TGFbeta signalling pathway in muscle regeneration : an important regulator of muscle cell fusion

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Sorbonne Université

Ecole doctorale Complexité du Vivant

Centre of Research in Myology
Signaling Pathways & Striated Muscles

TGFβ signalling pathway in muscle regeneration: an important regulator of muscle cell fusion

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Thèse de doctorat en Biologie Cellulaire et Moléculaire

Dirigée par Fabien LE GRAND

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SUMMARY

Muscle regeneration relies on a pool of muscle-resident stem cells called satellite cells (MuSCs). MuSCs are quiescent and can activate following muscle injury to give rise to transient amplifying progenitors (myoblasts) that will differentiate and finally fuse together to form new myofibers. During this process, a complex network of signalling pathways is involved, among which, Transforming Growth Factor beta (TGFβ) signalling cascade plays a fundamental role. Previous reports proposed several functions for TGFβ signalling in muscle cells including quiescence, activation and differentiation. However, the impact of TGFβ on myoblast fusion has never been investigated. In this study, we show that TGFβ signalling reduces muscle cell fusion independently of the differentiation step. In contrast, inhibition of TGFβ signalling enhances cell fusion and promotes branching between myotubes. Pharmacological modulation of the pathway *in vivo* perturbs muscle regeneration after injury. Exogenous addition of TGFβ protein results in a loss of muscle function while inhibition of the TGFβ pathway induces the formation of giant myofibres. Transcriptome analyses and functional assays revealed that TGFβ acts on actin dynamics to reduce cell spreading through modulation of actin-based protrusions. Together our results reveal a signalling pathway that limits mammalian myoblast fusion and add a new level of understanding to the molecular regulation of myogenesis.

RÉSUMÉ

La régénération musculaire s'appuie sur une réserve de cellules souches résidant dans le muscle appelées cellules satellites (MuSCs). Les MuSCs sont quiescentes et peuvent s'activer à la suite d'une blessure du muscle afin de former des progéniteurs amplificateurs (myoblastes) qui se différencieront et fusionneront pour former de nouvelles myofibres. Durant ce processus, un réseau complexe de voies de signalisation est impliqué, parmi lequel la signalisation du facteur de croissance transformant bêta (TGFβ) joue un rôle fondamental. Précédents rapports ont proposé de nombreuses fonctions pour la signalisation TGFβ dans les cellules musculaires, comme leur quiescence, activation et différenciation, mais l'impact de TGFβ sur la fusion de myoblastes n'a jamais été étudié. Dans cette étude, nous avons montré que cette signalisation réduit la fusion des cellules musculaires indépendamment de leur différenciation. Au contraire, l'inhibition de la signalisation TGFβ accroît la fusion cellulaire et favorise les ramifications entre myotubes. Une pharmaco-modulation de la voie *in vivo* perturbe la régénération musculaire après blessure. Une addition exogène de la protéine TGFβ conduit à une perte de fonction du muscle, tandis que l'inhibition de la voie induit la formation de myotubes géants. Les analyses transcriptomiques et fonctionnelles ont montré que TGFβ agit sur la dynamique de l'actine afin de réduire la diffusion cellulaire à travers une modulation des protrusions à base d'actine. Nos résultats ont donc révélé une voie de signalisation qui limite la fusion de myoblastes et ajoutent un nouveau niveau de compréhension sur la régulation moléculaire de la myogenèse.

LIST OF ABBREVIATIONS

ACVR2A	Activin receptor type-2A
ACVR2B	Activin receptor type-2B
ALK	Activin Receptor-Like Kinase
AMH	anti-Müllerian hormone
AMHR2	Anti-Mullerian hormone receptor type 2
ATP	Adenosine triphosphate
bHLH	Basic helix-loop-helix
BMP	Bone Morphogenetic Protein
BMPR2	BMP receptor type II
BrdU	Bromodeoxyuridine
c-Myc	Myelocytomatosis oncogene
Ca2+	Calcium
CAMKII	Calcium/Calmodulin-Dependent Protein Kinase-II
Cdc42	Cell Division Cycle 42
CDK	Cyclin-depended kinase
Co-SMAD	Common-mediator SMAD
CTX	Cardiotoxin
Cys	Cysteins
DMD	Duchenne Muscular Dystrophy
DOCK	Dedicator of Cytokinesis
DUF	Dumfounded
ECM	Extracellular Matrix
EDL	Extensor Digitorum Longus
EMT	Epithelial-mesenchymal transition
FAP	Fibro-Adipogenic Progenitor
FC	Founder Cell
FCM	Fusion-competent myoblast
FGF	Fibroblast Growth Factor
GDFs	Growth and Differentiation Factor

GEF	Guanine nucleotide Exchange Factor
Graf1	Rho-GTPase-activating protein
GSK3β	Glycogen Synthase Kinase 3 beta
GTP	nucleotide guanosine triphosphate
HGF	Hepatocyte Growth Factor
I-SMAD	Inhibitory SMAD
IFN-γ	Interferon-γ
IgSF	Immunoglobulin superfamily
IL-1β	Interleukin-1β
JNK	c-Jun N-terminal kinases
LAP	Latency Associated Peptide
Lef1	Lymphoid enhancer binding factor 1
LIMK1	LIM kinase 1
LLC	Large Latent Complex
LTBP	Latent TGFβ binding protein
MAPK	Mitogen-activated protein kinase
MD	Mature Domain
MEF2	Myocyte enhancer factor 2
MHC	Myosin heavy chain
MMP	Matrix Metalloproteinase
MRF	Myogenic Regulatory Factor
Mrtf	Myocardin-related transcription factor
Mstn	Myostatin (GDF8)
mTOR	mammalian Target of Rapamycin
MuSC	Muscle Stem Cell (Satellite Cell)
Myf5	Myogenic factor 5
Myh3	Embryonic myosin heavy chain
Mymk	Myomaker
Mymx	Myomixer
MyoD	Myogenic differentiation (Myod1)
NES	Nuclear Export Signal

NLS	Nuclear Location Sequence
NM-MHC	non-muscle myosin heavy chain
PAI-1	Plasminogen Activator Inhibitor-1
PAK	p21-activated kinase
PAR6	Partitioning-defective 6
Pax	Paired box
PI3K	Phosphatidylinositol-3-kinase
PS	Phosphatidylserine
R-SMAD	Receptor-activated SMAD
RhoA	Ras Homolog Family Member A
Rok	Rho kinase
Rspo1	R-spondin1
SARA	Smad Anchor for Receptor Activation
SBE	Smad-Binding Element
Scx	Scleraxis
SLC	Small Latent Complex
SLRP	Small leucine-rich proteoglycan
SMAD	Small Mother Against Decapentaplegic
SNS	Stick-and-Stones
SPC	Subtilisin-like proprotein convertase
Srf	Serum response factor
TA	Tibialis anterior
TGFβ	Transforming Growth Factor beta
TGFβR2	TGFβ receptor type II
TNF-α	Tumor Necrosis Factor alpha
TSP1	Thrombospondin
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
WASP	Wiskott-Aldrich Syndrome Protein
Wnt	Wingless-type MMTV integration site family

INTRODUCTION

I. Skeletal Muscle

Locomotion is a characteristic feature of all animals and is defined as the process of moving from one place to another. The ability to move in vertebrates is controlled by the nervous system and relies on the musculoskeletal system, a coordinated structure of bones, tendons, ligaments, joints and skeletal muscle. These heterogenous tissues constitute more than 50% of the human weight and are arranged throughout the entire body to provide internal support, correct posture, dynamic balance and allow motion to occur. Specifically, skeletal muscle is the most abundant tissue in human body and it does not account for locomotion only, but also for vital functions such as feeding, breathing, vision, reproduction, blood circulation and energy metabolism.

In vertebrates there are two types of muscles: smooth and striated. Smooth muscle is present in the walls of hollow organs, such as stomach, uterus, bladder, as well as in the walls of passageways (arteries and veins) and tracts of respiratory and reproductive system. Striated muscle features a highly organized internal structure with repetitive units that provide to the muscle a distinctive striated appearance. Striated muscles can be classified in cardiac muscle and skeletal muscle. Cardiac muscle is responsible for heart contraction and therefore it controls blood flow throughout the body, whereas skeletal muscle is anchored to bone by tendons and it controls skeleton movement and posture.

Adult skeletal muscle is a complex organ system mainly composed of long multinucleated cells, called myofibers. Under normal physiological condition skeletal muscle is a stable tissue, however, it has a remarkable capacity to adapt upon extrinsic stimuli and repair after injury. In example, changes in mechanic signals or nutrient availability result in modifications of muscle fibre size and metabolic properties. Moreover, the presence of muscle stem cells (MuSCs) allows muscle tissue to regenerate after injury.

In this chapter, after a brief overview of skeletal muscle formation and its structure, we will review the muscle regeneration process with a special regard on muscle stem cells and other muscle-resident cell populations.

1. Embryonic Myogenesis

1.1. **Skeletal muscle formation** (adapted from Girardi and Le Grand, 2018)

The process of muscle formation, termed myogenesis, is the result of a well-orchestrated coordination of transcriptional cascades and signalling pathways that spatio-temporally direct cell proliferation and differentiation, migration and morphological changes ((Bryson-Richardson and Currie, 2008).

Myogenesis initiates with commitment of pluripotent mesodermal cells into myoblasts. These proliferating myogenic progenitors activate a muscle-specific genetic program that restrict their cell fate to the myogenic lineage, exiting the cell cycle and differentiating into myocytes. Fusion between myocytes leads to the generation of multinucleated syncytial cells, called myotubes, which lastly will grow and form mature myofibers.

In vertebrates, skeletal muscles derive from the paraxial mesoderm, which segments into somites on either side of the neural tube and notochord (Christ and Ordahl, 1995). The ventral part of the somite, termed sclerotome, gives rise to the cartilage and bones of the vertebral column and ribs, whereas the dorsal region, the dermomyotome, contributes to the formation of the skeletal muscle of the body and limbs, as well as the overlying dermis. Moreover, two regions can be further distinguished in the dermomyotome: the epaxial domain, which gives rise to the back muscle, and the hypaxial domain, from which originate the body wall muscles and the limb muscles.

During embryonic and foetal development, myogenesis occurs in different sequential phases. In the first phase, post-mitotic dermomyotome border cells lose their epithelial organization and delaminate underneath the remaining dorsal dermomyotome (Figure 1 and 2). Here, myoblasts differentiate and form the primary myotome, a structure composed of aligned myocytes and myotubes that serve as scaffold for following waves of myogenesis, providing the positional cues for the muscle cells coming from the somite (Gros et al., 2004; Kahane et al., 1998) (Figure 1 and 2). In the second phase, called primary myogenesis, proliferating muscle progenitors migrate from the central region of the dermomyotome and differentiate

and fuse with the first myofibers thereby giving rise to skeletal muscles (Relaix et al., 2005) (Figure 1 and 2). Myogenesis in the limb will follow a similar process with a delay in developmental time since muscle progenitor cells first have to migrate from the dermomyotomal ventral lip to the limb bud anlagen. Secondary myogenesis occurs during foetal growth, when proliferating muscle progenitors continuously generate foetal myoblasts that fuse to form secondary myofibers. Primary and secondary fibres can be distinguished morphologically and also display differences in muscle gene expression. In example, secondary fibres acquire the characteristics of fast fibres as express only fast myosin, while primary fibres tend to become slow fibres (see I.2.4. Myofibre metabolism). Subsequently, the generated muscle masses undergo very extensive growth during foetal and postnatal period ending with the formation of the mature skeletal muscle tissue. At the end of foetal development, a proportion of myogenic progenitors locate underneath the basal lamina of myofibers, thereby giving rise to adult muscle stem cells (see I.3.1. Muscle regeneration)(Gros et al., 2005).

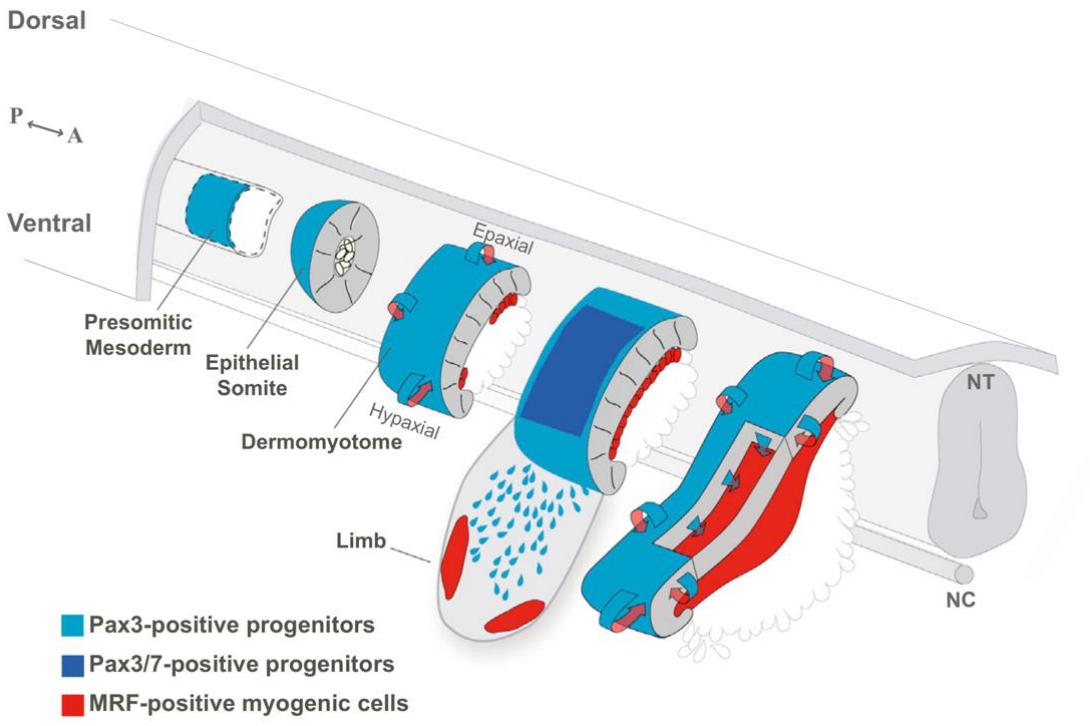

Figure 1. Embryonic Myogenesis. Schematic representation of the morphogenetic movements during primary and secondary myogenesis. Image adapted from Buckingham and Rigby, 2014.

1.2. Molecular regulators of embryonic myogenesis

As mentioned above, the acquisition of the myogenic lineage is achieved by the activation of muscle-specific genes in somatic pluripotent cells (Figure 2). These genes are a group of basic helix-loop-helix (bHLH) transcription factors referred to as MRFs that includes Myf5, MyoD, MRF4 and Myogenin (Asfour et al., 2018). Via their bHLH domains MRFs bind to specific consensus DNA sequences called E-boxes (CANNTG). E-boxes are ubiquitously found in promoter and enhancer regions of muscle specific genes (Berkes and Tapscott, 2005). Before DNA binding, MRFs form homodimers or heterodimers with others MRFs or E-proteins, which are ubiquitously expressed bHLH factors. Once bound to these regulatory regions, MRFs recruit cofactors, chromatin modifiers and the RNA transcription machinery to activate transcription of these loci. MRF expression is spatio-temporally controlled by transcription factors that mainly belong to the Paired Box Genes (Pax), gene family important in early development for the specification of tissues. Among the nine members of the PAX family, Pax3 and Pax7 play a major role during muscle formation.

Pax3 is diffusely detected as the first ever expressed myogenic transcription factor in paraxial mesoderm becoming restricted to the epithelial dermomyotome later during embryogenesis (Magli et al., 2013) (Figure 1). Pax3 commits undifferentiated mesodermal cells toward the myogenic lineage, defining the myogenic progenitor cells (MPCs) (Hutcheson et al., 2009) (Figure 2). Genetical ablation of Pax3 in mouse results lethal at embryonic day 15 due to compromised muscle formation (Bober et al., 1994). Before delamination, Pax3+ cells initiate the expression of the MRFs.

The first MRF to be activated is Myf5, which is transiently expressed from the presomitic mesoderm to the myotome. While in hypaxial dermomyotome Myf5 is directly activated by Pax3, in the epaxial region Myf5 acts independently of Pax3 (Bajard et al., 2006) (Figure 2). Although Myf5 expression does not result in the commitment of presomitic cells to the myogenic lineage, *in vitro*, this transcription factor is sufficient to induce myogenic program into mouse fibroblasts (Braun et al., 1989). Specifically, Myf5 has been shown to activate MyoD, the first MRF to be discovered. MyoD is referred to as the master regulator of the skeletal muscle, as its ectopic expression is able to transform multiple cell lines into myoblasts (Davis et al., 1987; Weintraub et al., 1989). While loss-of-function mutation of MyoD results

in apparently normal muscle development (Braun et al., 1992), combined Myf5 and MyoD mutation leads to a total lack of skeletal muscle formation, suggesting that either Myf5 or MyoD is required for the determination of myoblasts *in vivo* (Rudnicki et al., 1993).

