-null skeletal muscle. This is the first study which has investigated the transcriptome profile of Smad3-null skeletal muscle and sheds a new level of insight into the large-scale molecular changes in atrophied muscle due to loss of Smad3.

5.2 Materials and methods

5.2.1 Animals

Smad3^{+/-} mice were kindly gifted by Prof. Walter Wahli (University of Lausanne, Lausanne, Switzerland). The Smad3^{-/-} (Smad3^{ex8/ex8}) mice have been previously described and were generated by targeted disruption of *exon 8* of the *Smad3* gene through homologous recombination (297). Transgenic mouse lines (Smad3^{-/-}, Smad3^{+/+}, and Smad3^{+/-}) were produced through mating pairs of Smad3^{+/-} mice. Genotyping was performed according to the previous published study (297).

5.2.2 Microarray analysis

Microarray analysis was performed using *M. quadriceps* (Quad) muscle from 6-week-old Smad3-null and Wild-type male mice (3 mice for each genotype). Muscles were dissected and frozen immediately in liquid nitrogen and kept at -80 °C until RNA extraction was performed. Total RNA was isolated using TRIzol reagent (Invitrogen) and RNA integrity was monitored by electrophoresis. Whole-genome expression analysis was carried out using Illumina microarray. The labeled cRNA samples were hybridized to Illumina's Mouse MG-6 v2.0 Expression BeadChips according to the manufacturer's instructions (Illumina, Inc., San Diego, CA). Illumina BeadStudio software (version 3.3.7) was used to assess the signal intensity with background correction. Raw data with background subtracted were loaded from BeadStudio to *lumi* package (Bioconductor), especially designed for Illumina microarray data processing (63). Data was normalized using quantile normalization by *lumi* software. *Lumi* is also used to perform quality control of raw data, quality control after

normalization, and gene annotation, as well as all statistical analysis on the microarray data. Gene expression fold change of Smad3-null samples was calculated as the average signal value relative to Wild-type samples. Genes with a fold change ≥ 1.5 are considered to be up-regulated while genes with a fold change ≤ -1.5 are down-regulated. Student's T-test was used to determine significant differences in transcription profile between two genotypes. P-value of 0.05 was used as the cutoff value to define differentially expressed genes. For visualization purpose, a heat map was generated from fold change of differentially expressed genes using the microarray visualization tool, Orange Canvas 1.0 software (<http://orange.biolab.si>).

5.2.3 Gene ontology (GO) and functional network analysis

The significant overrepresentation of Gene Ontology (GO) terms among the differentially expressed genes was analyzed using the online tool from Mouse Genomics Informatics database (MGI) (<http://www.informatics.jax.org>). GO assignments of differentially regulated genes were performed according to the GO categories of biological processes or cellular components. Functional classification of genes with altered expression pattern in Smad3-null skeletal muscle was performed using tools including Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Mountain View, CA, USA) <http://www.ingenuity.com>, and Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resources <http://david.abcc.ncifcrf.gov/> (102-103).

5.2.4 Quantitative real time PCR

Isolation of total RNA from *M. quadriceps* (Quad) muscle was performed using TRIzol reagent (Invitrogen) according to the manufacturer's instruction. Subsequent cDNA synthesis was conducted according to iScript cDNA Synthesis kit (Bio-Rad). SsoFast EvaGreen supermix (Bio-Rad) and the CFX96 Real Time System (Bio-Rad) were used for quantitative real time PCR reactions. Gene expression fold change was calculated using the delta-delta Ct method. The housekeeping gene, *GAPDH*, was used as an endogenous control for normalization. Table 2.1 lists the gene-specific nucleotide sequences of oligonucleotides used in quantitative real time PCR. Pearson's correlation coefficient (R) was used to estimate the correlation of quantitative real time PCR results and microarray results by Excel.

5.2.5 Collagen concentration analysis

Half of the entire costal diaphragm muscle, without any tendinous material, was dissected according to the method of Shavlakadze *et al.*(245). Collagen concentration of diaphragm muscle ($\mu\text{g}/\text{mg}$) was quantitatively determined by hydroxyproline assay assuming that collagen contains 13.4% hydroxyproline by weight (35). Measurement of hydroxyproline content in diaphragm muscle was conducted using the methods of Switzer and Summer (256). Diaphragm muscle samples were firstly acid-hydrolyzed in 5M HCl (1ml 5M HCl / 10 mg of wet muscle weight) at 130°C for 12 h and two aliquots for each sample (50 μl per aliquot) were taken for measurement. Briefly, hydroxyproline in the hydrolysate of samples was oxidized with 0.2 M chloramines-T and then converted to pyrrole by boiling. Pyrrole was extracted in toluene and mixed

with Ehrlich's reagent for colorimetric assay at 560 nm. Hydroxyproline content in the samples was calculated from the standard curve (Sigma Aldrich, Singapore).

5.2.6 Statistical analysis

Statistical significance was performed using the two-tailed Student's T test and results were considered significant at $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***). Results were expressed as mean $\pm$ SEM (standard error of mean). All statistical analysis of the microarray data was conducted using *lumi* software.

5.3 Results and discussion

5.3.1 Significant gene expression changes were found in Smad3-null skeletal muscle

To uncover the role of Smad3 in the regulation of skeletal muscle growth, whole genome Illumina BeadArray microarray was utilized to assess the global gene expression patterns in Smad3-null skeletal muscle. The *M. quadriceps* (Quad) muscle was used in this study because of its mixed MyHC isoform expression, as well as its profound weight loss in Smad3-null mice when compared to that of Wild-type mice (see Chapter 3, Figure 3.1b). Illumina's Mouse MG-6 v2.0 Expression BeadChips, used in the present study allowed analysis of changes in mRNA levels of 45,281 mouse transcripts. With a maximal P value of 5%, we found a total of 65 transcripts showing differential expressions between the two genotypes (minimal Fold change of 1.5). These transcripts represent around 0.11 % of the total number of transcripts present on the Illumina BeadChip. Among these 65 transcripts, we found 36 genes that were up-regulated and 29 genes that were down-regulated significantly in Smad3-null skeletal muscle, compared to Wild-type. The differentially expressed genes (annotated) in Smad3-null skeletal muscle compared to Wild-type samples are summarized in Table 5.1 and highlighted in the heat map (Figure 5.1). Non-annotated genes, including Riken cDNAs and hypothetical proteins are listed in Table 5.2 and are also shown in the heat map (Figure 5.1).

Figure 5.1 Heat map showing the relative expression levels of differentially regulated transcripts in Smad3-null skeletal muscle

Heat map depicts the relative expression levels of the 65 differentially regulated transcripts in Smad3-null skeletal muscle ($P < 0.05$ and Fold change ≥ 1.5 or ≤ -1.5). Fold changes were mapped to a color gradient from down-regulated expressions (green color) to up-regulated expressions (red color). Genotypes of the samples are shown at the bottom of the map and the gene symbols are listed on the right side of the map in rows.