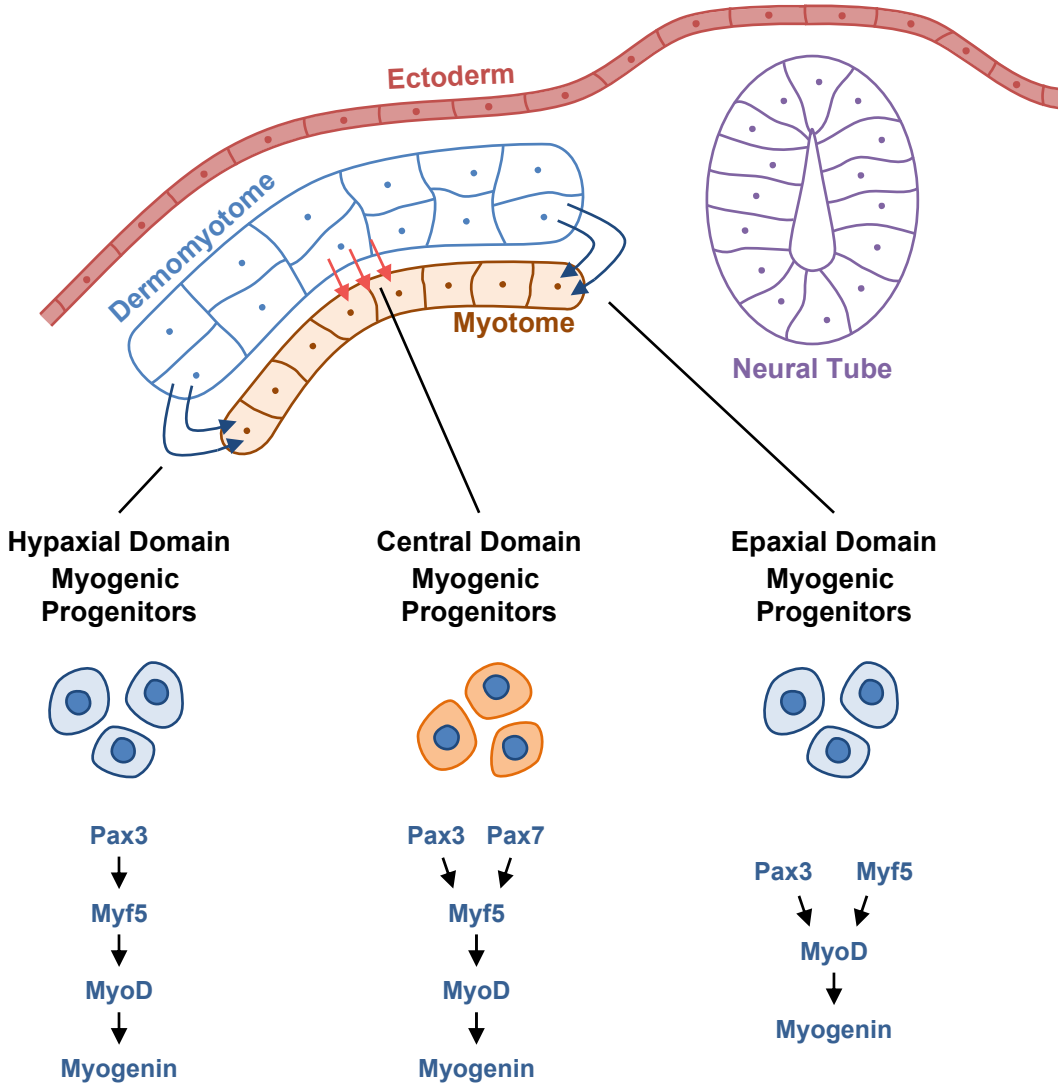

Figure 2. Genetic Hierarchy of MRFs. Schematic representation of embryonic myogenesis and different myogenic progenitors. In the first phase of muscle development, Pax3+ cells delaminate from the extremities of the dermomyotome (blue arrows) and form the myotome. In the second phase (primary myogenesis), Pax3/Pax7 positive cells migrate from the central region of the dermomyotome to complete skeletal muscle formation (red arrows). Importantly, Pax3 is required for Myf5 expression in hypaxial dermomyotome whereas it is dispensable in epaxial dermomyotome, where Myf5 can drive MyoD transcription independently of Pax3. Image adapted from Girardi and Le Grand, 2018.

Together, Myf5 and MyoD activate the last MRF Myogenin and this event correlates with irreversible cell cycle exit and the activation of terminal differentiation (Figure 2). Similarly to Myf5 and MyoD, forced expression of Myogenin is sufficient to convert fibroblast into myoblasts (Edmondson and Olson, 1989). Genetic ablation of Myogenin during mouse development does not impair somitogenesis or MyoD expression, suggesting that, together with many other genetic and biochemical analyses, Myogenin acts downstream of MyoD (and Myf5) in the genetic cascade that regulates myogenesis (Cheng et al., 1993).
In addition to the pivotal role of MRFs, myogenesis can be synergized by a further protein family termed myocyte enhancer factor 2 (MEF2). These transcription factors do not have myogenic potential by themselves, however, if present along with different MRFs they potentiate MRF transcriptional activity and thus myogenic differentiation (Jin et al., 2016).

Another important factor during muscle formation is Pax7, a paralog of Pax3. Whereas Pax3 is activated in all myogenic progenitor cells, Pax7 expression is restricted to the central domain of the dermomyotome and its function is limited to later development and adult myogenesis (Buckingham and Rigby, 2014) (Figure 2). Loss-of-function mutation of Pax7 leads to a milder phenotype than Pax3-null mice, with dramatic defect in muscle size and function and lethal only two weeks after birth. Indeed, while primary myogenesis is not altered, foetal and postnatal muscle growth is completely abolished (Seale et al., 2000).

2. Adult Skeletal Muscle Tissue

2.1. Skeletal muscle structure

In the human body there are more than 600 muscles, which vary considerably in size, shape, and arrangement of fibres. An individual skeletal muscle can be constituted of hundreds or even thousands of muscle fibres bundled together. These myofibre bundles are called fasciculi and they are wrapped in a connective tissue covering, termed perimysium (Figure 3). Several fasciculi form a muscle and are enclosed in an external layer of connective tissue called epimysium (Figure 3). Each myofibre has a specialized cell membrane referred to as sarcolemma and is surrounded by wispy and dense layer of extracellular matrix (ECM), called basal lamina. The basal lamina, together with capillaries and nerves, constitute the endomysium, which combines with perimysium and epimysium to create the collagen fibres of tendons, providing the connection between muscles and bones (Figure 3).

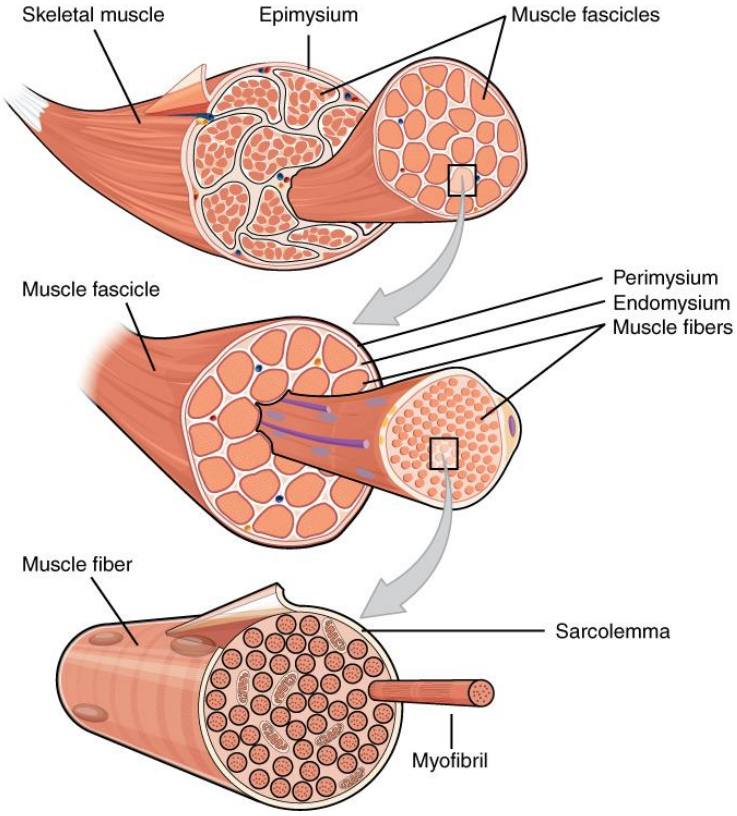

Figure 3. Skeletal Muscle Structure. Schematic representation of the skeletal muscle tissue components. Each muscle has three layers of connective tissue; epimysium, perimysium and endomysium. Myofibres are composed of multiple myofibrils and are bundled together forming muscle fascicle. Image adapted from the textbook “Anatomy and Physiology” (BC Campus Open Textbooks).

2.2. Sarcomere

The outcome of embryonic terminal muscle differentiation is the formation of myofibrils, the contractile structures within myofibers characterized by a highly order series of repetitive units called sarcomeres. Sarcomeres are the functional unit of the muscle fibres and their alternate organization provides the typical striated appearance of skeletal muscle (Ehler and Gautel, 2008) (Figure 4). Specifically, the repetitive pattern derives from the sequential arrangement of myofilaments of actin and myosin together with cytoskeletal elements such as α-actinin and titin. Each side of the sarcomere is delimited by a structure termed Z disc, in which the crosslinker protein α-actinin anchors the elastic filaments of titin to interdigitated and antiparallel filaments of actin and myosin. The Z-disc is localized in the I-band, region that appears darker in electron microscopy. The zone in between the two I-band is called A-band and mainly contains the myofilaments of actin and myosin. Finally, in the middle of the sarcomere is present a brighter region, called H-zone, in which only myosin filaments are present (Figure 4). Of note, in addition to structural proteins, sarcomeres also contain many accessory components, including proteins involved in transcriptional regulation and turnover control (Braun and Gautel, 2011).

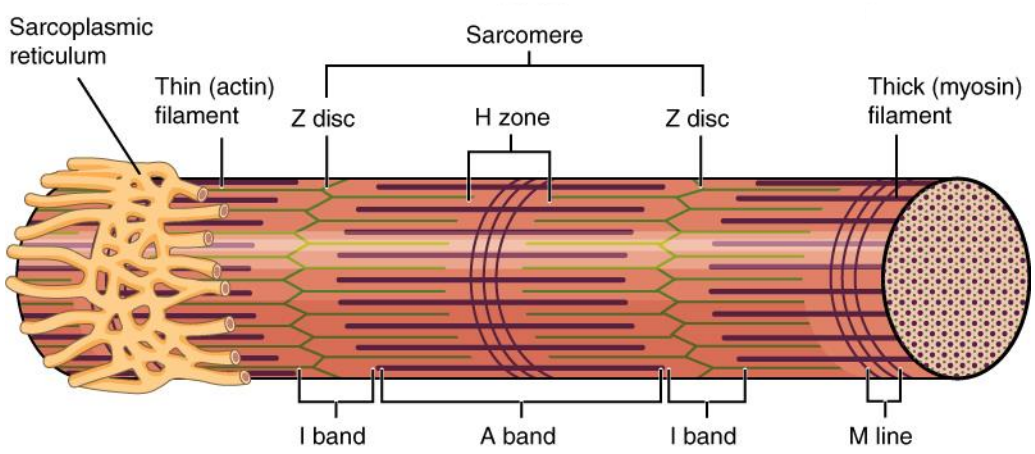

Figure 4. Sarcomere Structure. Sarcomeres are the functional unit of muscle fibres and are composed by a highly ordered series of myofilaments of actin (thin filaments) and myosin (thick filaments). Their sequential arrangement forms different regions, called bands, which give rise to the typical striated appearance of the muscle tissue. Image adapted from the textbook "Anatomy and Physiology" (BC Campus Open Textbooks).

2.3. Muscle contraction

The sequence of events that result in muscle fibre contraction begins with a signal from the motoneurons innervating the tissue. This stimulation fires an action potential through the sarcolemma causing a release of calcium ions (Ca^{2++}) from the sarcoplasmic reticulum, the specialized smooth endoplasmic reticulum of the muscle fibres. Muscle contraction is governed by the levels of calcium that, together with adenosine triphosphate (ATP), regulate the binding between myosin and actin filaments. This mechanism is also known as "sliding filament theory" and was originally proposed by Hugh Huxley in 1953 (Huxley, 1953). In this model, myosin filaments bind to actin filaments and after specific conformational changes the myofilaments slide along each other reducing the distance between the two Z-discs, thus generating muscle contraction. At a molecular level, released calcium interacts with shielding proteins and liberates the actin binding sites localized on myosin head. Myosin heads are associated with ADP and their subsequent binding to actin releases ADP leading to conformational change in myosin head, which consequently pulls actin filaments toward the sarcomere centre shortening the muscle fibre. At this step, myosin heads bind ATP, release actin filaments and ATP hydrolysis into ADP brings the myosin heads to the starting conformation. Additional cycles of ATP hydrolysis and conformational changes allow further myofilament sliding till complete muscle contraction. Importantly, ATP lysis produces heat increasing the body temperature. Indeed, skeletal muscles contribute to the maintenance of homeostasis in the body by generating heat.

2.4. Myofibre metabolism

Adult skeletal muscles are heterogenous in nature and each muscle is composed of specific mixture of fibres with different metabolic and contractile properties (Schiaffino and Reggiani, 2011). Based on these features, two types of myofibres can be distinguished: slow (type I) and fast (type II). In particular, the most physiologically relevant feature of muscle fibre types is the myosin heavy chain isoform (MHC) expressed. MHCs are the motor protein of myofibrils and their different properties directly determine speed, efficiency and power output of muscle contraction. Specialized fibre types are essential for generating diverse arrays of mechanical outputs and due to its plasticity skeletal muscle can adapt and change its contractile properties following physical exercise, muscle disuse or aging.

Type I fibres have an oxidative metabolism, thus producing the ATP required for contraction through the respiratory chain of mitochondria. Slow muscle fibres are more resistant to fatigue and specialized for more continuous activities. Molecular feature of type I fibres is the expression of myosin heavy chain type 1 (official gene symbol Myh7, coding for MYHC-1 protein).

Fast fibres are instead specialized for phasic activities and have a stronger, but short-lasting contraction force. Type II fibres express myosin heavy chain type 2, of which 3 isoforms exists. For these reasons, type II fibres can be further subdivided in type IIA (Myh2 expressing MYHC-2A), IIB (Myh4 expressing MYHC-2B) and IIX (MYH1 expressing MYHC-2X). While type IIB and IIX fibres have a glycolytic metabolism and exert strong and fast contractions, type IIA fibres generate quite rapid contraction, but possess an oxidative metabolism.

3. Muscle Stem Cells and Adult Myogenesis

3.1. **Muscle regeneration** (adapted from Girardi and Le Grand, 2018)

Skeletal muscle is a low-turnover tissue where the vast majority of myonuclei and muscle-resident cells are in growth-arrested G0-G1 states (Giordani et al., 2019). Myofibres are irreversibly post-mitotic, but the presence of muscle stem cells in intimate association with myofibres ensures their regeneration. This adult muscle stem cell population is also known as satellite cells (see I.3.2. Satellite cells: the muscle stem cell population) and are essential for skeletal muscle growth and reconstruction following injury (Lepper et al., 2011; Sambasivan et al., 2011). In response to myofibre damage, a multi-step regeneration process is activated to repair the tissue involving numerous cell types and events (Juban and Chazaud, 2017) (Figure 5). The first phase of muscle regeneration is characterized by an immediate necrosis of the damaged myofibres, together with an acute inflammation, immune cell infiltration and fibroblast expansion (Figure 5). Soon after, MuSCs leave their quiescent state and partially mimicking some sequences occurring during embryonic myogenesis they start to express a series of myogenic transcription factors that conduct their fate during muscle regeneration (Mashinchian et al., 2018). Muscle stem cells become activated and proliferate giving rise to a transient amplifying population expressing Myf5 and MyoD. At this stage they are referred to as adult myoblasts. These myogenic progenitors are able to differentiate into Myogenin+ myocytes and lastly fuse together to form new myofibres (Figure 5). In parallel, revascularization, reinnervation and deposition of new extracellular matrix ultimate the regeneration of the muscle tissue (Figure 5). Generally, 3-4 weeks after injury, skeletal muscle returns to a normal morphological and histological condition and are characterized in mice by centrally located nuclei. In common with all adult stem cells, a hallmark of the MuSCs is their ability to self-renew (Collins et al., 2005). During regeneration, a correct balance between self-renewal and differentiation is essential. Symmetric divisions allow the maintenance of the MuSC pool, while asymmetric expansion leads to the generation of myogenic progenitor cells (Kuang et al., 2007).

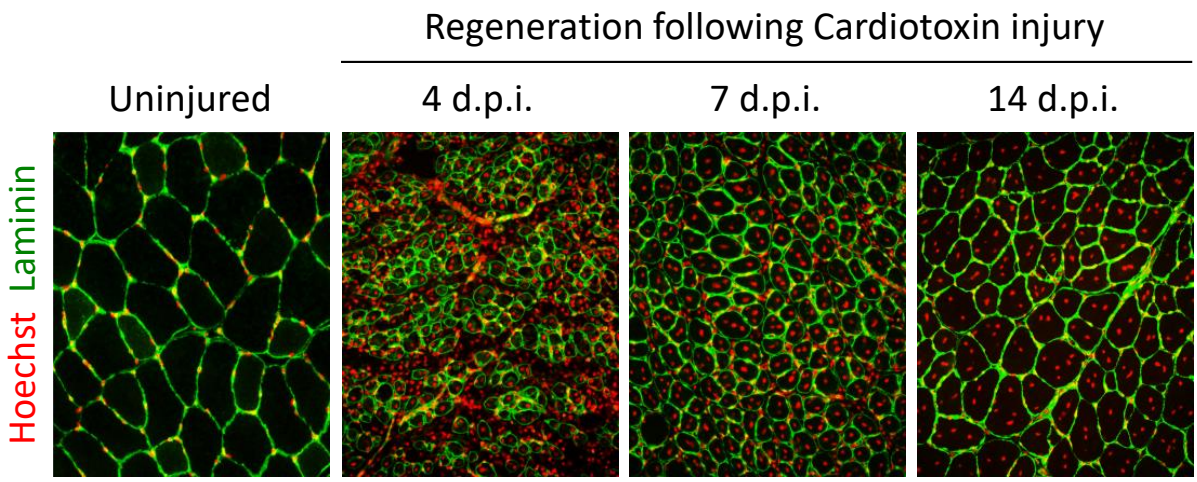

Figure 5. *Tibialis anterior* muscle regeneration time course. Immunofluorescence staining for Laminin at different time points, before and after Cardiotoxin-induced injury. Note that after a first period of necrosis and invasion of inflammatory cells and proliferation of myogenic cells (3 days), new myofibers are generated (7 days). 14 days post injury the tissue integrity is restored and newly formed myofibers can be distinguished by the presence of centrally located nuclei. Unpublished data and figure from Fabien Le Grand.

3.2. Satellite cells: the muscle stem cell population

Satellite cells are the muscle stem cells (MuSCs). They were discovered by Alexander Mauro, which observed a group of mononucleated cells at the periphery of myofibers by electron microscopy and named them based on their anatomic location (Mauro, 1961) (Figure 6). At that time, it was already well known that skeletal muscle regenerates and, due to the close association with myofibres, MuSC soon became good candidates for the cellular origin of new adult fibres.

The first evidence supporting this role of MuSCs was provided by Bischoff in 1975 using ex vivo single myofibres isolated from rat and cultured for several days. In this setup, continuous observation of MuSCs associated to the fibres revealed that these cells are able to synchronously divide after 22 hours and give rise to a progeny that formed multinucleated myotubes after 5 days *in vitro* (Bischoff, 1975). In 1977 the capability of MuSCs to build new fibres was also confirmed *in vivo*, using radioactive labelling and cell tracking of MuSCs during rat muscle regeneration (Snow, 1977).

Few years after, also the embryonic origin of MuSCs started to be elucidated. Early studies in chick-quail chimeric embryo provided the first experimental evidence that MuSCs derive from somites (Armand et al., 1983). However, the final confirmation arrived only in the 2000's, when, with the use of modern techniques and genetic mouse models, it was proven that MuSCs arise from a specific population of the delaminating dermomyotome. Indeed, at the end of foetal development, a proportion of Pax3+/Pax7+ myogenic progenitors locate underneath the basal lamina of myofibers, enter quiescence and maintain the expression of Pax7, thereby giving rise to the muscle stem cell population (Gros et al., 2005; Relaix et al., 2005) (Figure 6).

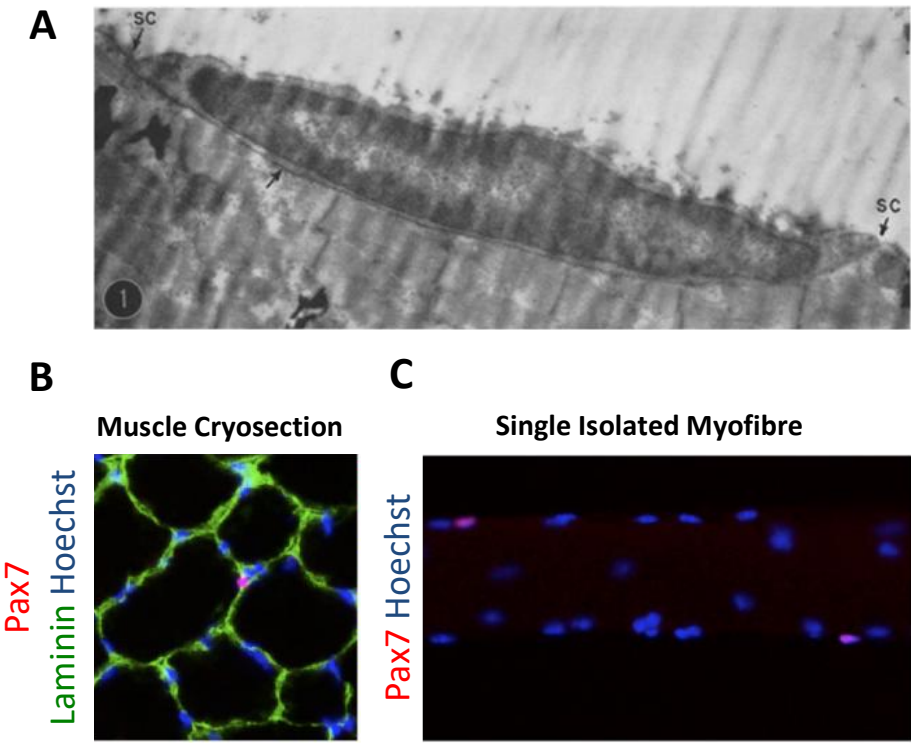

Figure 6. Satellite Cells. A. Electron microscopy photo showing a longitudinal view of a satellite cell in close contact with the host myofibre (Mauro, 1961). **B.** *Tibialis anterior* cross-section immune-stained with antibody against Pax7 (MuSC marker) and Laminin (Basal lamina component). **C.** Longitudinal view of a portion of a single myofibers isolated from *Extensor Digitorum Longus* (EDL) muscle and immuno-stained for Pax7. Adapted from A. Parisi's thesis.

3.3. Genetic program of adult myogenesis

MuSCs have been intensively studied in order to understand their molecular features. In the last decades multiple surface molecules and transcription factors have been found to be specifically expressed by MuSCs and/or their progeny.

Satellite cell membrane is characterized by several transmembrane proteins that are MuSC-specific, such as adhesion molecules, receptors and co-receptors. Frequently used markers are α7-integrin, M-Cadherin and CD34, which are expressed in both quiescent and activated MuSCs, as well as Syndecan-3 and -4 (Yin et al., 2013). These two transmembrane heparan sulphate proteoglycans are coreceptors and they facilitate the transduction of several signalling pathways (Cornelison et al., 2001). A specific marker for quiescent satellite cells only is Calcitonin Receptor that, as described below, plays an important role in controlling MuSC cell cycle entry (Fukada et al., 2007).

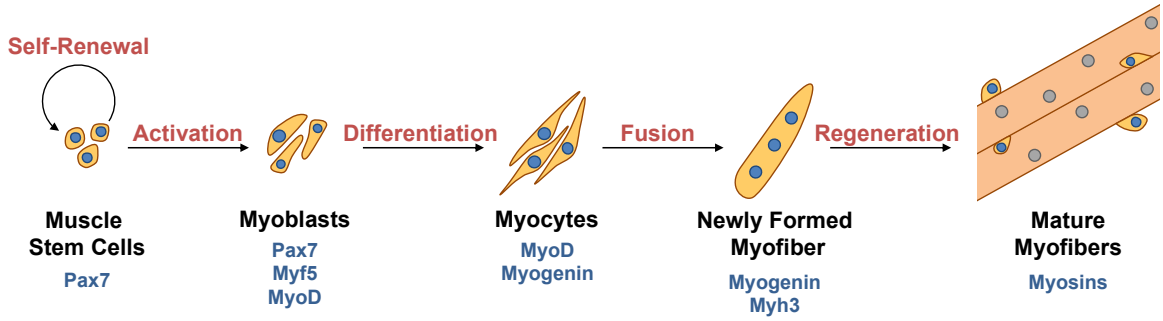

Figure 7. Genetic Program of Adult Myogenesis. Muscle regeneration relies on Satellite cells (MuSCs), quiescent muscle stem cell population characterized by the expression of Pax7. Upon injury, MuSCs get activated and give rise to an amplifying population termed myoblasts. These myogenic progenitors express Myf5 and MyoD, and initiate the myogenic program. Myoblasts differentiate into myocytes, which in turn express Myogenin and commit to terminal differentiation. Myocytes fuse together to form new fibres, which are characterized by the expression of the embryonic isoform of Myosin Heavy Chain (Myh3). Lastly, the newly formed myofibres express the contractile machinery compounds leading to fibre maturation and muscle regeneration. Importantly, during muscle regeneration MuSCs self-renew allowing the maintenance of the MuSC pool. Image adapted from Girardi and Le Grand, 2018.

Anyhow, the best MuSC marker is the paired-box transcription factor Pax7, which defines the MuSC pool and specifies their myogenic lineage (Seale et al., 2000) (Figure 7). Pax7-null mice are significantly smaller than wild-type animals and die within 2 weeks after birth, but most importantly they are characterized by the complete absence of MuSCs. Besides its role during muscle development, Pax7 is essential also during adult myogenesis, where conditional Pax7 gene inactivation dramatically perturbed muscle regeneration (Gunther et al., 2013; von Maltzahn et al., 2013). Further confirmation of Pax7 role in muscle repair derived from combined analyses of gene expression and genome-wide Pax7 binding-sites, which showed that Pax7 regulates a panel of genes responsible for proliferation and inhibition of differentiation (Soleimani et al., 2012).

As mentioned before, upon muscle injury, MuSCs leave their quiescence state and initiate a muscle-specific genetic program that largely recapitulates embryonic myogenesis with only minor differences (Figure 7). In fact, among Pax7 target genes, Myf5 is one of the most important and it is the first myogenic regulatory factor (MRF) expressed as in muscle development. Myf5 is a regulator of myoblast proliferation and its expression is followed by MyoD activation (Figure 7). MyoD is a MRF required for differentiation, thus a balance between Myf5 and MyoD determines myoblast behaviour (Yin et al., 2013). Then, MyoD induces the MRF Myogenin (Myog) expression and this event correlates with irreversible cell cycle exit and the activation of terminal differentiation (Figure 7). In this last phase, myocytes express Myosin Heavy Chain 3 (marker of terminal differentiation) and fuse together to form the new myofibres, which will mature and finally express and built all the contractile machinery compounds (Figure 7).

3.4. Muscle stem cell quiescence

In healthy adult muscles, MuSCs persist in a quiescent state (G0 phase) characterized by a highly condensed chromatin (Gunther et al., 2013) and a low metabolic and transcriptional activity (Cheung and Rando, 2013). Unfortunately, the study of quiescence has been hindered by a considerable technical limitation: MuSCs become rapidly activated when removed from their niche. To try to better examine the dormant state of MuSCs *in vitro*, Rando's group developed a biomimetic microenvironment with laminin-coated collagen-based artificial

myofibres in combination with a specialized medium (Quarta et al., 2016). In this engineered niche, murine and human MuSCs maintain their dormant state displaying key quiescent features. Notably, this optimized culture environment enhanced the MuSC self-renewal potential resulting in an improved engraftment *in vivo* compared to traditional conditions.
To gain insights on the transcriptomic and molecular state of MuSC in their native *in vivo* state, great technical advancements have been achieved in 2017, when 2 group designed new technical approaches to study MuSC quiescence (Machado et al., 2017; van Velthoven et al., 2017). To overcome the major transcriptional and epigenetic changes induced by traditional isolation protocols, Machado and colleagues adopted an in situ PFA fixation prior to muscle dissociation (Machado et al., 2017). In this setup, MuSC isolation, purity and yield were comparable to current isolation protocols, however, pre-fixed MuSC retained the physiological spindle shape that is typically lost during dissociation. Another way to circumvent the impact of isolation procedures came from Rando group, where they were able to analyze quiescent MuSC transcriptome through a MuSC-specific RNA labelling system (van Velthoven et al., 2017). In both setups, these approaches revealed expected differences between quiescent and early activated MuSCs. When removed from their niche, MuSCs display significant increase in transcription of immediate early genes for activation, as well as various cell cycle regulators and myogenic factors. On the other hand, quiescent MuSCs were characterized by upregulation of genes typically related to quiescent, such as fatty-acid metabolism and cilia.

Lastly, an important aspect of MuSC quiescence regards an interesting “alerting” mechanism adopted by MuSCs in particular contexts (Rodgers et al., 2014). Indeed, it has been shown that MuSCs in an uninjured muscle contralateral to an injured muscle exhibit specific status termed G_{alert} that slightly differs from the normally described G0 quiescent. MuSCs in G_{alert} display a phenotype characterized by a small but significant increase in metabolic and transcriptional activity, a more pronounced propensity to enter cell cycle and an enhanced tissue regenerative function.

3.5. Self-renewal and heterogeneity of muscle stem cells

The ability of adult stem cells to generate daughter cells with identical stem properties is called self-renewal. Transplantation of a single intact myofibre into a radiation-ablated muscles endogenously defective for myogenesis led to generation of hundreds of myofibres bearing thousands of myonuclei and over two hundred associated muscle stem cells, demonstrating that MuSCs self-renew and are self-sufficient as a source of regeneration (Collins et al., 2005) (Figure 7). Interestingly, MuSCs can self-renew by reverting their myogenic commitment. Ex vivo experiments on single isolated myofibres showed that, although all the MuSCs upregulate MyoD after 24 hours, a subgroup of myogenic progenitors (about 23%) loses MyoD expression at the third day and return to a quiescent state (Zammit et al., 2004). Moreover, close examination of MuSC divisions on isolated single myofibers revealed that MuSCs can undergo both symmetric and asymmetric division: symmetric planar divisions (division plane parallel to myofibre axis) produce two identical stem cells, whereas asymmetric apical-basal divisions (division plane perpendicular to myofibre axis) generate two different MuSCs. While symmetric expansion allows the maintenance of the stem cell pool, asymmetric division give rise to one stem cell and one committed MuSC. These cells display heterogeneity in terms of expression of MuSC markers and in the degree of commitment, and in fact, MuSCs are not a homogenous population (Kuang et al., 2007; Le Grand et al., 2009).

One of the most important differentially expressed genes is Myf5. Using lineage tracing experiments, it was shown that a subset of MuSCs (10%) never expresses Myf5 maintaining a more stemness identity (Kuang et al., 2007). Indeed, two subpopulation of MuSCs can be distinguished: a more committed subgroup that expresses both Pax7 and Myf5, and a more stem subgroup expressing only Pax7. Pax7+/Myf5- can give rise to both Pax7+/Myf5- and Pax7+/Myf5+, whereas Pax7+/Myf5+ only generate Pax7+/Myf5+. Moreover, when transplanted in MuSC-devoid muscles, Pax7+/Myf5- cells efficiently supports muscle regeneration, forming new myofibres and extensively contribute to the MuSC compartment. However, Pax7+/Myf5+ cells preferentially differentiate and form myofibres, but do not restore MuSC pool. Together, these results support the evidence that Pax7+/Myf5- population has a broader lineage potential and thus can truly defined stem cells.

3.6. MuSC niche: the basal lamina and the sarcolemma

MuSCs reside between the sarcolemma and the basal lamina that surrounds all the myofibres. These two elements are the major components of MuSC niche, the particular microenvironment that preserves stem cell features, such as self-renewal ability, tissue specificity and pluripotency. Notably, MuSC niche is highly polarized and characterized by cell-cell interactions with the sarcolemma on the MuSC basal pole and ECM interactions on apical pole (Figure 8).

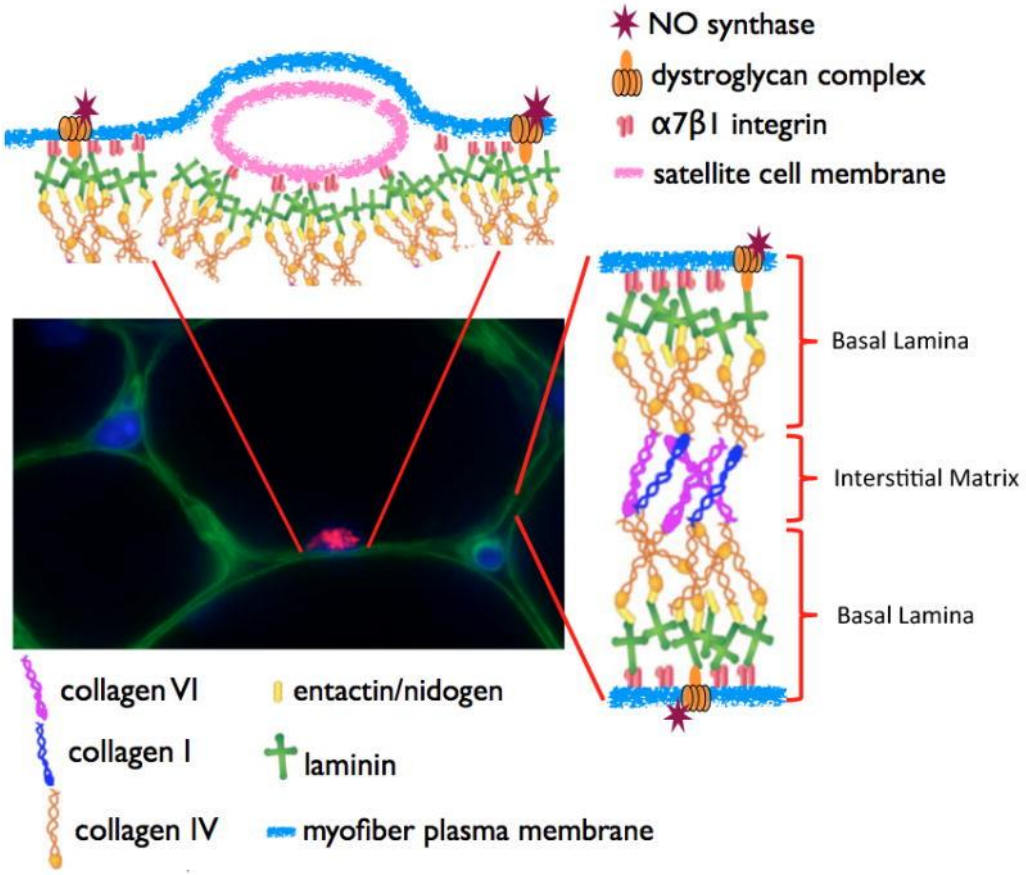

Figure 8. The Muscle Stem Cell Niche. MuSC (identified by immunofluorescent staining for Pax7) under the basal lamina (marked by Laminin immunofluorescent staining, in green) on a muscle cryosection. The schematic drawing illustrates the components of the extracellular matrix within the MuSC niche. From Lund and Cornelison, 2013.

The basal lamina is a thin layer of extracellular matrix that envelops the myofibres and is connected to the sarcolemma and the myofibre cytoskeleton through the dystrophin-glycoprotein complex (Michele and Campbell, 2003). Laminin and collagen IV are the main components of the basal lamina, which are secreted by the myofibres and muscle-resident fibroblasts respectively (Gillies and Lieber, 2011) (Figure 8). Moreover, a further layer of ECM composed of collagens and proteoglycans, named interstitial matrix, fills the space between myofibres. In addition to physical support, elasticity and mechanical force transduction during muscle contraction, the basal lamina and interstitial matrix provide a reservoir of signalling molecules ready to be released upon muscle injury and promote MuSC activation.
The linkage of MuSCs to laminin is established through the apically localized membrane receptors α7/β1-integrin (Rozo et al., 2016). Laminin and integrin play an important role in MuSC quiescence and behaviour, as their mutation significantly alters MuSC state. MuSC-specific loss of integrin β1 in adult mice results leads to a break in quiescence and cell cycle entry (Rozo et al., 2016), while laminin-α2-null mice fail to maintain the proper number of Pax7-positive MuSCs during secondary myogenesis (Nunes et al., 2017). Of note, MuSCs also participate to the remodelling of their niche by secreting the glycoprotein Fibronectin, which promotes MuSC expansion in Wnt-dependent manner (Bentzinger et al., 2013).

The sarcolemma chemically and electrically isolates MuSCs from the sarcoplasm, the myofibre cytoplasm. MuSCs and muscle fibres interact through CD34 and M-Cadherin (Beauchamp et al., 2000; Irintchev et al., 1994). While M-Cadherin-null mice display no overt muscle phenotype suggesting a compensation by other cadherins (Krauss, 2010), CD34-null mice show impaired entry into proliferation and delayed myogenic progression (Alfaro et al., 2011). Lastly, quiescent MuSCs express Calcitonin Receptor (Fukada et al., 2007), transmembrane protein which is able to indirectly sense electrical signals from innervated myofibers. Thus, electric stimuli might be involved in MuSC fate regulation, in fact, MuSC-specific loss of Calcitonin Receptor results in an aberrant cell cycle entry and extravasation into the interstitial space (Yamaguchi et al., 2015).

4. Skeletal Muscle Cell Heterogeneity

4.1. Skeletal muscle cellular composition

Skeletal muscle is not composed by myofibres only, in fact, several other components are present within this complex tissue and they actively participate to its functionality. In example, muscle tissue is in intimate association with the vascular and nervous system, as supported by the abundant presence of vessels and motoneurons among the myofibres. The broad network of blood capillaries provides the correct supply of nutrients and oxygen as well as the clearance of metabolic waste. Motoneurons are connected to the sarcolemma through a particular synapse called neuromuscular junction and are responsible for the transmission of signals required for muscle contraction.