Table 5.1 List of the differentially expressed annotated genes

Differentially expressed annotated genes in Smad3-null skeletal muscle when compared to Wild-type muscle. P value cutoff of $P < 0.05$ and fold change (FC) cutoff of $FC \geq 1.5$ or $FC \leq -1.5$ were used to filter differentially expressed genes.

Gene ID	Gene Name	Description	FC
Down-regulated			
241431	XIRP2	xin actin-binding repeat containing 2, transcript variant 1, mRNA.	-4.23
229228	NUDT6	nudix (nucleoside diphosphate linked moiety X)-type motif 6, mRNA.	-2.59
17841	MUP2	major urinary protein 2, transcript variant 1, mRNA.	-2.51
71687	TMEM25	transmembrane protein 25, mRNA.	-2.35
67582	SLC25A26	solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 26, nuclear gene encoding mitochondrial protein, mRNA.	-2.32
12643	CHAD	chondroadherin, mRNA.	-2.10
17840	MUP1	major urinary protein 1, mRNA.	-1.95
15439	HP	haptoglobin, mRNA.	-1.92
18746	PKM2	pyruvate kinase, muscle, mRNA.	-1.91
270192	RAB6B	RAB6B, member RAS oncogene family	-1.91
100689	SPON2	spondin 2, extracellular matrix protein, mRNA.	-1.85
14264	FMOD	fibromodulin, mRNA.	-1.77
21906	OTOP1	otopetrin 1, transcript variant b, mRNA.	-1.70
12845	COMP	cartilage oligomeric matrix protein, mRNA	-1.65
55991	PANX1	pannexin 1, mRNA.	-1.64
99887	TMEM56	transmembrane protein 56, mRNA.	-1.61
170942	ERDR1	erythroid differentiation regulator 1, mRNA.	-1.60
107765	ANKRD1	ankyrin repeat domain 1 (cardiac muscle), mRNA.	-1.60
69219	DDAH1	dimethylarginine dimethylaminohydrolase 1, mRNA	-1.58
78321	ANKRD23	ankyrin repeat domain 23, mRNA.	-1.57
12843	COL1A2	collagen, type I, alpha 2, mRNA.	-1.57
26450	RBBP9	retinoblastoma binding protein 9, mRNA.	-1.56

16012	IGFBP6	insulin-like growth factor binding protein 6, mRNA.	-1.56
235339	DLAT	dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex), mRNA.	-1.55
12753	CLOCK	circadian locomotor output cycles kaput, mRNA.	-1.53
Up-regulated			
20201	S100A8	S100 calcium binding protein A8 (calgranulin A), mRNA.	3.96
20202	S100A9	S100 calcium binding protein A9 (calgranulin B), mRNA.	2.51
14539	OPN1MW	opsin 1 (cone pigments), medium-wave-sensitive (color blindness, deutan), mRNA.	3.46
66402	SLN	sarcolipin, mRNA.	3.44
16002	IGF2	insulin-like growth factor 2, mRNA.	2.43
12700	CISH	cytokine inducible SH2-containing protein, mRNA.	2.18
227731	SLC25A25	solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 25, nuclear gene encoding mitochondrial protein, mRNA.	2.11
23882	GADD45G	growth arrest and DNA-damage-inducible 45 gamma, mRNA.	1.99
93694	CLEC2D	C-type lectin domain family 2, member d, mRNA.	1.93
12577	CDKN1C	cyclin-dependent kinase inhibitor 1C (P57), mRNA.	1.90
27273	PDK4	pyruvate dehydrogenase kinase, isoenzyme 4, mRNA.	1.88
18627	PER2	period homolog 2 (Drosophila), mRNA.	1.80
71706	SLC46A3	solute carrier family 46, member 3, mRNA.	1.77
74388	DPP8	dipeptidylpeptidase 8, mRNA.	1.76

11475	ACTA2	actin, alpha 2, smooth muscle, aorta, mRNA.	1.75
15130	HBB-B2	hemoglobin, beta adult minor chain, mRNA.	1.68
13386	DLK1	delta-like 1 homolog (Drosophila), mRNA.	1.67
67457	FRMD8	FERM domain containing 8, mRNA.	1.66
66797	CNTNAP2	contactin associated protein-like 2, transcript variant 1, mRNA.	1.64
74155	ERRFI1	ERBB receptor feedback inhibitor 1, mRNA.	1.63
17025	ALAD	aminolevulinate, delta-, dehydratase, mRNA.	1.61
15901	ID1	inhibitor of DNA binding 1, mRNA.	1.61
80884	MAGED2	melanoma antigen, family D, 2, mRNA.	1.59
53376	USP2	ubiquitin specific peptidase 2, transcript variant 2, mRNA.	1.59
71770	AP2B1	adaptor-related protein complex 2, beta 1 subunit, transcript variant 1, mRNA.	1.57
67087	CTNNBIP1	catenin beta interacting protein 1, mRNA.	1.54
18626	PER1	period homolog 1 (Drosophila), mRNA.	1.52
28000	PRPF19	PRP19/PSO4 pre-mRNA processing factor 19 homolog (S. cerevisiae), mRNA.	1.52
112407	EGLN3	EGL nine homolog 3 (C. elegans), mRNA.	1.52
98932	MYL9	PREDICTED: myosin, light polypeptide 9, regulatory, mRNA.	1.52
23849	KLF6	Kruppel-like factor 6, mRNA.	1.51

Table 5.2 List of the differentially expressed non-annotated transcripts

Differentially expressed non-annotated transcripts in Smad3-null skeletal muscle compared to Wild-type muscle. P value cutoff of $P < 0.05$ and fold change (FC) cutoff of $FC \geq 1.5$ or $FC \leq -1.5$ were used to filter differentially expressed transcripts.

Gene ID	Gene Name	Description	FC
Down-regulated			
5690181	LOC100048331	PREDICTED: Mus musculus similar to DnaJ (Hsp40) homolog, subfamily A, member 4, misc RNA.	-1.84
1850730	LOC620807	Mus musculus novel member of the major urinary protein (Mup) gene family, mRNA.	-1.79
6560296	2510002J07RIK	Mus musculus RIKEN cDNA 2510002J07 gene, mRNA	-1.78
3310672	LOC100047427	PREDICTED: Mus musculus similar to thyroid hormone receptor, mRNA.	-1.54
Up-regulated			
460100	LOC100043257	PREDICTED: Mus musculus similar to RNA binding motif protein 3, mRNA.	2.10
1850603	LOC100040353	PREDICTED: Mus musculus similar to splicing coactivator subunit SRm300, mRNA.	1.69
5550161	8430408G22RIK	Mus musculus RIKEN cDNA 8430408G22 gene, mRNA.	1.65
1190368	A530098C11RIK	Mus musculus RIKEN cDNA A530098C11 gene, mRNA.	1.64
2100687	4921517L17RIK	Mus musculus RIKEN cDNA 4921517L17 gene, mRNA	1.57

5.3.2 Gene Ontology (GO) analysis and functional categorization of differentially expressed genes

Firstly, to identify the predominant biological processes and cellular components enriched among the differentially expressed genes in Smad3-null skeletal muscle, Gene Ontology analysis was performed using the GO mapping tool of Mouse Genomics Informatics database (MGI) <http://www.informatics.jax.org>. The overrepresented Gene Ontology (GO) biological process and cellular component annotations from differentially expressed genes were illustrated in Figure 5.2a and 5.2b respectively. Absence of Smad3 in skeletal muscle led to dysregulated expression of genes involved in a broad range of biological processes. The most abundant groups of genes were relevant to “signal transduction” (15%) and “developmental processes” (15%), while “cell organization and biogenesis” (12%) was the next most enriched group (Figure 5.2a). In terms of cellular component locations, 25% of genes were located within the “nucleus”, while the second and third largest groups were associated with the “plasma membrane” (19%) and “extracellular matrix (ECM)” (15%) (Figure 5.2b).

Furthermore, to identify the functional groups which might be pertinent to the function of Smad3 in skeletal muscle growth, we carried out bioinformatic analysis. Differentially expressed genes were classified into functionally related gene groups, based on the combined analysis from Ingenuity Pathway Analysis (IPA) <http://www.ingenuity.com>, and Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resources <http://david.abcc.ncifcrf.gov/> (102-103). The details of these functional categories and their related genes were summarized in Table 5.3. Genes related to extracellular matrix (ECM) components, and glucose metabolism were down-regulated in Smad3-null skeletal muscle, whereas genes

associated with inflammatory response, and cell cycle inhibition were up-regulated. Interestingly, genes involved in circadian rhythm signaling were also dysregulated in skeletal muscle in the absence of Smad3. These different functional groups are discussed in detail in the following sections.

Figure 5.2 Summary of the Gene Ontology (GO) classification of the differentially expressed genes in Smad3-null skeletal muscle.

Pie charts show the enriched GO biological processes (**a**) and cellular component (**b**) annotations. The size of each slice is indicative of the percentage of differentially regulated genes in each enriched category.

a

b

Table 5.3 Functional categorization of genes differentially expressed in Smad3-null skeletal muscle derived from cDNA microarray.

Expression differences in Smad3-null skeletal muscle are expressed as fold change (FC) relative to Wild-type skeletal muscle. Gene names and GeneBank accession numbers are provided.

Gene Name	Gene ID	FC	Potential relevant function
Extracellular matrix (ECM)			
fibromodulin (Fmod)	14264	-1.77	ECM
Cartilage oligometric matrix protein (Comp)	12845	-1.65	TGF-beta signaling pathways Focal adhesion ECM-receptor interaction
collagen, type I, alpha 2 (Col1a2)	12843	-1.57	Focal adhesion ECM-receptor interaction
spondin 2, extracellular matrix protein (Spon2)	100689	-1.85	ECM
chondroadherin (Chad)	12643	-2.10	Focal adhesion ECM-receptor interaction
Inflammation			
S100 calcium binding protein A8 (calgranulin A) (S100a8)	20201	3.96	Inflammation
S100 calcium binding protein A9 (calgranulin A) (S100A9)	20202	2.51	Inflammation
Biological rhythms			
period homolog 1 (Drosophila) (Per1)	18626	1.52	Circadian rhythm
period homolog 2 (Drosophila) (Per2)	18627	1.80	Circadian rhythm
circadian locomotor output cycles kaput (Clock)	12753	-1.53	Circadian rhythm
Metabolism			
pyruvate kinase, muscle (Pkm2)	18746	-1.91	Pyruvate metabolism Glycolysis/Gluconeogenesis
pyruvate dehydrogenase kinase,	27273	1.88	Pyruvate metabolism

isoenzyme 4 (Pdk4)			
dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex) (Dlat)	235339	-1.55	Pyruvate metabolism Citrate cycle (TCA cycle) Glycolysis/Gluconeogenesis
major urinary protein 1 (Mup1)	17841	-1.95	Lipocalin family
major urinary protein 2 (Mup2)	17840	-2.51	Lipocalin family
Cell cycle			
cyclin-dependent kinase inhibitor 1C (P57) (Cdkn1c)	12577	1.90	Cell cycle
growth arrest and DNA-damage-inducible 45 gamma (Gadd45g)	23882	1.99	Cell cycle MAPK signaling pathway P53 signaling pathway
Contractile fiber			
actin, alpha 2, smooth muscle, aorta (Acta2)	11475	1.75	Smooth muscle contractile fiber Cytoskeleton
ankyrin repeat domain 1, cardiac muscle (Ankrd1)	107765	-1.60	Contractile fiber
ankyrin repeat domain 23, cardiac muscle (Ankrd23)	78321	-1.57	Contractile fiber
Miscellaneous genes			
EGL nine homolog 3 (C. elegans) (Egln3)	112407	1.52	Pathways in cancer, Renal cell carcinoma
PRP19/PSO4 pre-mRNA processing factor 19 homolog (S. cerevisiae) (Prpf19)	28000	1.52	Spliceosome, Ubiquitin mediated proteolysis
catenin beta interacting protein 1 (Ctnnbip1)	67087	1.54	Wnt signaling
contactin associated protein-like 2	66797	1.64	Cell adhesion molecules

(Cntnap2)			
inhibitor of DNA binding 1 (Id1)	15901	1.61	TGF-beta signaling pathway

5.3.3 Microarray data validation

To assess the validity of the microarray data, the changes in mRNA expression of 11 genes were quantified using quantitative real time PCR in samples prepared from independent groups of Wild-type and Smad3-null mice (3 mice for each group). These genes were selected from the four main groups which were defined as dysregulated functional categories in Smad3-null skeletal muscle (Table 5.3). We analyzed the expression of genes related to ECM (*Fmod*, *Colla2*), inflammation (*S100A8*, *S100A9*), metabolism (*Pdk4*, *Pkm2*, *Dlat*, *Mup1*, *Mup2*), and cell cycle (*P57*, *Gadd45g*). As shown in Table 5.4, our quantitative real time PCR results confirmed the expression pattern of the microarray data for all genes except *Gadd45g*. Thus, a strong correlation (Pearson's correlation coefficient, $R=0.77$) between quantitative real time PCR and microarray data was found.