Moreover, skeletal muscle is also populated by a variety of different mononuclear cell types that have been described during the past three decades. Importantly, in 2019 our laboratory determined the complete cellular composition of adult mouse skeletal muscle by combining single-cell transcriptomics and single-cell mass cytometry (Giordani et al., 2019). In this work, Giordani and colleagues defined each mononuclear cell type in the tissue identifying 10 distinct populations, of which 8 were previously described (MuSCs, FAPs, macrophages, neutrophils, endothelial cells, B cells, T cells, and glial cells) and 2 newly identified (SMMCs and Scleraxis+ tenocytes) (Figure 9). In the next paragraph we will shortly review the main muscle-resident cell type with specific regard to their contribution to muscle regeneration.

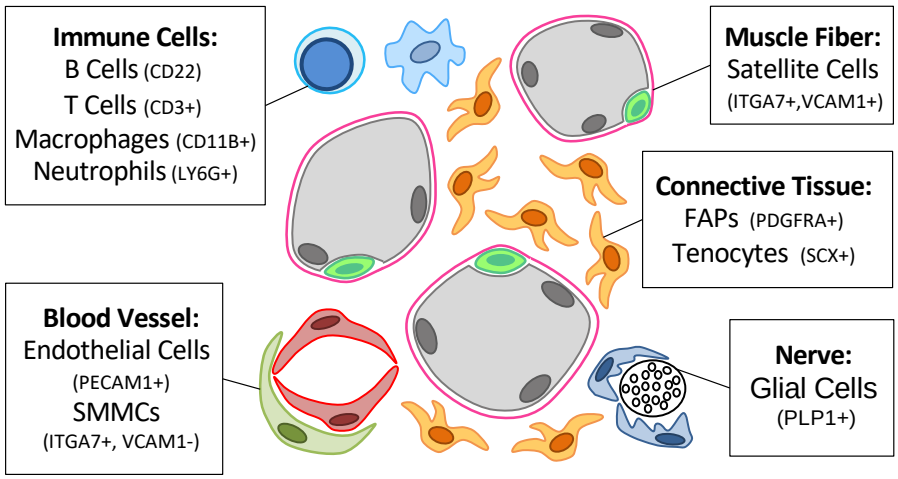

Figure 9. Muscle-Resident Cell Populations. Schematic representation of the 10 different mononuclear cell types in adult mouse muscle. Adapted from Giordani et al., 2019.

4.2. Muscle-resident cell types

Interstitial Cells

Interstitial cells are a heterogeneous population of cells intercalated between the fibres and in muscle tissue. The majority of interstitial cells in skeletal muscle are connective tissue fibroblasts, which express the specific marker Tcf4 (Murphy et al., 2011). These muscle-resident fibroblasts are responsible for extracellular matrix component secretion and play a fundamental role in fibrotic deposition in pathological conditions. Moreover, Tcf4+ fibroblasts are important for muscle regeneration, as their genetic ablation results in an impaired tissue repair process (Murphy et al., 2011). Indeed, fibroblasts transiently expand during adult myogeneis and control myoblast fate through a reciprocal and dynamic interplay. Fibroblasts promote myoblast proliferation and inhibit their differentiation, thus avoiding premature differentiation and support a proper muscle regeneration process. Reciprocally, proliferating myoblasts support the initial fibroblast expansion, whereas differentiated myocytes negatively regulate the number of fibroblasts in the later phases of regeneration.
Importantly, after the paper of Giordani et al. muscle fibroblasts definition has been revised (Giordani et al., 2019). Indeed, the high-dimensional single-cell cartography of the muscle revealed that the muscle fibroblast compartment is composed of two subpopulations, called fibro-adipogenic progenitors (FAPs) and Scleraxis+ (Scx+) tenocytes.

FAPs were previously identified by two independent groups in 2010 (Joe et al., 2010; Uezumi et al., 2010) and besides the expression of Tcf4, FAPs specifically express Sca1 and PDGFRα. These cells are generally non-myogenic and are mainly bipotent as they are able to differentiate along the fibrogenic and adipogenic lineages both *in vitro* and *in vivo* (Joe et al., 2010; Uezumi et al., 2010, 2011). However, it has also been shown that FAPs exhibit robust osteogenic activity in response to BMP2 stimulation or activin signals (Lees-Shepard et al., 2018; Wosczyna et al., 2012). The critical role of FAPs during muscle regeneration was recently proven in Rando's group, where they developed a genetic mouse model to specifically target and ablate PDGFRα-expressing cells, thus FAP lineage (Wosczyna et al., 2019). Depletion of FAPs results in a reduced MuSC and blood cell expansion and in a consequent regenerative deficit, suggesting the requirement of FAPs in the early stages of muscle regeneration.

Tenocytes are present in all developing tendons and ligaments, but also in the adult skeletal muscle interstitium. These interstitial cells express tendon cells markers and among them Scleraxis, a tendon cell-specific transcription factor that regulates tendon differentiation during development (Murchison et al., 2007). *In vitro*, muscle-derived tenocytes maintained their identity whereas *in vivo* were able to create collagen-rich environment between the myofibers following transplantation. Although their function needs to be fully elucidated, it is reasonable to postulate multiple roles for this interstitial population, such as extracellular matrix secretion and remodelling, tendon regeneration and myofibre-tendon attachment support.

Blood vessels

MuSCs are not randomly distributed throughout the tissue but display preferential localization in proximity to capillaries (Christov et al., 2007). Blood vessels are source of oxygen and nutrients thus supporting MuSC survival, as well as regulating their fate. Indeed, hypoxic conditions have been proposed to favour MuSC self-renewal, hence oxygen levels control the balance between commitment and self-renewal (Liu et al., 2012). In addition, vascular-proximity allows MuSCs to interact with CD31+ endothelial, which have been shown to secrete growth factors promoting MuSC proliferation and survival.

Immune cells

Although in healthy muscle tissue immune cells are barely detectable, after muscle damage numerous and various immune cell types invade the injured area in few hours. Early after injury, neutrophils enter the muscle tissue coming from the circulation (Saclier et al., 2013a), as well as macrophages, coming from the surrounding connective tissue (Brigitte et al., 2010). Other cell types reported to infiltrate the damaged muscle are eosinophils and regulatory T cells, which, although their numbers remain very low compared to macrophages, they contribute to the early steps of muscle regeneration by promoting FAP and myoblast expansion, respectively (Burzyn et al., 2013; Heredia et al., 2013).

Neutrophils are important for muscle tissue repair, as mice depleted of neutrophils show a deficient regenerative response (Teixeira et al., 2003). The number of neutrophils peaks immediately after damage and rapidly declines after 2/3 days post injury. In fact neutrophils contribute mainly in the first wave of the proinflammatory phase by releasing proinflammatory cytokines including TNF-α (tumor necrosis factor alpha), IFN-γ (Interferon-γ), and IL-1β (interleukin-1β) (Yang and Hu, 2018), as well as enzymes and oxidative factors to facilitate the clearance of the necrotic muscles (Nathan, 2006).

Early studies demonstrated macrophages are essential for muscle regeneration (McLennan, 1996) and more recent work confirmed these findings via selective ablation of macrophages (Arnold et al., 2007; Tidball and Wehling-Henricks, 2007). Macrophages are recruited by neutrophils shortly after injury and intensively accumulate when neutrophil number declines. Interestingly, macrophages switch their phenotype during regeneration processes and consequently exert dual and diverse functions (Biswas and Mantovani, 2010). At the early stages of injury macrophages are proinflammatory contributing to the phagocytosis of necrotic myofibres, but also preventing early myogenic differentiation. The second phase of muscle repair is instead characterized by anti-inflammatory macrophages, which, while dampening environmental inflammatory signals, they directly support adult myogenesis and fibre growth (Chazaud et al., 2009). Specifically, *in vitro* analyses showed that proinflammatory macrophages promote myoblast proliferation whereas anti-inflammatory macrophages stimulate both their commitment into terminal myogenesis and myotube formation (Saclier et al., 2013b). Moreover, macrophages prevent myoblast and myotube apoptosis through direct cell-cell contacts that specifically trigger anti-apoptotic signals and thus ensuring their survival until regeneration completion (Sonnet et al., 2006). Reciprocally, activated MuSCs have been shown to attract monocytes (macrophages precursors) to the damaged area and interplay with macrophages to amplifies chemotaxis (Chazaud et al., 2003).

II. Cell-Cell Fusion

Biological membranes define both the boundaries of the basic unit of cellular structure as well as the diversity of the internal compartments in eukaryotes. Membrane integrity safeguard cell organization and internal compartmentalization, therefore is necessary for life. However, controlled fusion of two membranes is essential for basic cellular functions and crucial for the development and maintenance of living organisms. Membrane fusion has been described in several organisms, ranging from yeast to human, and in multiple cell types, such as gametes, macrophages and myoblasts. The fusion process can occur intracellularly, as with synaptic vesicles, or intercellularly, as for mammalian fertilization, and these processes adopt different mechanisms and involve diverse array of specialized proteins (Chen and Olson, 2005) (Figure 10).

Intracellular fusion has been extensively studied and is dependent on SNARE proteins, membrane-embedded receptors localized on the vesicle (vesicle-anchored SNAREs, v-SNAREs) and target membranes (target-anchored SNAREs, t-SNAREs) (Figure 10). SNAREs interactions allow the recognition between the two membranes and the formation of a complex constituted of a bundle of α helices, termed SNAREpin. Lastly, SNAREpin brings the appose lipid bilayers together and promotes their fusion (Han et al., 2017).

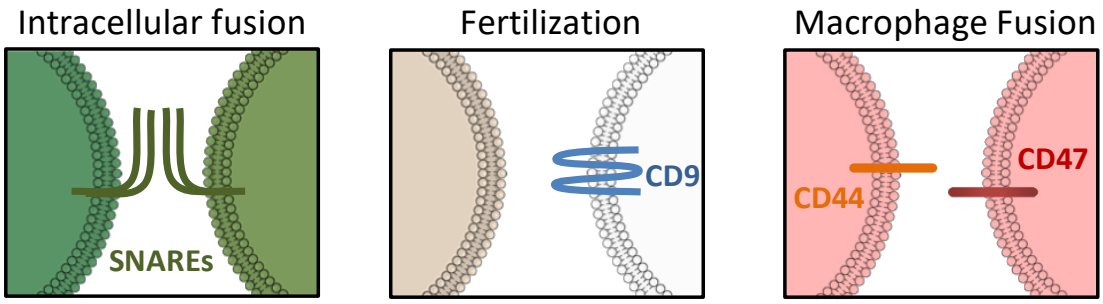

Figure 10. Mammalian Cell Fusion. Simplified version of the mammalian intracellular and cell-cell fusion processes. Intracellular vesicle fusion is mediated by SNAREs, whereas macrophage fusion events require the transmembrane proteins CD4 and CD47. Mammalian fertilization is dependent on the tetraspanin CD9 localized on the egg membrane.

Despite the well-established mechanism of intracellular fusion, little is known about the mechanism underlying intercellular fusion. Importantly, experimental analyses of cell-cell fusion revealed that this process is independent of SNAREs and thus involve alternative mechanisms and specialized molecules, depending on the cell type and fusion events. The most studied cell-cell fusion processes are mammalian fertilization, macrophage activity and myotube formation.

The fertilization process in mammals consists in a series of complex events that finally concludes with the membrane fusion of the two gametes, sperm and egg (Primakoff and Myles, 2002). Prior to fusion, the sperm has to penetrate the outer layer of the oocyte, secrets specialized enzymes and reaches the inner layer of the oocyte, called zona pellucida. Only when the sperm is within the zona pellucida, the membranes of the two gametes fuse together. Many proteins have been associated to this process in the last decades. However, to date, the only protein that has been shown to be essential for sperm-egg fusion is the Tetraspanin CD9 (Figure 10). Female mice carrying CD9 mutation are sterile because their eggs are defective in membrane fusion although they interact with the sperm normally (Kaji et al., 2002).

Macrophages are another cell type able to fuse. Interestingly, these cells can generate two different multinucleated cell types depending on the fusion partner. Macrophages differentiate and fuse with other macrophages to form the hallmark giant cells of inflammation, important for immune responses, but they also undergo a complex fusion process that leads to their conversion into osteoclasts, critical for bone remodelling (Vignery, 2000). In macrophages, the proteins implicated in the fusion process are CD44 (also known as macrophage fusion receptor MRF) and CD47 (Figure 10). These transmembrane proteins have been shown to function through a similar mechanism of the cell surface protein involved in myoblast fusion in *Drosophila* (see II.1. Myoblast Fusion during *Drosophila* Development), suggesting that different cell-cell fusion events may share common features.

One of the most used experimental systems for the analysis of cell-cell fusion is myoblast fusion, which in the last decades started to be intensively studied mainly in *Drosophila* and in mouse. In the following paragraphs, we will provide an overview of the current understanding of myoblast fusion in both animal models.

1. Myoblast Fusion during *Drosophila* Development

In *Drosophila* embryos, mononucleated myoblasts fuse together leading to the formation of larval body wall muscles, equivalent to skeletal muscle in vertebrates. Myoblast fusion in *Drosophila* occurs between two types of muscle cells, termed founder cells and fusion-competent myoblasts (FCMs) (Rochlin et al., 2010) (Figure 11). *Drosophila* larva muscles are constituted by a single myofibre, which arises from the fusion of one founder cell with several FCMs. Following the initial fusion, additional rounds of fusion continue between the developing myotube and FCMs until the final muscle size is achieved. While features of the founder cell determine muscle properties, the number of FCMs that fuse within the nascent myotube defines muscle size and mass. These two populations derive from the same mesodermal lineage and they are in close proximity within *Drosophila* embryo. Despite the small distances, cell migration is an essential prerequisite to achieve embryonic myogenesis.

1.1. Interaction between founder cell and fusion-competent myoblasts

Myoblast migration in *Drosophila* is dependent and mediated by transmembrane proteins belonging to the immunoglobulin superfamily (IgSF), such as Dumfounded (Duf) and Stick-and-Stones (Sns) (Figure 11). In particular, founder cells specifically express Duf (Ruiz-Gomez et al., 2000)(Ruiz-Gomez et al., 2000), while Sns is found on the cell surface of FCMs (Bour et al., 2000). Briefly, Duf functions to attract FCMs, which are able to migrate toward founder cells in a Sns-dependent manner (Kocherlakota et al., 2008; Ruiz-Gomez et al., 2000). Duf and Sns are not only important for migration and play an essential role also in myoblast recognition and adhesion, two critical steps in cell-cell fusion. FCMs need to recognize founder cells and myotubes, but not other FCMs. Accordingly, Sns does not promote homotypic interaction between Sns-expressing cells but physically associate with Duf (Galletta et al., 2004). This interaction allows the formation of an adherent structure between founder cell and FCM, called “fusogenic synapse” (Sens et al., 2010) (Figure 11).

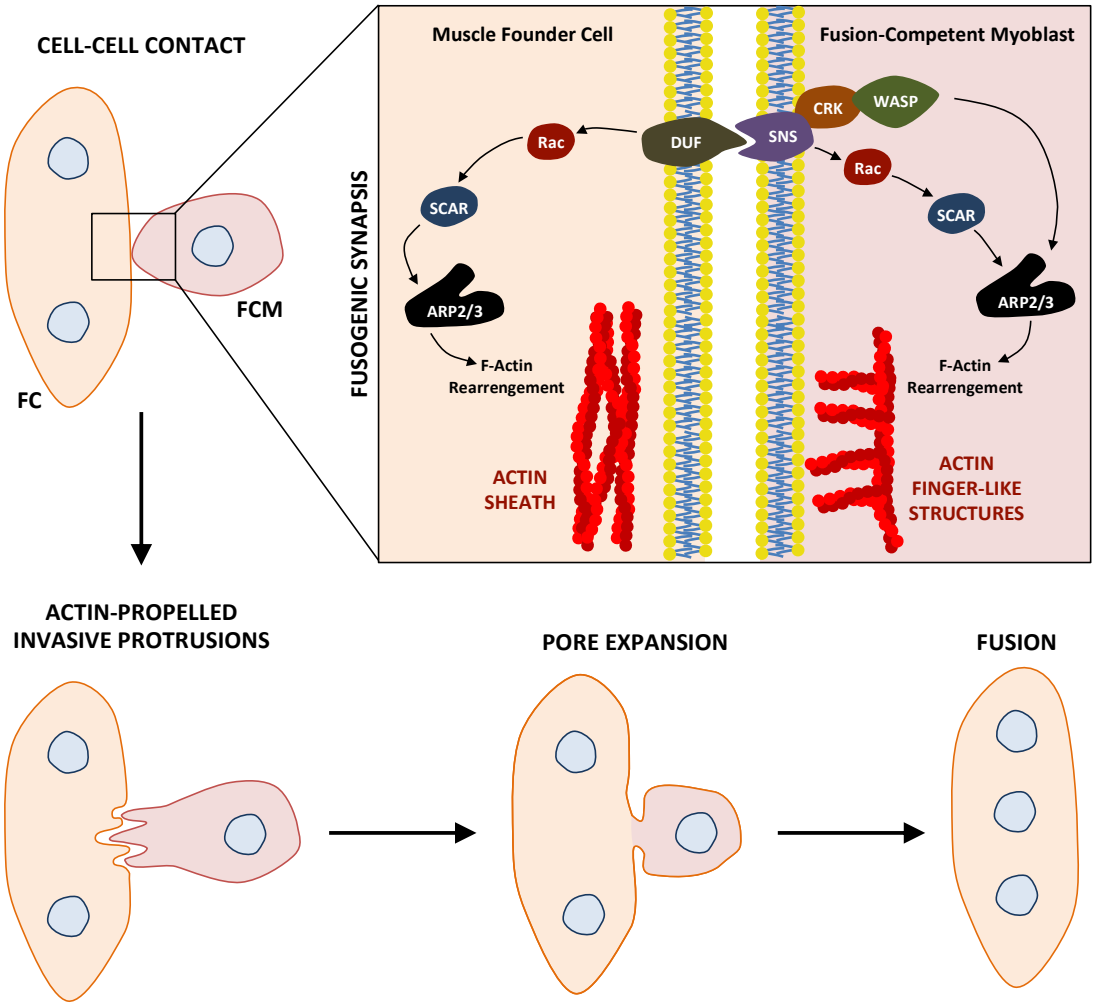

Figure 11. Muscle Cell Fusion in *Drosophila*. Myoblast fusion in *Drosophila* occurs between two types of muscle cells, termed founder cells (FCs) and fusion-competent myoblasts (FCMs). These muscle progenitors are characterized by the expression of Duf and Sns, respectively. Once in close proximity, Duf-Sns interaction initiates numerous intracellular events that result in a highly asymmetric actin-remodelling. FCs build a thin sheath of F-actin at the level of the fusogenic synapse, whereas FCMs form invasive actin finger-like structures. Briefly, FCMs invade the receiving FC with actin-propelled membrane protrusions leading to the pore formation and finally to complete fusion.