Genes	FC microarray	FC RT- PCR
<i>Fmod</i>	-1.77	-1.30
<i>Colla2</i>	-1.57	- 4.0
<i>S100A8</i>	3.96	15
<i>S100A9</i>	2.51	19
<i>Gadd45g</i>	1.99	-1.42
<i>P57</i>	1.90	4.31
<i>Pkm2</i>	-1.91	-1.77
<i>Pdk4</i>	1.88	2.47
<i>Dlat</i>	-1.55	-1.64
<i>Mup1</i>	-1.95	-1.23
<i>Mup2</i>	-2.51	-1.85

Table 5.4 Microarray data validation by quantitative real time PCR

Fold change of gene expression in *Smad3*-null skeletal muscle when compared to Wild-type muscle obtained through microarray and quantitative real time PCR (RT-PCR).

5.3.4 The essential role of Smad3 for ECM formation in skeletal muscle

Out of 65 differentially expressed transcripts, 5 genes encoding extracellular matrix (ECM) components were significantly down-regulated in Smad3-null skeletal muscle, including *Collagen type I alpha 2 (Col1a2)*, *Fibromodulin (Fmod)*, *Cartilage oligomeric matrix protein (Comp)*, *Spondin 2 (Spon2)*, and *Chondroadherin (Chad)*. We verified the expression of *Col1a2* and *Fmod* by performing quantitative real time PCR analysis, and the results confirmed the significant down-regulation of these genes in Smad3-null muscle (Table 5.4). Collagen is a major component of muscle ECM (153) and our results showed that Smad3-null skeletal muscle has decreased collagen gene expression. Therefore, the total collagen content of the diaphragm muscle from both genotypes (n=3) was quantified and the results suggested that Smad3-null diaphragm muscle had, indeed, significantly lower collagen content when compared to Wild-type muscle (Figure 5.3). The diaphragm muscle, rather than Quad muscle, was used for collagen measurement because the tendon of the Quad muscle cannot be easily removed.

ECM is an integral part of skeletal muscle, mainly consisting of collagen (collagen type I/III/IV) and a group of proteoglycans known as small leucine rich repeat proteoglycans (SLRPs), such as *Fmod* and *Chad* (6). Smad3 is well accepted as a required downstream signaling mediator for the activation of pro-fibrotic genes stimulated by TGF- β , which plays a central role in tissue fibrogenesis (43, 261, 298). Over-expression of Smad3 in human dermal fibroblasts leads to the up-regulation of ECM-related genes, whereas transfection of dominant-negative Smad3 blocks this up-regulation (277). Blocking of TGF- β /Smad3 signaling confers animals resistance to various tissue fibrosis, including skin, lung, liver, kidney and intestine (144). Therefore, it was not surprising that we found down-regulated expression of genes

encoding both fibrillar collagen and proteoglycans in *Smad3*-null muscle. Previous studies indicated that TGF- β promotes *Colla2* and *Fmod* expressions through a *Smad3*-dependent pathway (149, 277). The reduced expression of *Colla2* and *Fmod* detected in *Smad3*-null skeletal muscle is in agreement with these studies.

Myostatin is another important pro-fibrotic regulator, predominantly expressed in skeletal muscle. It directly stimulates fibroblast proliferation (151) and type I collagen synthesis (185). Absence of Myostatin results in down-regulated mRNA levels of collagen genes in skeletal muscle (285), as well as reduced scar tissue formation during muscle regeneration (173). However, there is a paucity of studies regarding whether *Smad3* is required or not for the pro-fibrotic function of Myostatin. Our previous data shows significantly enhanced *Myostatin* expression in *Smad3*-null skeletal muscle (81). It was hypothesized that higher *Myostatin* levels in skeletal muscle might promote fibroblast function and stimulate expression of ECM genes in *Smad3*-null muscle. However, reduced expression of ECM-related genes found in *Smad3*-null muscle suggests that Myostatin may stimulate ECM gene expression through a *Smad3*-dependent mechanism. In other words, *Smad3* deficiency may block the pro-fibrotic functions of Myostatin in muscle.

In addition to providing scaffolding in muscle, ECM strongly influences other functions of skeletal muscle. In fact, the ECM makes up the niche for skeletal muscle stem cells, satellite cells (SCs). Interaction between ECM and SCs regulates SC activation, proliferation, differentiation and migration (84). Furthermore, SCs are also subjected to regulation by various extracellular growth factors which are associated with proteoglycans in the ECM. These proteoglycans initiate communication between cells and the surrounding ECM (110, 197). Therefore, the altered ECM environment in *Smad3*-null muscle might have an impact on SC functionality. Furthermore, both

TGF- β and Myostatin activities are strongly inhibited by decorin, a proteoglycan component of muscle ECM (189). By sequestering these negative regulators in the ECM, decorin promotes myogenic proliferation and differentiation (132, 150). Interestingly, a recent study describes high affinity between Fmod and Myostatin and hypothesizes that Fmod, a proteoglycan in muscle ECM, may modulate Myostatin activity by a mechanism similar to that of decorin (190). In this study, we found that *Fmod* expression was down-regulated in Smad3-null muscle. Therefore, reduced Fmod levels in Smad3-null muscle ECM may make Myostatin more accessible to its cell surface receptor complex, resulting in exaggerated Myostatin signaling in Smad3-null muscle. Together with increased *Myostatin* level found in Smad3-null muscle (81), this increased Myostatin activity, due to reduced levels of inhibitors in the ECM, may contribute to the muscle atrophy observed in Smad3-null mice.

In summary, this study offers new evidence for the essential role of Smad3 in skeletal muscle fibrogenesis. Figure 5.4 illustrates the schematic representation of our hypothesis that Myostatin/TGF- β promotes fibrous tissue formation via a Smad3-dependent mechanism and Myostatin activity might be enhanced in Smad3-null muscle due to down-regulation of *Fmod* in the ECM.

Figure 5.3 The total collagen content of *Smad3*-null diaphragm muscle was significantly lower than that of Wild-type muscle.

Collagen content was quantified by the colorimetric determination of hydroxylproline concentration (µg/mg) in muscle. Values represent the means $\pm$ SEM (n=3). Statistical difference is indicated, $P < 0.001$ (***).

Figure 5.4 *Smad3* is required for the pro-fibrotic functions of both TGF- β and Myostatin.

Upon TGF- β /Myostatin binding to their respective cell surface receptor complex, the activated receptor recruits and phosphorylates Smad3 and the co-Smad, Smad4. Activated Smad3 and Smad4 form a heteromeric complex and translocate into nucleus to initiate expression of genes encoding extracellular matrix (ECM) components. Some proteoglycans in ECM, such as Decorin and Fmod, inhibit TGF- β /Myostatin activities by sequestering them in ECM.

5.3.5 Genes involved in metabolic pathways

Several recent studies suggest a role for Smad3 as a regulator of glucose and lipid metabolism (154, 259, 292). Smad3-null mice have less adiposity, due to inhibited adipogenesis, increased fatty acid (FA) uptake and FA β -oxidation in white adipose (WAT) tissue (260, 292). Skeletal muscle accounts for the largest energy consumption and is one of the major metabolically active tissues. Microarray data in this study revealed dysregulated expression of genes related to glucose metabolism, as well as genes associated with general energy-metabolism. However, it is noteworthy to mention that a study from Tan *et al.* found no significant alteration of expression of genes involved in the β -oxidation and lipolysis in Smad3-null muscle (259).

Three genes involved in glucose metabolism, pyruvate kinase muscle isozyme (*Pkm2*), pyruvate dehydrogenase kinase 4 (*Pdk4*), and dihydrolipoamide S-acetyltransferase (*Dlat*), were dysregulated in Smad3-null muscle. The *Pkm2* gene encodes two isoenzymes of pyruvate kinase, PKM1 and PKM2 by alternative splicing. Both enzymes catalyze the rate-limiting step of glycolysis, converting phosphoenolpyruvate (PEP) to pyruvate (251). Pyruvate could be further converted into acetyl-CoA (pyruvate oxidation), which can be oxidized in the tricarboxylic cycle (TCA). This step is catalyzed by the pyruvate dehydrogenase complex (PDC) and its activity is tightly regulated by Pdk. Although there are 4 isoenzymes of Pdk (Pdk1-Pdk4) present in mammalian tissue, Pdk4 is believed to be the key controller, inactivating PDC by phosphorylation of dehydrogenase (254). *Dlat* encodes the E2 subunit of PDC. As an integral part of the complex, DLAT is required for PDC activity (98). In our microarray data, decreased expression of *Pkm2* and *Dlat*, and increased *Pdk4* expression was found in Smad3-null skeletal muscle. Given the fact that *Pkm2* regulates one of the rate-limiting steps in glycolysis, decreased *Pkm2* expression in

Smad3-null muscle suggests that glycolysis in skeletal muscle is inhibited and thus less pyruvate is produced. As for the catabolism of pyruvate, with up-regulated *Pdk4* expression and down-regulated *Dlat* expression, it is also reasonable to expect inhibited pyruvate oxidation in Smad3-null skeletal muscle. Therefore, pyruvate produced is directed either to lactate or alanine, rather than oxidized (125).

On the other hand, dysregulation of *Pkm2*, *Pdk4* and *Dlat* gene expression may also contribute to a catabolic fuel shift in Smad3-null muscle. Skeletal muscle uses both glucose and fatty acids as fuels to produce energy. Both energy systems are strictly controlled by different catabolic pathways to meet different energy demands during exercise (82). Pyruvate oxidation is the critical reaction linking two major catabolic pathways, glycolysis and the tricarboxylic cycle (TCA) (275). As mentioned above, this reaction is catalyzed by PDC. Therefore, PDC activity and Pdk are important targets for the metabolic switch between glucose and lipid oxidation in skeletal muscle (255). *Pdk4* expression is usually up-regulated to inhibit carbohydrate oxidation when carbohydrate availability is low. For example, during prolonged exercise or insulin deficiency *Pdk4* expression markedly increases in skeletal muscle (100, 215). One study indicates that prolonged inhibition of PDC leads to increased β -oxidation, converting fatty acids to acetyl-CoA (286). Therefore, increased *Pdk4* expression triggers a possible metabolic fuel shift from carbohydrate catabolism to fatty acid catabolism. Up-regulated expression of *Pkm2* and down-regulated expression of *Pdk4* was discovered in skeletal muscle of animals in hibernation. By coordinating the expression of these catabolic genes, animals during hibernation undergo energy catabolism shifts from carbohydrates to fatty acids (293). Similar expression patterns of *Pkm2* and *Pdk4* genes were also found during PPAR α agonist treatment of human white adipocytes (220). PPAR α is a master regulator of fatty acid oxidation and

PPAR α agonist treatment strongly reduces the glycolysis and glucose oxidation in human white adipocytes. (220). Therefore, with increased *Pdk4* expression and decreased *Pkm2* expression, Smad3-null muscle is likely to have a catabolic fuel shift from glucose oxidation to lipid oxidation. However, the question still remains, whether or not lipid catabolic pathways such as lipolysis and fatty acid β -oxidation are indeed up-regulated in Smad3-null muscle. Recent studies suggest that Smad3-null mice have less adiposity and are resistant to diet-induced obesity due to increased fatty acid β -oxidation in white adipose tissue (WAT) (260, 292). Given the significant contribution of skeletal muscle to systemic lipid metabolism, increased fatty acid β -oxidation in skeletal muscle, in addition to WAT, might also contribute to the decreased fat mass and smaller adipocyte size observed in Smad3-null mice. Although a study from Tan *et al.* suggests that the expression of genes involved in lipid uptake (*LPL*), fatty acid oxidation (*Acadl*, *ACS*, *PPAR β/δ* , *Acadvl*), as well as mitochondria function (*CPT1b*, *Cox5b*) in Smad3-null muscle remained unchanged (259); fatty acid oxidation and mitochondria function, specifically in skeletal muscle of Smad3-null mice deserves to be analyzed in further detail.

Our previous study found increased ubiquitin-mediated proteolysis in Smad3-null muscle due to enhanced expression of MuRF1. MuRF1 has long been known as a muscle-specific E3 ubiquitin ligase which promotes myofibrillar protein degradation through the ubiquitin-proteasome system during muscle wasting (42). Interestingly, recent studies suggest a role of MuRF1 in regulation of systemic glucose and protein metabolism (96, 136). MuRF1 physically interacts with key enzymes of the catabolic pathways to target these enzymes for proteasome-dependent degradation. Over-expression of MuRF1 chronically inhibits carbohydrate catabolism and muscle energy consumption to preserve glucose or amino acids in skeletal muscle. It is worth noting

that PKM2 and PDC subunits are among those enzymes targeted by MuRF1 (96). Therefore, up-regulated MuRF1 expression found in Smad3-null muscle might promote degradation of PKM2 and PDC subunits. Together with the reduced mRNA level of these genes found in the microarray data, Smad3-null skeletal muscle might have reduced glucose catabolism, including glycolysis and pyruvate oxidation. Figure 5.5 outlines our hypothesis of dysregulated glucose metabolism in Smad3-null muscle. To prove this hypothesis, protein levels of these key enzymes should be estimated and more importantly, pyruvate concentration and pyruvate oxidation in Smad3-null muscle needs to be measured.