1.2. Asymmetric actin cytoskeleton rearrangement

Importantly, both Duf and Sns trigger downstream signalling that drives fusion event through a drastic cytoskeleton remodelling (Figure 11). Duf cascade involves specific adaptor proteins and guanine nucleotide exchange factors (GEFs), and leads to a Rac-mediated activation of Scar (Brugnera et al., 2002; Chen and Olson, 2001) (Figure 11). Scar promotes nucleation of actin filaments by Arp2/3 complex (from Actin Related Proteins) and they are both required for myoblast fusion (Richardson et al., 2007). Similarly, Sns activates the Scar-Arp2/3 axis, but it also binds to CT10 regulator of kinase (Crk), which in turn recruits the actin polymerizing machinery composed of Wiskott-Aldrich syndrome proteins (WASPs) and related regulators (Kim et al., 2007) (Figure 11).

Although these molecular events involve common proteins, such as Scar and Arp2/3, the asymmetrical recruitment of WASP in FCM only results in two specific rearrangement of the actin cytoskeleton in the two different muscle cells. Indeed, dynamic generation and disruption of F-actin foci was visualized in correspondence of the fusogenic synapse in live embryos (Richardson et al., 2007), but more specific analyses revealed distinct actin organization in the two muscle populations both *in vitro* (Haralalka et al., 2011) and *in vivo* (Sens et al., 2010). Founder cells form a thin sheath of F-actin underlying the fusogenic synapse, while in FCMs, F-actin-enriched invasive podosome-like structures are present at the point of cell-cell contact (Sens et al., 2010) (Figure 11). These observations, together with several other studies in different systems, suggests that fusion is a highly asymmetric process both at molecular and at morphological level.

Importantly, also the non-muscle myosin IIA and IIB (NM-MHC) have also been shown to be essential for the morphological rearrangements of myoblasts prior to fusion (Swailes et al., 2006) and their inhibition strongly attenuated the formation of the characteristic actin wall of founder cells together with their fusion (Duan and Gallagher, 2009). NM-MHCIIs are actin binding proteins and intracellular effectors of mechanosensory responses, which can be activated by surface proteins such as integrin, Rho GTPase and Rho kinase (Rok) (Amano et al., 1996) and have been shown to participate in myoblast fusion (Kim et al., 2015). Mechanistically, the “attacking” FCM invades the “receiving” founder cell with actin-propelled membrane protrusions (Shilagardi et al., 2013), whereas the founder cell mounts a NM-MHCII-

mediated mechanosensory response (Kim et al., 2015) (Figure 11). In detail, the actin finger-like structures provide an active driving force causing an accumulation of NM-MHCII in correspondence of the fusogenic synapse, as well as the activation of Rho and Rok signalling, which increases the amount of activated NM-MHCII and generates additional cortical tension. The pushing force from the actin-propelled membrane protrusions, together with the NM-MHCII-induced resisting force, brings the cell membranes into close proximity under high mechanical tension and ultimately promotes cell membrane fusion (Kim et al., 2015).

Additionally, it has recently been shown that α/β_H-spectrin participates in the generation and regulation of the dynamic forces that controls membrane fusions. Spectrin is a membrane skeletal protein that associates with actin and other structural proteins to maintain cellular shape and provide mechanical support for plasma membranes (Machnicka et al., 2014). The specific isoform expressed in *Drosophila* α/β_H-spectrin accumulates at the fusogenic synapse of the receiving fusion partner in response to mechanical stimuli generated by the FCM invasive protrusions. The resulting spectrin-enrichment has a dual function. First, in the founder cell α/β_H-spectrin maintains and recruits additional Duf protein at the synapse level, restricting Duf diffusion and stabilizing its interaction with Sns. Second, Spectrin accumulation locally blocks future protrusion from the FCM, forcing new protrusion to invade proximal spectrin-free areas and thus triggering additional spectrin accumulation. This positive feedback loop of Spectrin accumulation, together with the previously discussed mechanisms, increases the mechanical tension at the fusogenic synapse and leads to membrane merger.
Finally, a critical step of membrane fusion is pore formation. Although in the late 90's electron microscopy studies suggested the presence of multiple membrane pores between fusing cells (Doberstein et al., 1997), a more recent work has proposed that a single pore forms at the tip of one invasive protrusion and expands to engulf the fusing FCM (Sens et al., 2010). However, fusion pore formation remains to be fully elucidated.

2. Myoblast fusion in murine skeletal muscle development and regeneration

Mammalian muscle cell fusion is a complex multistep process that occurs during skeletal muscle development, post-natal growth and regeneration. The complexity of mammalian musculature, together with the elevated number of myoblasts involved, makes essential a thigh regulation of the fusion process, in which the accurate cell types and the appropriate number of cells fuse with correct timing and precise localization. As in *Drosophila*, myoblast migration, adhesion, recognition and membrane merger as well as cell signalling and cytoskeletal reorganization are all crucial steps for proper and efficient fusion (Figure 12).

2.1. Myoblast migration

Live imaging of satellite cells in regenerating muscle provided the first direct evidence for myoblast migration *in vivo* (Ishido and Kasuga, 2011), however, most of our knowledge about muscle stem cell migration capabilities have been inferred by *in vitro* studies. Time-lapse videomicroscopy of satellite cells on single-isolated myofibre showed an extensive migratory behaviour of the muscle stem cell population (Siegel et al., 2009). Neutralization of the laminin-binding integrin α7β1 significantly reduced satellite cell motility, suggesting a role for laminin in migration. Moreover, muscle stem cells and primary cultures express several receptors for chemoregulatory molecules, and they are able to migrate in response to a variety of factors, ranging from chemokines to growth factors (Corti et al., 2001; Griffin et al., 2010; Siegel et al., 2009). Migratory behaviour changes greatly during differentiation as myocytes display less motility compared to myoblasts and additionally exhibit different responses to migratory factors (Griffin et al., 2010). Interestingly, myoblast fusion can be enhanced by both positive and negative regulators of cell migration. Whereas positive migratory factors promote cell fusion by increasing the probabilities of myoblasts being close to one another, negative migratory factors may enhance fusion by acting as a brake on cell movements to facilitate cell-cell contact and adhesion (Bondesen et al., 2007). Thus, the net balance between these two classes of migratory regulators would be crucial for modulating myoblast fusion.

2.2. Myoblast cell-cell contact: adhesion and recognition

Once in close proximity, myoblasts contact each other through numerous cell-cell adhesion molecules (Figure 12). In mouse, many more adhesion proteins have been identified than in *Drosophila*, suggesting a higher level of complexity in mammalian. Interestingly, the only conserved adhesion molecule in both systems is Nephrin, homolog of Sns (Sohn et al., 2009). Nephrin expression was detected in developing mouse skeletal muscle and its presence was shown to be essential for mononucleated myoblasts to fuse into myotubes. Indeed, whereas wild type myoblasts were able to fuse into Nephrin-null myotubes, myoblasts lacking Nephrin provide little or no contribution to the nascent wild type myotubes (Sohn et al., 2009). Other proteins involved in myoblast adhesion and recognition are muscle-cadherins (M-Cadherins), integrins and a disintegrin and metalloprotease 12 (ADAM12) (Brzoska et al., 2006; Cifuentes-Diaz et al., 1995; Lafuste et al., 2005), as their removal or inhibition leads to the inhibition of myotube formation *in vitro* and also *in vivo* (Charrasse et al., 2006; Schwander et al., 2003).

Another important family of adhesion protein involved in fusion is the transmembrane 4 superfamily proteins, also known as Tetraspanins (Figure 12). Tetraspanins build a network referred to as "tetraspanin web", where they directly interact with other molecules forming primary complexes, which in turn associate to form higher ordered complexes (Charrin et al., 2009). In particular, CD9 and CD81 tetraspanins associate together with their major molecular partner CD9P-1 forming a super complex that negatively regulate MuSC fusion. As a consequence, muscle regeneration in mice lacking either CD9 or CD81 exhibit impaired muscle regeneration associated by the formation of giant dystrophic myofibers resulting from excessive fusion (Charrin et al., 2013). However, the mechanisms by which these molecules negatively control MuSC fusion remain to be determined.

The large variety of adhesion proteins involved in myoblast cell-cell contact underlines the great complexity of the mammalian system controlling fusion. All these molecules not only participate in myoblast adhesion and recognition, but also control and trigger specific signalling pathways that in turn drive molecular responses preparing the cell for fusion. An example of cascade activated by cell-surface engagement of adhesion proteins is Rac1, which during myoblast fusion get activated in a M-Cadherins-dependent manner (Charrasse et al., 2007) (Described below). Rac1 mediates local actin polymerization and its GTPase activity has been shown to be essential for myoblast fusion.

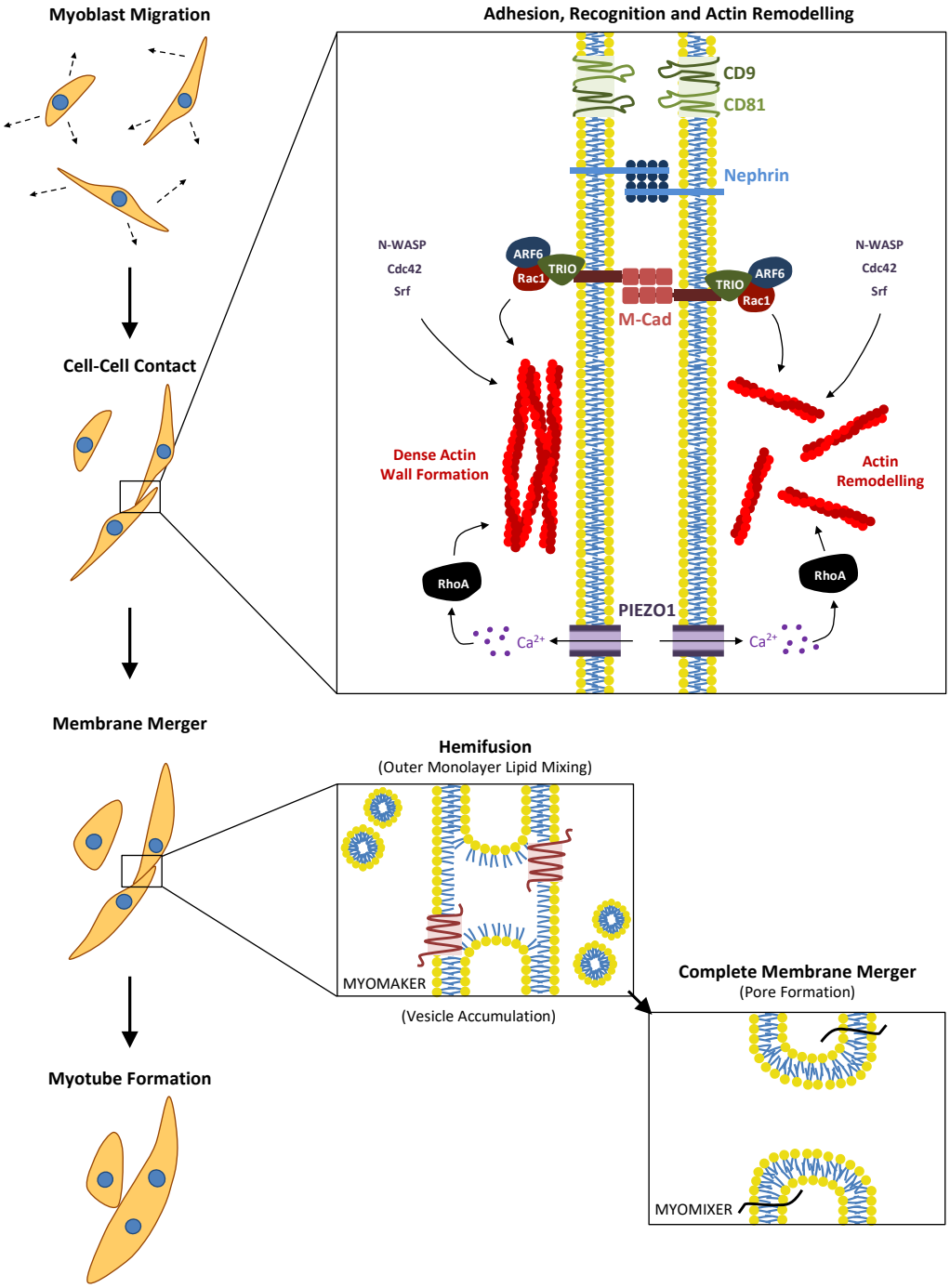

Figure 12. Mammalian Myoblast Fusion. Myoblast fusion in mouse is a complex multistep process. Muscle progenitors need to migrate and contact each other. Once in close proximity, myoblasts interact through numerous adhesion molecules, such as tetraspanins (CD9 and CD81), Nephrin and M-Cadherins. Myoblast recognition triggers several intracellular events that lead to a substantial actin cytoskeleton remodelling in both cells and to the formation of a dense actin wall uniquely in one cell. As fusion proceed, vesicles start to accumulate at the level of the fusion site and membrane merger initiates. Firstly, the outer monolayers of lipids merge together in a process called hemifusion, which is governed by Myomaker. Secondly, Myomixer completes membrane merger generating fusion pores which expand and finally result in fusion completion. Refer to the text for a more detailed description of the murine fusion system.

2.3. Actin cytoskeleton remodelling during myoblast fusion

Filamentous actin reorganization

Actin cytoskeleton rearrangements are crucial for fusion and occur before, during and after membrane merging (Fulton et al., 1981). Before fusion, differentiating myoblasts extend lamellipodia and filopodia contacting neighbouring cells and creating cell-cell contact sites in which adhesion proteins and signalling molecules accumulate (Abramovici and Gee, 2007; Mukai et al., 2009). These regions primarily contribute in triggering intracellular responses that prepare the cell for fusion, but it has also been proposed that additionally filopodia may provide mechanical forces by pulling the cells in close contact to one another, thus promoting membrane merging (Abramovici and Gee, 2007). Extensive actin reorganization occurs during the fusion process and these cytoskeletal rearrangements have been visualized in fusing myoblast *in vitro* (Duan and Gallagher, 2009; Swailes et al., 2006) (Figure 12). Imaging of prefusion aligning myoblasts via transmission electron microscopy revealed a dense actin bundle enrichment paralleling the long axis of the aligned myoblast (Swailes et al., 2006). This highly organized actin wall is hypothesized to provide cortical tension needed for membrane merging and also to temporarily regulate the fusion process (Duan and Gallagher, 2009).

Subsequent to the assembly of cortical actin bundles, membrane vesicles accumulate in both aligned myoblasts at the level of the fusion site (Figure 12). As fusion progresses, gaps within the actin wall appear, allowing membrane vesicles to pair between juxtaposed myoblast. Following close apposition of the membranes, localized fusion pores are formed and expand laterally. Importantly, the actin-binding protein Non-muscle myosin II (NM-MHCII) is associated with the cortical actin filaments and its function is essential for the actin wall formation, vesicle accumulation and membrane merging as its inhibition completely blocks all these processes (Duan and Gallagher, 2009). Consistently, pharmacological impairment of actin polymerization results in reduced myoblast fusion (Nowak et al., 2009).

Molecular regulation of actin cytoskeleton in muscle cells

Actin remodelling factors are essential in mammalian myoblast fusion. Molecules involved in the regulation of actin dynamics identified in *Drosophila*, such as the 4 guanine exchange factors (GEFs), Brag2, Dock1, Dock5 and Trio, have an evolutionally conserved role in mouse

fusion machinery, as their mutation result in defective fusion both *in vitro* and *in vivo*. Specifically, Brag2 or Dock1 are activated by Arf6 and Rac1 respectively and their functional impairment leads to the formation of myotubes with few nuclei *in vitro* (Pajcini et al., 2008). The relevance of Dock1 was also confirmed *in vivo*, where the generation of Dock1 and Dock5-null mice revealed a strong deficiency in myoblast fusion during muscle development (Laurin et al., 2008).

Finally, Trio is a Rho-GEF that takes part of one of the best-described signalling involved in actin remodelling (Charrasse et al., 2007) (Figure 12). *in vitro* Trio knockdown results in reduced myotube formation (Charrasse et al., 2007), while *in vivo*, Trio-deficient mice showed impaired secondary myofibre formation during development (O'Brien et al., 2000). Interestingly, during cell-cell contact M-Cadherin–dependent adhesion activates Trio, which in turn mediates Rac1 GTPase activation, thus leading to actin rearrangements (Charrasse et al., 2007). The formation of this M-Cadherin/Trio/Rac1 complex is driven by Arf6, a small GTPase originally discovered in *Drosophila* fusion machinery with conserved role also in mouse (Figure 12). Arf6 is essential for Trio/Rac1 association with M-Cadherin and also important for myoblast fusion. Indeed, dominant-negative Arf6 *Drosophila* showed impaired embryonic myofibre formation, while RNA interference against Arf6 expression reduced myoblast fusion in murine cultures.

An important role in murine fusion has been also proposed for N-WASp, a ubiquitous nucleation-promoting factor of branched actin filaments (Gruenbaum-Cohen et al., 2012). Interestingly, N-WASp regulates fusion without impacting myoblast differentiation, motility and adhesion, as its disruption *in vitro* and *in vivo* results in a significant impairment of the fusion process, without perturbing the capacity of myoblasts to migrate, contact and differentiate. Recently, Tsk5 and Dnm2 (Dynamin 2) have been suggested to regulate actin organization in podosomes, membrane-bound actin-enriched invasive structures that are responsible for cell adhesion, migration and also myoblast fusion (Chuang et al., 2019). Tsk5 is a tyrosin kinase able to recruit actin regulators to the membrane hence promoting the formation of podosomes, while Dnm2 assembly around actin-bundles strengthening them providing the required rigidity for membrane fusion.

As proper actin regulatory molecules are indispensable for myoblast fusion, the signalling upstream these molecules consequently play a key role in myotube formation. Besides, Trio-activated Rac1 signalling, also Rho, Cdc42 and Srf (serum response factor) have been implicated in controlling actin remodelling during muscle cell fusion (Doherty et al., 2011; Randrianarison-Huetz et al., 2018; Vasyutina et al., 2009). While Graf1 (Rho-GTPase-activating protein)-induced RhoA down-regulation leads to a robust fusion *in vitro* (Doherty et al., 2011), Rac1 and Cdc42 mutant myoblasts shows severe deficits in fusion (Vasyutina et al., 2009), confirming the high complexity of the crosstalk in between all the different pathways involved in actin remodelling during fusion. Finally, Srf controls the expression of target genes involved in cytoskeletal organization and, together with its cofactor Myocardin-related transcription factor (Mrtf), generates a feedback system to ensure that actin levels are appropriate to support the actin dynamics required for cell behaviours (Randrianarison-Huetz et al., 2018). Consequently, Srf-mutant primary cultures fail to fuse due to a misregulation of actin organization with an impaired formation of finger-like actin-based protrusions, which have been proposed to be functionally required for efficient fusion.

2.4. The last step of fusion: membrane merger

The plasma membrane actively contributes to myoblast fusion

Besides all actin rearrangements and the complex protein network regulating fusion, another essential role in this process is played by the plasma membrane. Indeed, multiple events occurs also at the level of the phospholipid bilayer. For example, lipid rafts containing cholesterol transiently accumulate at the cell contact sites providing the required membrane rigidity for cell adhesion and accumulation of adhesion molecules (Mukai et al., 2009). However, after cell-cell contact, the fusion site undergoes a dynamic lateral dispersion of lipid rafts resulting in increased membrane fluidity and destabilization of lipid bilayer, prompting membrane merger. Another lipid modification consists in phosphatidylserine (PS) exposure at the cell-cell contact areas (van den Eijnde et al., 2001). PS is normally localized to the inner leaflet of the plasma membrane but can be exposed at the cell surface during early apoptosis and myoblast fusion. Although PS exposition is mainly related to apoptosis, its exposure during myotube formation is governed by a different mechanism and independent from

programmed cell death processes (van den Eijnde et al., 2001). In primary cultures, addition of exogenous PS strongly enhances myotube formation, while PS pharmacological masking abrogates myoblast fusion (Jeong and Conboy, 2011). Interestingly, modulation of the PS receptor, Stabilin-2, has parallel effects; Stabilin-2 overexpression in myoblast is associated with an increased fusion, conversely, Stabilin-2-deficient myoblasts lose the capacity to fuse (Park et al., 2016).

A potential mechanism for PS has been recently described by Tsuchiya and colleagues, which proposed a role in fusion for PS flippase (transmembrane lipid transporter protein). When localized to the outer membrane layer, PS inhibits the mechanosensitive Ca^{2+} channel Piezo1, while flippase-mediated inward translocation of PS allows its activation. Once activated, Piezo1 mediates Ca^{2+} influx that promotes RhoA/Rok-mediated actomyosin assembly and prevents uncontrolled fusion of myotubes (Figure 12). Accordingly, genetical removal of Piezo1 using Crispr/Cas9 system *in vitro* results in excessive fusion and aberrant myotube formation. Taken together, these observations suggest that cell surface flippase and translocation of PS form one leaflet of the membrane to the other are critical regulators of muscle cell fusion.

Myomaker and Myomixer: master regulators of muscle fusion

Although a large variety of molecules is involved in fusion, no muscle-specific or nodal regulators of mammalian myoblast membrane fusion were described until very recently. However, in 2013 Millay and colleagues discovered Myomaker (Mymk), the first dominant muscle-specific fusion factor (Millay et al., 2013). Identified through bioinformatic in silico searches, Mymk is a multi-pass transmembrane protein able to provide fusogenic capabilities in otherwise non-fusogenic cells. Overexpression of Mymk in murine fibroblast promotes fusion with myoblasts strongly indicating that Mymk participate in membrane merger reaction (Millay et al., 2013). Notably, forced expression of this membrane protein did not induce fusion between fibroblast themselves, highlighting the likelihood that additional myogenic fusion factors existed. Indeed, three independent groups recently identified a second muscle-specific fusion protein named Myomixer (Mymx, also known as Myomerger or Minion), which when co-expressed with Mymk is sufficient to induce fusion in non-fusiogenic fibroblasts (Bi et al., 2017; Quinn et al., 2017; Zhang et al., 2017). Therefore, Mymk is required symmetrically

(on both fusing cells), whereas Mymx asymmetrically (only on one cell of the couple). These observations were further confirmed by knockout experiments in myogenic cells *in vitro*, where Mymx-deficient myoblasts efficiently fused to wild-type myoblasts, but Mymk-null muscle cells did not (Bi et al., 2017; Zhang et al., 2017). However, during adult muscle growth *in vivo*, Mymk is necessary only in satellite cells and not in myofibres, indicating a more complex mechanism depending on the physiological and developmental context (Goh and Millay, 2017). Generation of Mymk or Mymx loss-of-function mice results in lack of skeletal muscle formation and death at birth, however, numerous myosin$^+$ cells were detected in both mouse models, indicating no alteration in the differentiation process. Interestingly, both Mymk and Mymx expression levels are regulated by MRFs, as numerous E-box elements have been found upstream of those genes. Accordingly, these fusion factors are not expressed in proliferating myoblasts, but strongly induced upon differentiation (Millay et al., 2014).

Although the functions of Mymk and Mymx are well-characterized, the precise mechanism behind these muscle-specific fusion factors remains not fully elucidated. Recently, Leikina and colleagues proved that Mymk and Mymx work independently controlling two different stages of fusion (Leikina et al., 2018). In fact, fusion can be subdivided in two distinct steps. The first, named hemifusion, consists only in the outer lipid monolayer mixing (Chernomordik and Kozlov, 2005); the second involves pore formation and cytoplasm mixing thus considered the actual fusion (Figure 12). Mymk is required for hemifusion, whereas Mymx drives the subsequent pore formation and ultimate fusion (Figure 12). Indeed, while Mymx-deficient myoblasts stall at hemifusion stage, Mymk-null myoblasts are not capable to reach the hemifusion step. Moreover, previous studies reported that annexins, receptors of PS, are important for the outer membrane leaflet mixing step and the subsequent stages of fusion are dependent on Dnm2 (Leikina et al., 2013). Therefore, although no supporting data have been reported yet, it is intriguing to consider a potential collaboration between Mymk, Mymx and PS, annexins and dynamin.

Further insights for a precise mechanism are elusive. Myomerger physically associates with cytoskeleton-related proteins and pharmacological inhibition of actin polymerization blocks fibroblast fusion induced by co-expression of Mymk and Mymx (Zhang et al., 2017). It is thus tempting to speculate a correlation between F-actin and the fusion factors, however, no experimental evidences have been reported yet.

III. TGFβ Signalling Pathway

The Transforming Growth Factor beta (TGFβ) superfamily is comprised of at least 33 secreted factors, including the prototypic members TGFβ isoforms, Activins, Nodals, Bone Morphogenetic Proteins (BMPs), Growth and Differentiation Factors (GDFs) and anti-Müllerian hormone (AMH). This cytokine family is present in all the metazoan described to date and is ubiquitously expressed in all the mammalian tissues from the earliest stages of development to the adult animal (Weiss and Attisano, 2013). Originally, TGFβ was identified as "transforming polypeptide" on the basis of its ability to stimulate cellular transformation and anchorage-independent growth of non-neoplastic cell line in culture (Roberts et al., 1981). However, many other functions and even opposite activities were ascribed to the TGFβ proteins in the following years. Despite exhibiting pronounced structural similarities and employing similar mechanisms of signal transduction, TGFβ members play widespread and diverse roles in both embryonic development and adult homeostasis. A hallmark of TGFβ pathway is in fact the capability to exert different functions depending on the physiological conditions, cell type and cellular background. This context-dependent nature of TGFβ activity, together with the vast diversity of functions, made TGFβ cascade one of the most studied pathway with at least 70,000 publications at this point.

In this chapter we will review the TGFβ signalling pathway, starting by exploring molecular mechanism of signal transduction before delving into TGFβ biological functions, with a specific regard on its activities in skeletal muscle.

1. TGFβ Ligand Maturation

1.1. TGFβ ligands

The TGFβ superfamily emerged at the onset of multicellular life (metazoan) and then expanded through gene duplication from primitive species to invertebrates and vertebrates (Huminiecki et al., 2009). To date, they have been described 5 TGFβ related ligands in *Caenorhabditis elegans*, 7 in *Drosophila melanogaster* and up to 33 in mammalian genome. Specifically, in addition to the prototypic members TGFβ1, 2 and 3, the human genome features 11 GDFs, 10 BMPs, 5 Activins and Inhibins, 2 leftys, Nodal and AMH. In general, the TGFβ superfamily is classified into two main categories: the TGFβ-like group (including TGFβs, Activins, Inhibins and Nodals) and the BMP-like group (including BMPs and GDFs).

Despite the several subfamilies identified, most of the TGFβ members share common features. All TGFβ ligands are translated as large precursors that require a cleavage step to release the mature dimeric protein. Once mature, most of them are dominated by a specific conformation stabilized by disulfide bonds named cystein knot (Galat, 2011). Solving TGFβ crystal structures have revealed an ordered set of 7 highly conserved cysteins (Cys) in the C-terminal region of numerous family members; 6 Cys residues form 3 intramolecular disulfide bridges, while the seventh cysteine mediates dimerization via an intermolecular disulfide bond to form the mature dimeric growth factor. Of note, while most ligands are homomeric, various combinations of heterodimers have been described (Morikawa et al., 2016). In addition to structural similarities, most of TGFβ ligands are united by the ability to specifically bind TGFβ receptors type I and II, to be latent when interacting with Latency Associated Peptide (LAP) and to covalently bind Latent TGFβ binding proteins (LTBPs) (discussed below) (Robertson and Rifkin, 2013).

1.2. TGFβ ligand secretion and storage

All TGFβ ligands are initially synthesized as dimeric pro-peptides with a large amino-terminal pro-domain called Latency Associated Peptide (LAP) and a highly conserved carboxy-terminal region named mature domain, which comprises the active ligands (Figure 13). The pro-domain is required for proper folding and peptides dimerization, and it is cleaved in the Trans Golgi by

proteases of the subtilisin-like proprotein convertase (SPC) family (i.e. Furin). After intracellular cleavage, a noncovalent association persists between LAP and the conserved region (Figure 13). This assemblage takes the name of Small Latent Complex (SLC) and is retained in the cytoplasm until it is covalently bound to a single Latent TGFβ binding protein (LTBP) (Figure 13).

LTBPs are a group of secreted proteins (LTBP-1, -2, -3 and -4) with several binding domains and multiple functions. Originally identified by their association with latent TGFβ ligands (Kanzaki et al., 1990), LTBPs contribute to the proper folding of TGFβ precursors (Brunner et al., 1989) and its secretion (Robertson et al., 2015), but most importantly play a fundamental role in TGFβ sequestration and extracellular activity. Except LTBP-2, all the LTBPs covalently bind to LAP via disulfide bridge and this interaction leads to the formation of the so called Large Latent Complex (LLC) (Saharinen and Keski-Oja, 2000) (Figure 13). This association is essential for TGFβ functionality, as proven by *in vivo* impairment of LAP-LTBP interaction which phenocopied TGFβ1-null mice (Yoshinaga et al., 2008). Once assembled, LLC is consequently secreted in the Extracellular Matrix, where it links with specific components of the ECM thanks to the multiple binding domains of LTBP. Particularly, LTBP-1 and -4 interact with Fibrillin-1 (Massam-Wu et al., 2010; Ono et al., 2009) and Fibronectin (Fontana et al., 2005; Kantola et al., 2008), while LTBP-3 interactions with the ECM components is to date not well characterized (Figure 13). These interactions occur through transglutaminase-dependent cross-linking of LTBP and matrix protein (Nunes et al., 1997), and the inhibition of transglutaminase abrogates TGFβ activation (Kojima et al., 1993). In general, the association of Large Latent Complex and the ECM proteins is essential for TGFβ bioavailability and activation (described below).

Other groups of extracellular TGFβ binding proteins with secondary or more restricted roles have also been described. Small leucine-rich proteoglycans (SLRPs) are a large family of glycoproteins able to regulate multiple signalling pathways. Many SLRP members, such as decorin, biglycan and fibromodulin, are able to bind and sequester TGFβ ligands (Hildebrand et al., 1994) with consequent modulation of their activities (Horiguchi et al., 2012). Noteworthy, also the TGFβ receptor type III is part of this proteoglycan family (described below).

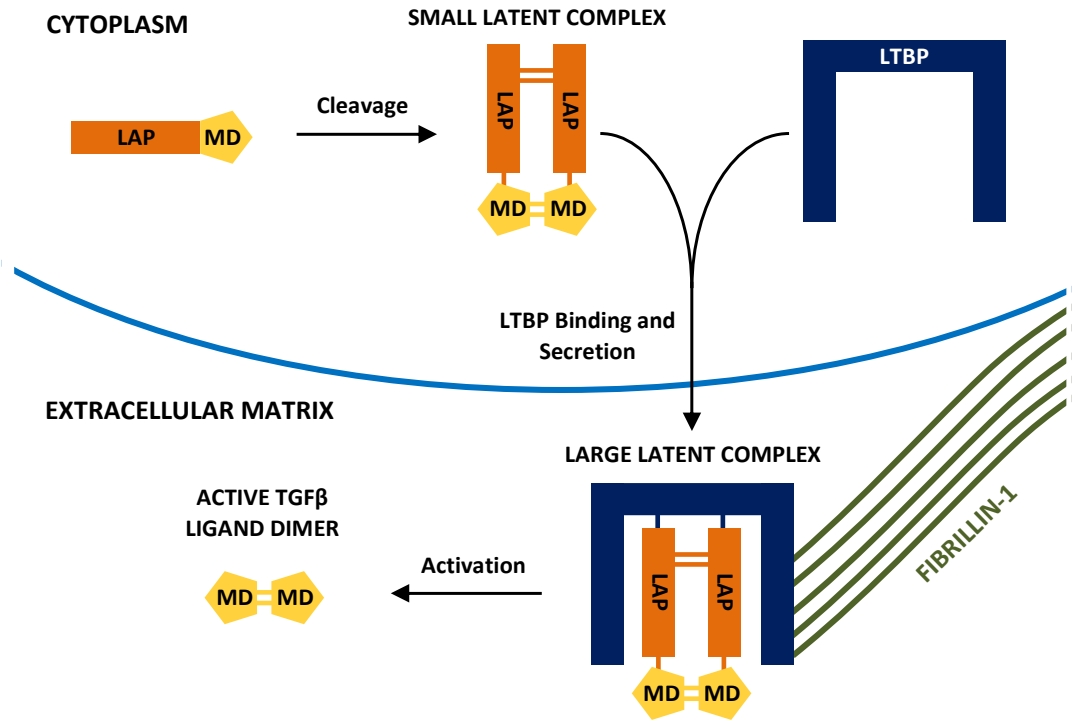

Figure 13. TGFβ Ligand Maturation. TGFβ ligands are translated as large precursors containing the Latency Associated Peptide (LAP) and the Mature Domain (MD). After intracellular cleavage, LAP and MD remain non-covalently associated forming the Small Latent Complex (SLC). SLC covalently binds to a single Latent TGFβ binding protein (LTBP) and is secreted into the extracellular matrix (ECM). This super complex is also referred to as Large Latent Complex (LLC) and associates with ECM components, such as Fibrillin-1. Here, different mechanisms lead to the disruption of the complex with consequent release of the mature and active TGFβ ligand dimer. Refer to the text for a more detailed description of the storage, secretion and activation of TGFβ ligands.

1.3. Activation of TGFβ ligands

When sequestered in the ECM, TGFβ ligands are held in a latent state (Figure 13). LAP interaction is sufficient to confer latency on many TGFβ members by shielding the TGFβ receptor binding sites (Lawrence et al., 1984). Therefore, the release of TGFβ from LAP is a critical regulatory step for TGFβ function and activity. Indeed, the trigger of TGFβ signalling cascade is represented by liberation of TGFβ ligand from its latency complex, more than is actual secretion. In 2003, Annes and colleagues proposed a new concept according to which LLC constitutes a “extracellular sensor” (Annes et al., 2003). In this model, the TGFβ mature ligand functions as “effector”, LTBP as “localizer” and LAP as “detector”. The reservoir for

latent TGFβ is in fact localized in the matrix thanks to LTBP interactions. Here, mature TGFβ ligands wait the appropriate activation signal that will act on the detector LAP to release the active form of the effector. The extracellular concentration of TGFβ activity is thus established by the conversion of latent TGFβ to the active form, and, the conversion rate is determined by the interplay between ECM components, LLC and TGFβ activators. Once activated, TGFβ does not persist in the ECM and is rapidly cleared from the extracellular space, although the mechanisms mediating this clearance are not well understood. Many different activation mechanisms have been described, ranging from molecular to physiochemical factors (Robertson and Rifkin, 2013).

Integrins

Integrins are dimeric transmembrane receptors composed of α and β subunits that are involved in many cellular processes, including TGFβ activation. Specifically, integrins αvβ6 and αvβ8 are well-established activators of TGFβ (Annes et al., 2002; Mu et al., 2002; Munger et al., 1999). Other isoforms have also been described to interact with latent TGFβ, however not all of them lead to TGFβ ligand release. TGFβ-integrin association depends upon the RGD amino-acid sequence present in LAP, which is a recognition consensus sequence for integrins. Genetical alteration of RGD sequence in mouse (with consequent impairment of TGFβ-integrin association) results in a TGFβ1-null phenocopy, suggesting that most (or all) of TGFβ1 activation *in vivo* requires interaction with integrins. TGFβ liberation via integrins can be protease-dependent or protease-independent (Yang et al., 2007).

Protease-independent TGFβ activation was originally discovered in a αvβ6 integrin over-expression system, in which latent TGFβ1 was activated even in the presence of protease inhibitor. In particular, αvβ6 integrin interacts with LAP RGD sequence leading to conformational changes of LAP with consequent release of TGFβ1 ligand. This mechanism relies on the interplay between the LAP, LTBP and the matrix. The traction generated between the cell surface and the ECM by these components is transmitted to LLC, deforming LAP (Wipff et al., 2007). Thus, the association between LAP and LTBP, and the interaction of LTBP with the matrix are essential to obtain TGFβ activation (Annes et al., 2004).

TGFβ activation can occur also in a protease-dependent mechanism. Specifically, integrin αvβ8 has been shown to activate TGFβ1 through a coordinated interaction with LLC and matrix metalloproteinase (MMP) on the cell surface. Recruitment of the MT1-MMP by integrin facilitates LAP cleavage realising active TGFβ, and this process can be blocked *in vitro* by the administration of MMP inhibitors, or *in vivo* through genetical disruption of MT1-MMP (Mu et al., 2002).

Proteases

Several classes of proteases have been shown to activate TGFβ *in vitro*, including cysteine, aspartyl, serine and metalloproteinases (Jenkins, 2008; Maeda et al., 2001). The most studied are serine proteases and metalloproteinases. Implicated in many TGFβ-related pathological conditions, these two classes of proteases release TGFβ ligands *in vitro* via different mechanisms. However, defining protease relevance *in vivo* results extremely challenging as genetical impairment of proteases in mouse causes only mild effect to TGFβ signalling state. Although this might suggest a minor role of protease-dependent TGFβ activation, these *in vivo* observations could also reflect the general ability of numerous proteases to cleave multiple substrates, thus generating a significant redundancy *in vivo*.