Altered glucose metabolism in skeletal muscle significantly affects systemic carbohydrate metabolism. Several studies have suggested that Smad3-null mice have higher serum insulin levels (hyperinsulinemia) and lower blood glucose levels (hypoglycemia) (154, 260). Different mechanisms have been hypothesized. Lin *et al.* hypothesized that Smad3 directly represses insulin gene transcription upon TGF- β stimulation (154), while Tan *et al.* suggested that an improved glucose uptake into peripheral tissues, including skeletal muscle, might contribute to this increased insulin sensitivity and glucose tolerance (260). Interestingly, Hirner *et al.* found that over-expression of MuRF1, specifically in skeletal muscle, leads to strikingly increased serum insulin levels and hypothesized that insulin secretion might be stimulated to antagonize the down-regulated glucose metabolism observed in MuRF1-over-expressing skeletal muscle (96). Therefore, up-regulated MuRF1 found in Smad3-null muscle could be another possible mechanism for the increased insulin level found in Smad3-null mice. Given the role of MuRF1 in both muscle wasting and systemic carbohydrate metabolism regulation, up-regulated MuRF1 level found in Smad3-null muscle might be a link between muscle atrophy and regulation of insulin secretion.

Two family members of the major urinary protein family (Mups), Mup1 and Mup 2, were markedly down-regulated in Smad3-null skeletal muscle. MUPs belong to a lipocalin family, which are secreted proteins mainly produced in liver (180). These proteins function as pheromone-binding proteins and circulating MUPs are also involved in systemic metabolism regulation (306). Although the exact molecular mechanism for their function as metabolic controllers remains to be elucidated, recent studies suggest that MUP-1 stimulates energy expenditure and inhibits gluconeogenesis and lipogenesis in the liver (305). Mup1 mRNA level in the liver of obese mice is down-regulated by about 30-fold (105), whereas drug-mediated enhancement of insulin sensitivity significantly up-regulates the expression of both Mup 1 and Mup 3 (18). Mup1 promotes energy expenditure by increasing mitochondria function and biogenesis in skeletal muscle. At the same time, Mup1 down-regulates expression of both gluconeogenic and lipogenic genes in liver (305). Although Mups are predominantly expressed in liver, the detectable expression of Mups in skeletal muscle, as found in this present study, suggests that Mups may have a role in regulating muscle metabolism.

Figure 5.5 *The Glucose metabolic pathway might be down-regulated in Smad3-null skeletal muscle.*

(a) The *Pkm2* gene encodes pyruvate kinase isoforms which catalyze the rate-limiting step of glycolysis. Pyruvate oxidation, conversion from pyruvate to acetyl-CoA, is the reaction connecting glycolysis and tricarboxylic cycle (TCA). It is catalyzed by the pyruvate dehydrogenase complex (PDC). *Dlat* encodes the E2 subunit of PDC. Activity of PDC is strongly inhibited by *Pdk4*. Down-regulated expressions of *Pkm2* and *Dlat*, as well as up-regulated expression of *Pdk4* were found in *Smad3*-null skeletal muscle in this study. (b) Previous studies suggest that *Smad3*-null skeletal muscle has increased Myostatin level, which can also up-regulate MuRF1 expression (81). MuRF1 is a muscle-specific E3 ubiquitin ligases and targets PKM2 and PDC units for protein degradation mediated by the ubiquitin-proteasome system (96). Therefore, genes involved in the glucose metabolic pathway might be down-regulated in *Smad3*-null skeletal muscle, which possibly results in reduced glucose metabolism.

5.3.6 Genes encoding master regulators of circadian rhythms

This study identified differentially expressed genes encoding master regulators of peripheral circadian rhythm, including circadian locomotor output cycles kaput (*Clock*), and *Period1/2* (*Per1/2*), in Smad3-null skeletal muscle. Mammals exhibit innate behavioral and physiological circadian rhythms, which are regulated by a network of rhythmic gene expressions over a 24 h period. This endogenous circadian clock enables mammals to participate in daily activities in relation to external environmental cycles. In addition to the central nervous system, peripheral tissues including skeletal muscle are controlled by this biological clock. Locomotor activity in skeletal muscle shows endogenous circadian rhythm (165), and in fact more than 200 genes in skeletal muscle show a strong circadian expression pattern (171). *Clock* and *Period* genes are critical core-clock components and regulate the expression of genes outside the core named clock-controlled genes (CCGs), which exhibit circadian rhythm expression pattern. *Myod1*, the master regulator of myogenesis, is one of the CCGs in skeletal muscle, whose expression is subjected to the transcriptional regulation of CLOCK (11). CLOCK and BMAL1, function as a transcription factor heterodimer and initiate the expressions of CCGs (31). *Period* genes function as the major negative feedback loop for CLOCK/BMAL1. CLOCK/BMAL1 initiate the expression of *Per1/2/3* and *Cryptochrome* (*Cry1/2*). PER/CRY proteins in turn, translocate to the nucleus to repress CLOCK/BMAL1-mediated transcription. Three *Per* family members are found in mammals, *Per1*, *Per2* and *Per3*. Down-regulated expression of master regulator, *Clock*, and up-regulated expression of inhibitors of CLOCK/BMAL1 activity, *Per1/2*, are likely to be an indicator of repressed rhythmicity in Smad3-null skeletal muscle. This in turn may trigger the dysregulated circadian expression of muscle specific genes, such as *Myod1*.

It is known that environmental stimuli, such as feeding and light, influence this endogenous rhythm. However, there is still a lack of evidence showing the effects of TGF- β or Myostatin, the initiators of Smad3 signaling, on the regulation of circadian gene expression in skeletal muscle. This is the first study to demonstrate that Smad3 deficiency leads to dysregulated expression of circadian rhythm genes in skeletal muscle; indicating the possible functions of TGF- β super-family members and Smad3 signaling in the regulation of circadian cycling in skeletal muscle. Furthermore, a list of genes including *Slc25a25*, *Usp2*, *Pdk4*, and *Egln3* highlighted in this study have been shown to have circadian expression pattern in adult skeletal muscle (211). Therefore, alternations in the expression of these genes might be due to the dysregulated expression of critical clock genes (*Clock*, *Per1/2*), rather than as a direct result of the absence of Smad3.