Serine proteases localize in the extracellular matrix via binding to specific cell surface receptors. Here, they release the active form of TGFβ through different mechanisms. The first serine protease to be described as TGFβ activator was Plasmin, which liberates TGFβ ligand from the latent complex via proteolytic cleavage of LAP (Lyons et al., 1990). Another example is represented by Thrombin, Serine protease that activate TGFβ proteins through a similar mechanism (Taipale et al., 1992). However, the significance of both Plasmin- and Thrombin-mediated activation *in vivo* remains poorly understood.

Matrix metalloproteinases are a family of endopeptidase with a conserved cys residue in the pro-domain and a conserved zinc molecule in the catalytic domain. These degrading enzymes are able to process all kinds of matrix protein, including TGFβ latent complex. As described above, MT1-MMP participates in TGFβ activation cooperating with integrin, however other MMPs, such as MMP2 and MMP9, are able to act independently form integrins. MMP2 and MMP9 preferentially cleave LAP of TGFβ2 and 3 *in vitro*, with minor effect on TGFβ1.

Moreover, both MMPs are overexpressed in caveolin-null mice and this leads to an enhanced TGFβ signalling, providing evidences of their activator role also *in vivo* (Sotgia et al., 2006). Another well-established TGFβ activator *in vitro* is MMP13, but its role *in vivo* remains not fully elucidated (D'Angelo et al., 2001).

Finally, an example of protease that releases TGFβ ligand without acting on LAP is represented by BMP1, which belongs to peptidase M12A family. Indeed, BMP1 is a metalloprotease able to cleave LTBP-1, but not LAP, and its activity does not lead to a direct activation of TGFβ ligands. Rather, LTBP-1 cleavage liberates LLC from ECM and consequently allows MMP-mediated cleavage of LAP, resulting in TGFβ activation (Ge and Greenspan, 2006). In addition, BMP1 metalloproteinase family has been implicated in activation of GDF8 (Myostatin) and GDF11 (Wolfman et al., 2003). Together, these observations suggest that BMP1 is an important regulator of different TGFβ ligand activation.

Thrombospondin

Another protein reported as TGFβ activator is Thrombospondin (TSP1), a large homotrimeric molecule secreted by many cell types. Interestingly, TSP1 does not release TGFβ through proteolytic cleavage, but by interacting with the N-terminal region of LAP and causing a conformational change that makes TGFβ1 accessible to its receptor (Schultz-Cherry et al., 1995). *in vivo*, both TSP1 knockout and pharmacological inhibition lead to a phenotype partially overlapping phenotype of TGFβ1 null mice, demonstrating TSP1 significance *in vivo* as TGFβ activator (Crawford et al., 1998). TGFβ can be liberated by its latency also by Spondin-1 (SPON1, also known as F-spondin), a protein structurally related to TSP1 being composed of six thrombospondin domains. By specifically impairing Spondin-1 TSP domain functionality in cartilage explants cultures (Either by using a neutralizing antibody against TSP domain or by deleting it), TGFβ signalling activity was reduced (Attur et al., 2009). Although the precise link between Spondin-1 and TGFβ activation has not been described yet, these results prove the essential role of the TSP domain and suggest parallels with activation mechanism adopted by TSP1.

2. TGFβ Signalling Transduction

The intracellular events triggered by the binding of TGFβ ligand to its receptors and transduction of the signal to the nucleus, are well-conserved among species. The main transducers of TGFβ signalling cascade are SMAD family members (from Mothers against decapentaplegic homologs). SMADs are intracellular mediators that get phosphorylated by the activated receptor, transduce the signal into the nucleus and execute the downstream response by directly regulating gene expression (Figure 14). Although intracellular signal transduction of the TGFβ pathway seems straightforward at first glance, the combinatorial interactions in the heteromeric receptor and SMAD complexes, receptor-interacting and SMAD-interacting proteins, and cooperation with sequence-specific transcription factors grant versatility to TGF-β family responses, thus resulting in high intricacy of the elicited biological responses (Schmierer and Hill, 2007).

2.1. TGFβ Receptors

TGFβ ligands transduce their signal by binding single-pass transmembrane receptors with kinase activity present at the cell surface of the target cell. These receptors are structurally related to serine/threonine kinases, but early studies showed that TGFβ receptors are able also to phosphorylate tyrosine residues indicating their dual specificity (Lawler et al., 1997). TGFβ receptors are always found in dimeric form and can be functionally classified in two groups (Figure 14). In vertebrates, TGFβ receptor family is composed by 12 members; 7 type I receptors and 5 type II receptors. Type II receptors are constitutively active kinases, while type I receptors are active only in presence of TGFβ ligand. As discussed below (see III.2. TGFβ Signalling Transduction), dimers of receptor type I and type II receptors pair up together to receive the ligand and phosphorylate the downstream SMAD effectors.

Historically, receptors were named based on their first reported ligand (i.e. Activin type I receptor, ACVR1B), however it is now well-established that one receptor can bind several ligands. The 7 type I receptors are also named Activin Receptor-Like Kinases (ALKs), while type II receptors keep the early nomenclature based on the ligands. Besides ALK1-7, we find TGFβ receptor type II (TGFβR2), BMP receptor type II (BMPR2), Activin receptor type-2A and 2B (ACVR2A and B) and Anti-Mullerian hormone receptor type 2 (AMHR2).

ALKs determine which SMADs are phosphorylated (Figure 14). Precisely, ALK4, ALK5 and ALK7 phosphorylate SMAD2 and SMAD3, whereas ALK1, ALK2, ALK3 and ALK6 activate SMAD1, SMAD5 and SMAD8. The specificity for the ligand is instead given by the combination of both receptors: specific TGFβ ligands can bind only to certain couple of receptors. For instance, TGFβ isoforms 1, 2 and 3 binds exclusively to ALK5 and TGFβR2. On the other hand, Activin signals through ALK4 when associated with ACVR2B, which in turn can receive BMP2 if coupled with ALK3 or BMP7 with ALK2. In general, the 12 TGFβ receptor members pair up in several combination to mediate the signal of more than 30 TGFβ members. This evidence highlights an evident ligand-receptor promiscuity, but also implies further downstream regulatory systems to obtain the variable and versatile nature of TGFβ signalling.

Lastly, also β-glycan actively participates in the reception of the TGFβ ligands in some specific cell types. Although it acts as a co-receptor, β-glycan is also known as TGFβ receptor type III. As mentioned before, type III TGFβ receptors are proteoglycan transmembrane proteins able to bind latent TGFβ complexes. Its role consists in recruiting TGFβ ligands at the cell surface and consequently present them to the receptors, facilitating the initiation of the cascade.

2.2. The intracellular mediator of the TGFβ signalling: SMAD proteins

SMADs are a well-conserved family of proteins that not only transduce the signal from the membrane into the nucleus, but also regulate gene expression determining the TGFβ signalling outcome. Mammalian genome encodes for 8 SMADs that can be subdivided into three functional classes (Figure 14). SMAD1, 2, 3, 5 and 8 are the so-called Receptor-activated SMADs (R- SMADs), which interact and get phosphorylated by type I receptors. They can be further classified into TGFβ-activated SMADs (SMAD2 and 3) and BMP-activated SMADs (SMAD1, 5 and 8) depending on the TGFβ ligand that activates them. Following their phosphorylation, R-SMADs associate with common-mediator SMAD4 (Co-SMAD4) and these complexes will translocate in the nucleus. The last group of SMAD are the inhibitory SMAD6 and 7 (I-SMADs). SMAD7 negatively regulates TGFβ signalling by competing for the binding to the type I receptor or by promoting receptor and SMAD degradation (Itoh and ten Dijke, 2007), while SMAD6 competes with R-SMADs for Co-SMAD4 binding. Notably, SMAD7 transcription is positively regulated by TGFβ signalling cascade, indicating the presence of a negative feedback loop.

SMADs consist of two globular regions, called MH1 and MH2, connected by an unstructured linker region. The N-terminal MH1 domain contains a β-hairpin structure with DNA-binding ability and thus is responsible for SMAD-DNA interaction. The MH2 domain is instead located in the C-terminal region and is conserved in all the SMAD classes. Thanks to its versatile binding abilities, MH2 module mediates multiple interactions with receptors, other SMADs, transcription factors, co-activator and co-repressors. Moreover, a nuclear location sequence (NLS) is present in the MH2 domain, which is exposed only when SMADs are phosphorylated allowing their nuclear translocation (Xu et al., 2002). Finally, the linker region is not only involved in connecting the two MH domains.

Indeed, this region contains several phosphorylation sites that allow interactions with other proteins and crosstalk with other pathways, and a PY motif that is bound by SMURF proteins (Alarcon et al., 2009). SMURFs are E3 ubiquitin ligases that stimulate proteasomal degradation of free SMADs or SMAD-associated receptors when recruited by SMAD7. Of note, the linker region of Co-SMAD4 possess a nuclear export signal (NES), essential for shuttling SMADs back to the cytoplasm.

In general, SMADs represent the core of the TGFβ signalling pathway, being the primary mediator of TGFβ signal transduction. The modulation of SMAD activity and the different arrays of SMAD-binding proteins present in the cell, are one of the most critical steps in the determination of the TGFβ response.

2.3. TGFβ signalling cascade: from cell membrane to the nucleus

Despite the complexity of the responses induced by TGFβ pathway, its signalling cascade is surprisingly simple and straightforward (Figure 14). Once activated, dimeric TGFβ ligands bind to their receptors, which consequently form a super complex comprised of two type I receptors and two type II receptors. Different ligands bind to different receptors depending on their affinity: BMP subfamily has high affinity for the type I receptor and low for the type II, while TGFβ subfamily vice versa. However, in both cases the ligand-receptor complex assembly is based on a two-step model. Firstly, the ligand binds to the receptor with higher affinity, and thanks to the consequent conformational changes, they are able to associate with the other receptor type forming the ultimate complex (Groppe et al., 2008). Within this heteromeric complex, type II receptors phosphorylate type I receptors activating their kinases activity (Horbelt et al., 2012). This process leads to the phosphorylation and consequent activation of R-SMAD proteins.

Different R-SMADs are activated depending on the ligand (Figure 14). Specifically, SMAD2 and 3 are phosphorylated downstream of TGFβ isoforms, Nodal or Activin, whereas SMAD1, 5 and 8 are activated in response to BMPs or GDFs stimulation. Independently from the ligand, all phosphorylated R-SMADs form homo- or heterodimers and associate with a single Co-SMAD4. This trimeric complex is targeted by importin β, which binds to the NLSs and drives the R-SMAD/Co-SMAD4 complex into the nucleus. Additionally, the receptor-mediated R-SMAD phosphorylation decreases SMAD affinity for their cytoplasmic anchors and increases the affinity for nuclear factors, facilitating their translocation (Shi and Massague, 2003).

In the nucleus, R-SMADs and Co-SMAD4 are able to bind DNA, targeting specific promoters or enhancers. Precisely, R-SMADs recognize the DNA motif CAGAC, commonly known as Smad-Binding Element (SBE), but SMAD1 and 5 can additionally bind to GC-rich sequences (Korchynskyi and ten Dijke, 2002). Anyway, their affinity for DNA is low, thus the association with high-affinity DNA binding proteins is strictly required. SMAD complexes regulate transcription together with other transcription factors, co-activators, co-repressor and chromatin remodelling factors (Ross and Hill, 2008). Finally, TGFβ signalization is ended via SMAD dephosphorylation or degradation.

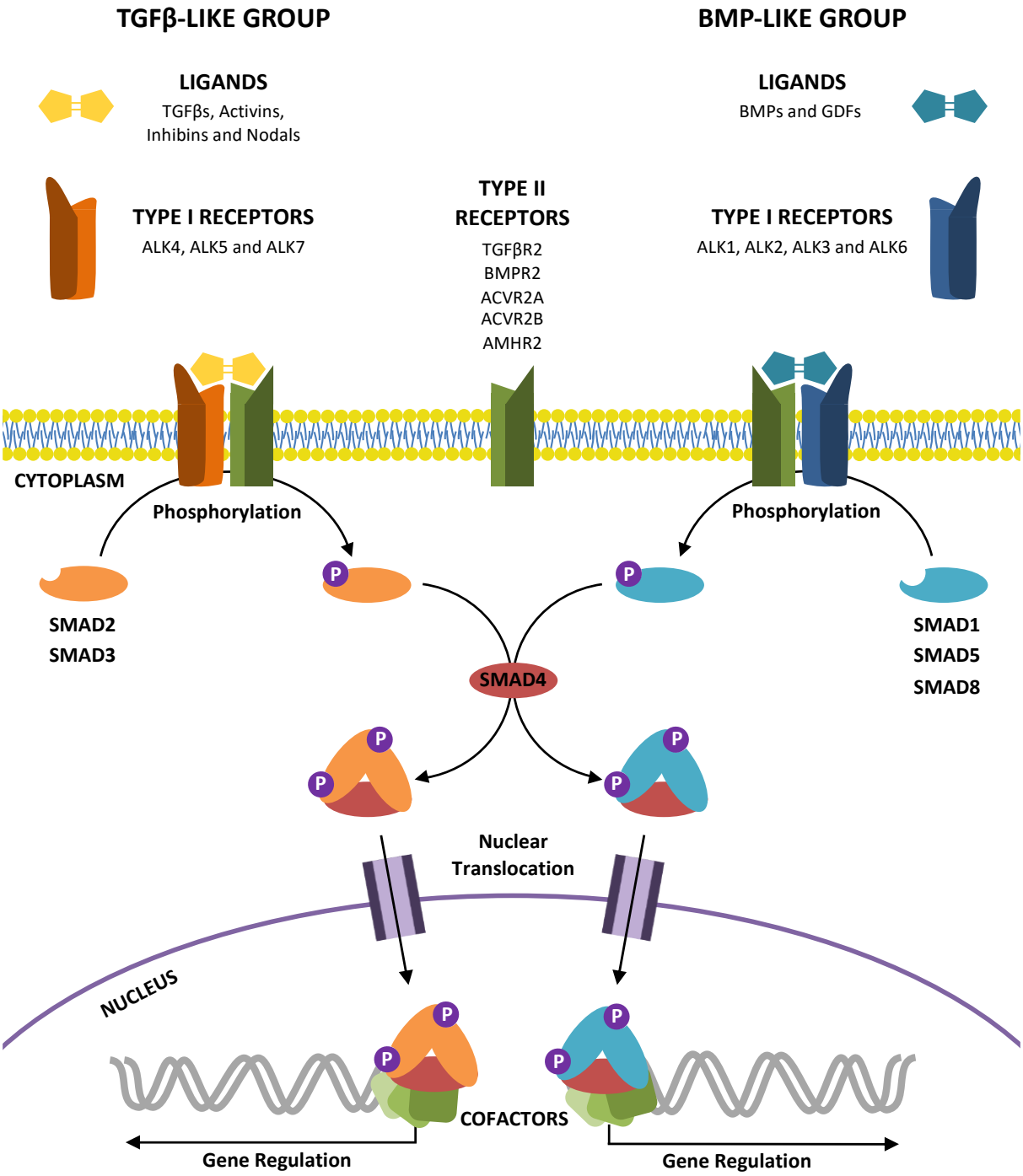

Figure 14. TGFβ Signalling Pathway. TGFβ superfamily is classified into two main categories: the TGFβ-like group (including TGFβs, Activins, Inhibins and Nodals) and the BMP-like group (including BMPs and GDFs). The binding of the ligand leads to the formation of a super complex comprised of two type I receptors, two type II receptors and the dimeric ligand itself. This heteromeric complex triggers the intracellular transduction of the signal through SMAD phosphorylation. Phospho-SMADs form dimers, associate with a single SMAD4 and translocate into the nucleus where in collaborations with several cofactors execute the downstream response regulating gene expression. Refer to the text for a more detailed description of the signalling cascade.

3. TGFβ Signalling Regulation and Complexity

As previously anticipated, the downstream events triggered by TGFβ ligand-receptor binding are relatively simple compared to the diverse range of responses elicited by the TGFβ pathway. However, multiple regulating factors act at different levels during the molecular cascade, defining the specificity, duration and strength of the response and thus determining the final output of the signalization.

3.1. Extracellular regulation of the TGFβ pathway

In all the signalling pathways involving secreted ligands, source and target cells represent the first determining step. The subtypes of ligands released by the source and the array of receptors exposed on the receiving cell define the signalling outcome. Although the concentration of the released ligand is also an important parameter, the bioavailability of TGFβ ligands is mostly determined by their activation as intensively discussed before (see III.1.3. Activation of TGFβ ligands). Therefore, the context-specific factors present in the niche of the source play an essential role in regulating the amount of active TGFβ ligand released, as well as timing and duration of its activation. However, in the ECM are present also proteins able to reduce TGFβ availability, which are termed ligand-trapping proteins. This protein family is comprised of various members that mask specific residues in the ligand, barring its access to the receptor. In example, Decorin and α2-macroglobulin binds to free TGFβ proteins, while Chordin, Noggin, and Cerberus block BMPs activity. Importantly, these inhibitors are essential for the formation of ligand gradients during embryogenesis and storage in adult tissues (Zakin and De Robertis, 2010). Additional negative regulators of TGFβ ligand activity are the ligands themselves. Indeed, antagonistic ligand have been described: for example, inhibin competes with activin for the same receptor, while lefty antagonizes Nodal binding (Lewis et al., 2000; Muller et al., 2012). Another important regulatory element at the extracellular level consists in membrane-anchored proteins that facilitate the interaction between ligands and receptors. In this category we find β-glycan that works as co-receptor presenting TGFβ isoforms to its receptor. Notably, β-glycan negatively regulates activin facilitating inhibin (activin antagonist) access to the receptors (Lewis et al., 2000). Other members of this class are Cripto, essential for Nodal, BMP6 and GDF1 activities (Cheng et al., 2003; Rosa, 2002), and DRAGON, co-receptor of BMP2 and 4 (Samad et al., 2005).

3.2. Intracellular regulation of the TGFβ pathway

SMAD phosphorylation

Although the intracellular regulation of TGFβ signalling is mainly based on SMAD-modulation, also the receptor intracellular domain activity is finely controlled. For example, FKBP12 binds in proximity to the kinase domain of type I receptors when unphosphorylated, preventing ligand-independent activation (Huse et al., 1999). On the other hand, activated TGFβ receptors are negatively regulated by SMAD7, which, in competition with R-SMADs, binds to the receptors triggering their degradation. The access of SMADs to the receptor is also regulated. For example, SMAD2 and 3 recognition by the receptors is facilitated by the auxiliary protein Smad anchor for receptor activation (SARA) (Tsukazaki et al., 1998). SARA possess two binding domains that allow its association with SMADs and with membrane phospholipids (Wu et al., 2000). These interactions permit SARA to localize SMAD2 and 3 in proximity of the membrane, improving the efficiency of their receptor-mediated phosphorylation.