5.3.7 Up-regulation of inflammatory cytokines in *Smad3*-null skeletal muscle

Remarkably increased expression of inflammatory cytokines, *S100A8* and *S100A9*, was found in *Smad3*-null skeletal muscle. *S100A8* and *S100A9* are inflammatory molecules which form a heterodimer named calprotectin, and are predominantly expressed in myeloid cells (253). The findings from a previous study also suggest that skeletal muscle, as an endocrine organ, up-regulates expression of these cytokines and promotes their secretion into circulation after exercise (194). Many chronic inflammatory conditions, such as obesity, inflammatory bowel and lung diseases, result in increased plasma calprotectin levels (195). Moreover, they are also important stromal chemokines in various cancers. Significantly increased levels of both proteins are also found in the plasma of colorectal cancer patients, and in muscle invasive

bladder cancer (MIBC) patients (128, 188). Importantly, the Smad3-null mice used in this study develop chronic infection in organs like the nasal mucosa, colon, and small intestine (297). Therefore, the up-regulated expression of inflammatory cytokines in Smad3-null skeletal muscle may be due to the systemic inflammation in Smad3-null mice.

5.3.8 Conclusion

In conclusion, this is the first study to analyze the global gene expression changes in Smad3-null skeletal muscle. Alteration in the expression of genes involved in ECM, metabolic pathways and inflammation were observed in Smad3-null muscle. This study also for the first time reports that Smad3 might be involved in controlling circadian rhythm genes in skeletal muscle.

Chapter 6 Prospective

Myostatin belongs to the TGF- β superfamily and acts as a potent negative regulator of skeletal muscle growth and development (120, 183). Since the discovery of the *Myostatin* gene, extensive research has been undertaken in our lab to unveil the molecular mechanism underlying Myostatin signaling in skeletal muscle (142, 174, 176, 179, 266). Growing evidence about Myostatin function, suggested a role for Smad3 protein, as a canonical downstream signaling molecule (142, 151, 174). However, the exact molecular mechanism of Smad3 signaling in muscle growth, development and regeneration has not yet been fully elucidated.

6.1 Role of Smad3 in pre-natal myogenesis

In this thesis I have described the molecular mechanism behind Smad3 function in post-natal myogenesis. Specifically, increased Myostatin levels, due to absence of Smad3, triggers ubiquitin-mediated proteolysis in muscle tissue, as well as inhibition of SC self-renewal, myogenic proliferation and differentiation of myoblasts. However, the role of Smad3 in pre-natal myogenesis still remains unclear. Although a study from Yang *et al.* claimed that no phenotypic difference was found between Smad3-null mice and controls at birth (297), I observed that neo-natal Smad3-null mice were smaller than controls, which suggests a possible regulatory function of Smad3 during embryogenesis. Moreover, the abundant Smad3 expression found in mesoderm at later periods of embryogenesis (E13-E15) in Wild-type mice, also suggests a possible role of Smad3, specifically in pre-natal muscle development (160). Therefore, future work will need to be conducted to clarify the role of Smad3 in skeletal muscle development

during mouse embryogenesis. Firstly, I will quantify muscle weight, muscle fiber size and fiber number, as well as muscle fiber type in neo-natal Smad3-null mice to address the question whether loss of Smad3 influences pre-natal myogenesis. Secondly, I will address the question whether pre-natal disruption of the *Smad3* gene results in increased Myostatin expression during skeletal muscle development. Previous studies have already revealed the critical developmental role that Myostatin plays in regulation of embryonic myogenesis in different mammalian species (208). Specifically, Myostatin controls myoblast population's growth during embryonic myogenesis (8), which leads to excessive muscle hyperplasia at birth in Myostatin-null mice. Myostatin also promotes slow myofiber formation through MEF2C-dependent signaling during primary and secondary myofiber formation (208). Therefore, spatiotemporal expression of Myostatin during different gestational ages in the developing Smad3-null embryo will be analyzed using either *in situ* hybridization or immunohistochemical techniques. Pax7-expressing cells, derived from dermomyotome, give rise to a pool of SCs during pre-natal myogenesis (36). In this study, I observed reduced SC self-renewal capacity and reduced SC number in Smad3-null mice. Although declined SC self-renewal is a contributing factor in the decreased SC number observed in Smad3-null mice, SC specification, during embryonic myogenesis, may also account for reduced SC number. Therefore, the question remains as to whether loss of Smad3 contributes to any defect in SC specification during pre-natal myogenesis. Cre-expressing transgenic mice, under the control of the *Pax7* promoter, will be crossed with mice carrying floxed alleles of the *Smad3* gene for conditional deletion of *Smad3* in Pax7-expressing progenitor cells (198). Furthermore, to define the activity and migration of myogenic progenitors during myogenesis in Smad3-null embryos, mice, in which progenitor cells can be detected

by GFP expression (driven by *Myf5* or *Pax7*) will be crossed into the *Smad3*-null genetic background. These studies will help us gain a better understanding on the regulatory function of Smad3 signaling in overall muscle development and growth.

6.2 Role of Smad3 in post-natal myogenesis

SCs are the main source of muscle precursor cells during post-natal muscle growth and regeneration (46). Due to defective SC functionality, *Smad3*-null mice have atrophied musculature and impaired *in vitro* myogenesis. To validate the role of Smad3 in SC function and post-natal myogenesis, I have used a notexin-induced muscle regeneration model. As expected, impaired muscle regeneration was found in *Smad3*-null mice, which suggested impaired *in vivo* post-natal myogenesis. Another model of muscle regeneration will be used in future work. *mdx* mice harboring a mutation in the *dystrophin* gene (39), undergo repeated cycles of muscle degeneration and regeneration (249). Dystrophin is a membrane cytoskeletal protein and loss of Dystrophin leads to the absence of the dystrophin-glycoprotein complex (DGC) (68). DGC links the extracellular matrix and the intracellular cytoskeleton to protect muscle sarcolemma from contraction-induced lesions. Therefore, to expand our understanding on the role of Smad3 in post-natal myogenesis, *mdx/Smad3*-null DKO mice will be generated to provide a model, where we can study multiple rounds of muscle regeneration in the *Smad3*-null genetic background. Smad3 will also be over-expressed in *mdx* genetic background to determine whether the muscle regeneration will be enhanced by Smad3 over-expression.

Smad3-null mice used in this study expressed a non functional allele of Smad3, in which *exon 8* of the *Smad3* gene (*Smad3^{ex8/ex8}*) was deleted in all tissues (297). This

Smad3-null mouse develops chronic infection in organs like nasal mucosa, colon, and small intestine (297). Increased susceptibility to colon cancer triggered by bacteria is also found in this Smad3-null mouse model (162). Both chronic inflammation and cancer are often accompanied by muscle wasting (62, 270). Therefore, an inducible, skeletal muscle-specific *Smad3* knock-out mouse will be generated to minimize the possibility of secondary effects on muscle growth and homeostasis. By crossing Cre-expressing transgenic mice, with mice that carry floxed alleles of the *Smad3* gene, I will be able to selectively inactivate the *Smad3* gene. Using Cre-expressing transgenic mice, driven by the *mef2c* skeletal muscle-specific promoter, the function of Smad3 specifically in skeletal muscle can be defined (92).

6.3 The role of Smad3 in regulation of Myostatin expression and activity

In this study, increased Myostatin expression in Smad3-null skeletal muscle was found, which suggests that regulation of Myostatin expression may be deregulated, due to the absence of functional Smad3 protein. Therefore, one of the questions that remains to be addressed is what mechanism is responsible for the up-regulation of Myostatin in Smad3-null skeletal muscle.

Unpublished data from our lab has revealed that Myostatin expression is regulated at the post-transcriptional level, through microRNA-27a/b, via a Smad3-dependent mechanism. microRNAs are small RNAs which target the 3' untranslated region (3'-UTR) of specific mRNA to repress translation or initiate the degradation of the target mRNA in a deadenylation-dependent mechanism (17). A Smad3 binding site was found in the promoter region of microRNA-27a/b and the expression of miRNA-27a/b

was induced by Smad3 (Arigela *et al.* ⁶). Therefore, it was hypothesized that Smad3 deficiency would down-regulate miRNA-27a/b expression, contributing to the increased level of Myostatin in Smad3-null muscle. Thus this study may reveal a novel mechanism for the negative feedback auto-regulation of Myostatin expression. Previous work from our lab has revealed that Myostatin expression, like other members of the TGF- β superfamily, is auto-regulated by a negative feedback loop, which in the case of Myostatin is Smad7-dependent (75). Figure 6.1 illustrates the two independent mechanisms which may contribute to the negative feedback auto-regulation of Myostatin expression.

Interestingly, recent studies have identified conserved SMAD-binding elements (SBE) in a number of pri-miRNAs. Smad2/3 proteins bind to these so called R-SBE (Smad binding element in primary transcripts of miRNAs) motifs to stimulate miRNA processing by recruiting the microprocessor complex to pri-miRNAs (55-56, 271). These new findings, together with previous studies suggest that Smad proteins regulate gene expression via two mechanisms, transcriptional and post-transcriptional. Firstly, Smad proteins function as transcription factors, which initiate or repress gene expression through binding SBE motifs located in the enhancer or promoter regions of target genes. Secondly, Smad proteins function as anchor proteins required for miRNA processing, through binding R-SBE motifs located in the stem of miRNAs to stimulate the conversion of pri-miRNAs (primary miRNA transcripts) into their respective pre-miRNAs (miRNA precursors). Therefore, Smads proteins are involved in both the transcription and maturation steps of miRNA production. Although around 20 pri-miRNAs have been identified to contain R-SBE motifs, the specific miRNAs whose maturation is regulated by Smad3 are still unknown (56). Therefore, it would be

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interesting to compare miRNA expression profiles between Smad3-null myoblasts and Wild-type controls treated with TGF- β /Myostatin, using miRNA microarray technology. This study would enable us to identify the set of miRNAs which are specifically up-regulated by TGF- β /Myostatin in a Smad3-dependent manner, either through transcriptional activation or through stimulated processing. Screening possible R-SBE motifs in these target miRNAs will enable us to further identify the set of miRNAs whose processing is Smad3-dependent.

Apart from regulating Myostatin expression, Smad3 may also be involved in the regulation of Myostatin activity. This study has provided several lines of evidence suggesting that Smad3 is necessary for the synthesis of ECM components in skeletal muscle. Microarray data suggested that a group of ECM-related genes was significantly down-regulated in Smad3-null skeletal muscle. Smad3 deficiency also led to remarkably reduced scar tissue formation in regenerated TA muscle. Interestingly, Myostatin activity is subjected to modulation by ECM components, such as decorin. Previous work from our lab indicates that decorin blocks the inhibitory effect of Myostatin on myoblast proliferation, through immobilizing Myostatin in the ECM (189). In addition, a recent study suggests a high binding affinity between Myostatin and Fibromodulin (Fmod), another ECM component (190). Thus it would be interesting to study whether Fmod blocks Myostatin-mediated inhibitory function on muscle growth, in a similar fashion to Decorin. It is worth noting that significantly decreased expression of *Fmod* was found in Smad3-null skeletal muscle (See Chapter 5 Section 5.3.4). Therefore, future work aimed at clarifying Fmod regulation of Myostatin activity would address the question, whether or not Myostatin signaling is further exaggerated in Smad3-null skeletal muscle due to reduced inhibitory proteins present in the ECM.

Since Myostatin is a potent negative regulator of skeletal muscle growth, both expression and activity of Myostatin are subjected to strict regulation. Therefore, further characterization of the role of Smad3 in regulation of both Myostatin expression and activity warrants further investigation.

6.4 Muscle functional study in Smad3-null mice

Due to increased Myostatin expression and atrophied myofibers in Smad3-null muscle, it is highly possible that Smad3-null muscle is weaker than Wild-type control. A study by Tan et al. suggests that Smad3-null mice show around 70% reduced physical activity (260), with similar food and water consumption as Wild-type mice, and this reduced activity might be due to muscle weakness. Therefore, muscle functions of Smad3-null mice will be analyzed in future work.

6.5 The role of Smad3 in the regulation of systemic metabolism

Recent studies demonstrate an important role of Smad3 in the regulation of systemic metabolism. In this study, gene expression analysis also suggests that Smad3 deficiency leads to dys-regulated expression of genes relating to metabolic pathways, which might contribute to reduced glycolysis, pyruvate oxidation, and enhanced lipid oxidation in skeletal muscle. Therefore, in future work, kinetic metabolic studies using radioactively labeled amino acids, carbohydrates and fatty acids will be very useful to analyze the protein, carbohydrate, and fat metabolism in Smad3-null mice.

Figure 6.1 Proposed model for the mechanism underlying auto-regulation of Myostatin expression

Previous work, together with unpublished data indicates that two mechanisms may be involved in the auto-regulation of Myostatin expression. Firstly, Myostatin expression is regulated post-transcriptionally by miRNA-27a/b, through Smad3-dependent signaling (Arigela *et al*⁷). Secondly, Myostatin expression is auto-regulated at the transcriptional level through a Smad7-dependent mechanism (75).

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a

b

Conclusion

In conclusion, this is the first study which has systematically analyzed the role of Smad3 signaling in post-natal myogenesis. My data indicates that absence of Smad3 leads to impaired post-natal myogenesis, and further unravels the molecular mechanisms underlying this defect. Furthermore, a better understanding of the specific role of Smad3 in regulation of Myostatin expression and Myostatin signaling will be useful for effectively developing Myostatin-targeted treatments; to potentially combat human muscle wasting, associated with gene mutations (Duchenne muscular dystrophy, DMD), physical inactivity (Atrophy), aging (sarcopenia) and chronic diseases (cachexia).

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