SMAD transcriptional cofactors

Once in the nucleus, SMAD complexes regulate transcription by associating with diverse DNA-binding factors. The DNA-binding activities of the complex components cooperatively generate a high-affinity interaction with target promoters that contain the cognate sequences (Massague et al., 2005). Since many of these transcriptional partners are tissue-specific, these associations play a crucial role in determining the TGFβ signalling outcome. Depending on the particular R-SMAD/Co-SMAD4/partner combination, only specific set of TGFβ target genes will be modulated. The different arrays of transcriptional partners present in different cell types will generate unique responses even if triggered by the same ligands or receptors. Therefore, SMAD-associated proteins determine target gene specificity and transcriptional effects (activation or repression) in a cell-dependent manner, providing a basis for the breadth of TGFβ transcriptional responses.

The first SMAD-interacting protein identified was FoxH1 (Chen et al., 1996) and it was described in Xenopus. In response to activin/nodal signals, this forkhead family member associates with SMADs and target the promoter region of Mix2 enhancing its transcription.

Nowadays, the list of SMAD-associated factors comprises numerous members belonging to multiple DNA-binding protein classes. Homeobox, E-box, Jun/Fos, Runx CREBP and E2F are only some example of the large variety of SMAD collaborators (Shi and Massague, 2003). In general, SMADs have an intrinsic transcription-inducing activity thus exert a positive regulation of their target genes. However, at least 25% of the TGFβ target genes are repressed when the signalling is active (Zavadil et al., 2001). An example of SMAD-mediated gene down-regulation is the inhibition of c-Myc expression, in which SMAD proteins elicit a negative transcriptional activity via association with the co-repressor p107 (Chen et al., 2002). Interestingly, some of the TGFβ transcriptional partners act also independently of SMADs. In these specific circumstances, SMADs act as co-modulators since they do not provide a primary signal, but rather a secondary regulation of the activated factor. Taken together, these observations indicate that SMADs can act as a gene activators or repressors, but also, as modulators of other transcription factors. Of note, SMADs might also exert their functions independently from other associated proteins. Indeed, it has been shown that SMADs can bind to promoters that contain enough clustered copies of the SBE, such as SMAD7 promoter region (Denissova et al., 2000). However, it remains unclear if this SMAD-only complexes DNA-interaction leads to an actual gene regulation.

SMADs can also repress gene expression without binding to the DNA, but through direct inhibition of other transcription factors activity. For example, TGFβ signalling has been reported to negatively regulate the transcriptional activity of Myod1 and this effect results from a physical interference between SMAD3 and the muscle regulatory factor (see III.4. TGFβ Superfamily Biological Functions in Skeletal Muscle) (Liu et al., 2001).

SMAD post-translational modifications

SMAD activity is finely orchestrated by diverse post-translational modifications, adding a further regulatory level of the TGFβ signalling cascade. In example, SMAD phosphorylation can lead to different effect. C-cyclin-depended kinase 8 (CDK8) and cyclin T-CDK9 phosphorylate SMAD linker region allowing the association with YAP and promoting SMAD transcriptional functions (Alarcon et al., 2009). On the other hand, G1 cyclin-dependent kinases CDK2 and 4 have been shown to decrease SMAD3 activity phosphorylating distinct sites of the linker region (Matsuura et al., 2004). A similar effect has been reported for the

phosphorylation of SMAD2 by Calcium/Calmodulin-Dependent Protein Kinase-II (CAMKII) (Wicks et al., 2000). Importantly, SMAD phosphorylation plays a fundamental role in SMAD turnover. Proteasome-mediated degradation controls the levels of SMADs both in the cytoplasm and into the nucleus, and this process is triggered by post-translational phosphorylations. In particular, Glycogen Synthase Kinase 3 / (GSK3β) phosphorylates SMADs creating binding sites for SMURF proteins, which in turn target SMAD for proteasomal degradation via polyubiquitination (Fuentealba et al., 2007). However, only a small fraction of SMADs is degraded, in fact, activated SMADs are normally dephosphorylated and relocated to the cytoplasm (Inman et al., 2002).

Another example of post-transcriptional modification of SMADs is the sumoylation. Specifically, several reports shave shown that Small ubiquitin-like modifiers (SUMOs) are involved in the regulation of Co-SMAD4, which presents sumoylation sites in the MH1 domain and in the linker region (Lee et al., 2003; Long et al., 2004). However, the role of sumoylation remains unclear since both increased and decreased Co-SMAD4 activity have been reported (Lee et al., 2003; Long et al., 2004).

Crosstalk with other signalling pathways

Numerous cascades reportedly crosstalk with TGFβ pathway at multiple levels and SMADs serve as an essential hub for the integration of other signalling pathway inputs. Indeed, thanks to their dynamic binding-abilities and target motifs present in their linker region, SMADs combine and integrate several signals deriving from different pathways. As previously mentioned, GSK3β participates in SMAD turnover, but it is also one of the main molecular character of the Wnt signalling cascade. Specifically, Wnt signals through the inhibition of GSK3β, which, among other things, results in the prolongation of SMAD1 activity (Fuentealba et al., 2007). Moreover, in response to growth factors or under stress conditions, Mitogen-activated protein kinases (MAPKs) phosphorylate SMAD linker region, as well as CDK4 during cycle progression (Matsuura et al., 2004; Sapkota et al., 2007). These examples, together with many others here not reported, indicate the essential role of SMADs in the integration of combinatorial signals, which often produce context-, time-, and location-dependent biological outcomes that are critical for development.

3.3. Non-SMAD TGFβ signalling pathway

Although TGFβ ligands signal primarily through SMAD proteins, TGFβ cascade activates several other intracellular pathways that are collectively referred to as “non-canonical” TGFβ signalling. Some of these pathways elicit responses unrelated to transcription, others instead, regulates gene expression and as well as SMAD activity. Altogether, TGFβ non-canonical pathways substantially contribute to the diversification of the TGFβ cascade outcomes.

Non-canonical cascades can be directly triggered by type II receptors. BMPR2 interacts directly with LIM kinase 1 (LIMK1) in response to BMP ligand binding. LIMK1 is an inhibitor of Cofilin, which is an actin-depolarizing factor. Thereby, BMPR2-bound LIMK1 blocks cofilin activity and stabilizes the filamentous actin cytoskeleton (Foletta et al., 2003). Another example is TGFβR2, which phosphorylates partitioning-defective 6 (PAR6), a scaffold protein regulating cell polarity (Ozdamar et al., 2005). Once activated, PAR6 recruits SMURF1 in proximity of tight junctions prompting degradation of the small GTPase RhoA. This process leads to the dissolution of the tight junctions and is essential for epithelial-mesenchymal transition (EMT). In contrast, TGFβ-induced activation of RhoA has also been reported. RhoA regulates multiple intracellular processes and plays a fundamental role in cytoskeletal organization and TGFβ-mediated EMT. TGFβ rapidly activates RhoA in a SMAD-independent manner to induce stress fibre formation and mesenchymal characteristics (Bhowmick et al., 2001). It is thus possible that non-canonical TGFβ pathways regulate RhoA by two different steps. While in EMT early phase TGFβ stimulates a rapid activation of RhoA, in later stages it inhibits RhoA function. Both regulatory systems are essential for a proper EMT (Zhang, 2009).

Besides RhoA, non-canonical TGFβ cascade activates also Cdc42 GTPase (Wilkes et al., 2003). This cytoskeleton modulator is activated by TGFβ signalling independently of SMADs, since blocking SMAD2 and 3 phosphorylation does not impair its activation. Cdc42 interacts with multiple proteins forming a super-complex also involving TGFβ receptors (Barrios-Rodiles et al., 2005). Among Cdc42-associated proteins we find p21-activated kinase (PAK), which phosphorylates LIMK1. Cdc42 is thus involved in the regulation of actin cytoskeleton via LIMK1-mediated Cofilin inhibition, as well as BMPR2. These observations suggest potential convergence and collaboration in between signalling activated by different TGFβ ligands.

Non-SMAD pathways involves also MAPK pathways, JNK/p38 and phosphatidylinositol-3-kinase (PI3K)/AKT pathways. As previously described, MAPKs regulates SMAD activity phosphorylating the linker region. In turn, TGFβ receptors can modulate various branches of the MAPK pathways. The molecular links between TGFβ and MAPK are complex and involve multiple factor depending on the cell type and the cellular context (Massague, 2012). However, the precise mechanism by which TGFβ controls MAPK activities remains unknown. TGFβ cascade was shown to regulate also phosphatidylinositol-3-kinase (PI3K). While SMAD-dependent pathway was reported to down-regulate PI3K/AKT activity (Valderrama-Carvajal et al., 2002), non-canonical cascade rapidly activates this signalling. TGFβR2 receptor is constitutively associated with p85 (regulatory subunit of PI3K) and its kinase activity is essential for TGFβ-induced PI3K activation (Yi et al., 2005). PI3K is involved in actin cytoskeleton organization and cell migration. Specifically, PI3K contributes to the TGFβ-mediated EMT, and this effect might be mediated by the downstream effector of PI3K/AKT, mammalian Target of Rapamycin (mTOR). Lastly, TGFβ cascade can regulate JNK signalling in a SMAD-independent manner, however, JNK cascade works in conjunction with SMAD proteins to shape the overall TGFβ response. Besides regulation of SMAD activity, TGFβ-induced JNK cascade participates in multiple cellular functions involving several other intracellular factors (Zhang, 2009).

4. TGFβ Superfamily Biological Functions in Skeletal Muscle

TGFβ family plays key roles in many processes, ranging from development to adult homeostasis. At a cellular level, TGFβ proteins regulate fundamental processes like proliferation, differentiation, apoptosis and migration. Although it was originally considered a cell growth-promoting factor, it was soon observed that TGFβ cascade inhibits proliferation in most cell types and even induces apoptosis (Moses, 1992). TGFβ superfamily regulates also cell differentiation, especially during embryonic development where TGFβ ligands control cell fate in various circumstances (Hyytiainen et al., 2004).

Moreover, TGFβ signalling is a potent inducer of ECM components synthesis and fibrotic deposition. TGFβ stimulation upregulates numerous ECM protein-coding genes, such as fibronectin and collagen (Keski-Oja et al., 1988; Roberts et al., 1986), as well as proteinase inhibitors and ECM-degrading enzymes (Keski-Oja et al., 1991). A well-established target gene of the TGFβ cascade is the plasminogen activator inhibitor-1 (PAI-1), which drives ECM accumulation. TGFβ stimulates also integrin expression, which, together with the induction of ECM component secretion, substantially increases cell adhesion.

Another key activity of TGFβ concerns the regulation of immune system cells. Virtually all cell types of the immune system are under the control of TGFβ signalling, which, depending on the context, cell type and differentiation stage, exerts positive and negative effects. A striking example is represented by the genetic removal of TGFβ1 in mouse. This mutation results lethal due the massive in infiltration of lymphocytes into tissues (Shull et al., 1992).

Consistent with these crucial activities, aberrant TGFβ signalling is associated with a wide range of human pathologies, including fibrotic diseases and cancer (Weiss and Attisano, 2013). Since it would be untenable to consider all biological functions of all TGFβ family members, in the next paragraphs we will focus only on the roles of selected TGFβ family ligands in skeletal muscle, specifically Myostatin, BMP and TGFβ proteins.

4.1. Myostatin: negative regulator of muscle mass

A dominant regulator of skeletal muscle plasticity is Myostatin (Mstn), a potent negative regulator of skeletal muscle mass described for the first time during a screen for novel mammalian members of TGFβ (McPherron and Lee, 1997). Mstn *in vivo* relevance was clear from the early phase of its discovery. Both lab-generated Mstn-null mutations in mice and fish, as well as naturally occurring Mstn mutations in cattle showed a massive hypermuscularity (Kollias and McDermott, 2008). In contrast with the widespread biological functions of other TGFβ members, Mstn plays a restricted role in skeletal muscle development and homeostasis. In mice, Mstn gene starts to be expressed at embryonic day 9.5 post conception in the myotome and continues throughout muscle development until adult muscle tissue. However, its expression pattern is not only restricted to skeletal muscle, as it can be found also in heart and adipose tissue (Sharma et al., 1999). Genetic ablation of Mstn in mouse results in an excessive growth of the skeletal muscle, and this phenotype is attributed to both hyperplasia (increase in muscle fibre number) and hypertrophy (increase in muscle fibre size). Furthermore, a robust change in fibre type distribution has been described. Indeed, Mstn-null mice are characterized by a greater proportion of type IIb fibres, which consequently results in altered contractile properties (Mendias et al., 2006). In addition to muscle alteration, Mstn-null mice are characterized by a reduced store of adipose tissue (McPherron and Lee, 2002).

Interestingly, quantification of muscle strength and of other functional aspects of the Mstn-driven phenotype have raised contrasting opinions. While Mendias and colleagues described an increased force generated by EDL muscle in Mstn-null mouse, another physiological study reported no significant differences between wild type mice and the mutant model (Amthor et al., 2007; Mendias et al., 2006). However, both reports showed that the specific maximum tetanic force (muscle force corrected for cross sectional area) was reduced in mice lacking Mstn. In concert with this observation, a more recent work specifically showed that single isolated myofibres from Mstn-null mice, although characterized by a bigger size, do not exhibit an increased force compared to the healthy muscle fibres (Mendias et al., 2011). Altogether, these results support the idea that Mstn-driven hypertrophy do not actually result in a greater muscle force.

While the role of Mstn *in vivo* has been intensively explored, its intracellular mechanisms is less characterized and what it is known has been mainly inferred by *in vitro* studies using C2C12 cell line. Mstn negatively regulates myoblast proliferation and differentiation through ACVR2B-mediated SMAD2/3 phosphorylation (Lee and McPherron, 2001). Mstn signalling activation arrests myoblast cell cycle by up-regulating the cyclin-dependent kinase inhibitor p21 and down-regulating cyclin-dependent kinase-2 (Cdk2) levels and activity (Thomas et al., 2000). Moreover, Mstn specifically blocks myoblast differentiation by inhibiting the expression of MyoD, Myf5 and Myogenin (Langley et al., 2002). In addition to MRF gene modulation, Mstn has been shown to increase gene and protein levels of the E3 ubiquitin ligase Atrogin-1 both in *in vitro* and *in vivo* (McFarlane et al., 2006; Mendias et al., 2011). Atrogin-1 drives protein degradation in skeletal muscle and its downregulation in the Mstn-null context leads to the accumulation of damaged and misfolded proteins. The accumulation of non-functional proteins has been proposed to be responsible, at least in part, of the increased fibre size and lack of force amelioration.

Interestingly, also the myostatin homolog GDF11 plays an important role in skeletal muscle. GDF11 shares a high degree of structural similarity with myostatin and signals via SMAD2/3 (Fan et al., 2017). Despite common features and similar functions, these closely related TGFβ members exhibit different expression patterns during development and elicit also different functions. While GDF11 is widely expressed in embryo and it is essential for mammalian development and aging in multiple tissues (Nakashima et al., 1999), myostatin expression and role is restricted to the skeletal muscle as described above. Importantly, GDF11 function in muscle tissue remains controversial. Although early studies revealed that GDF1-null mice do not display significant alteration at skeletal muscle level (McPherron et al., 1999), ectopic administration of GDF11 in chick embryo limbs led to reduced myogenesis, similarly to myostatin effects (Gamer et al., 2001). A more recent work proposed instead a positive role for GDF11 in muscle tissue, specifically in aged mice. In this study, Wagers's group showed that GDF11 levels decline during aging and its systemic administration in old mice ameliorates their muscle strength, regeneration and MuSC genomic integrity. In contrast, the following year Glass's laboratory contradicted every aspect of the prior study, where they observed that GDF11 actually increases during aging and has a negative impact on MuSC expansion, myoblast differentiation and muscle regeneration (Egerman et al., 2015). These contrasting results are mainly related to technical difficulties in the detection of GDF11 protein levels, however, although contradictory, the role of GDf11 in skeletal muscle appears essential.

4.2. BMP signalling pathway: from development to adult myogenesis

BMP pathway is fundamental during embryonic development and is involved in a large variety of processes, including body axis patterning, gastrulation, mesoderm formation, skeletal development and organogenesis (Wu and Hill, 2009). Originally discovered by their potential to induce ectopic bone formation (Urist, 1965), BMPs are now well-established morphogens that, among the others, play a key role in early muscle formation. In general, BMP proteins are considered negative regulators of embryonic myogenesis, however different contexts can shape the BMP-mediated response (Biressi et al., 2007; Frank et al., 2006; Tajbakhsh, 2002). For example, BMP signalling controls the cell fate of Pax3-expressing myogenic progenitors determining location and timing of their proliferation and differentiation (Amthor et al., 1998). Low concentration of BMP proteins originating from the ectoderm maintains muscle precursors in a Pax3-expressing proliferative state, thus delaying their differentiation. In contrast, high BMP concentration compromises muscle formation, restricting muscle growth through apoptosis induction. These dose-dependent activities are finely orchestrated by BMP helpers and antagonists that shape the BMP gradient in the embryonic tissues. In particular, Follistatin binds BMP2 and 7 and accumulates them in muscle facilitating BMP-mediated muscle growth, while, on the other hand, Noggin antagonizes BMP functions (Amthor et al., 2002). BMP signalling plays a key role also during foetal skeletal muscle growth, where is active specifically at the muscle-tendon interface. Here, BMP cascade regulates the number of MuSCs during development, as well as the number of myogenic progenitors and fibres (Wang et al., 2010). The pivotal role of BMP pathway is confirmed also during post-natal muscle growth. Indeed, abrogation of BMP signalling severely diminish MuSC proliferation and consequently impairs the generation of the adult satellite cell pool (Stantzou et al., 2017).

BMP signalling plays an important role also in adult skeletal muscle. BMP proteins induce osteogenic program in proliferating and infiltrating cells leading ectopic bone and cartilage formation (Lounev et al., 2009). As in embryonic development, BMP cascade regulates also myogenic cell fate and activity. BMP stimulation in MuSCs retained in their niche on isolated myofibres increases the number of proliferating cells and decreased their commitment (Ono et al., 2011). Moreover, while BMP2 administration to cultured myoblasts reduces their differentiation, inhibition of BMP cascade during muscle regeneration results in a rapid and

early differentiation (Aoyama et al., 2011; Ono et al., 2011). Together, these results indicate that BMP signalling initially maintain MuSC proliferation by preventing premature differentiation. Also, in this case, Noggin expressed by differentiated muscl cells participates by antagonizing BMP activity and thus allowing myoblast differentiation and fusion.

Significantly, two independent groups have identified BMP signalling as a dominant regulator of myofibre size and muscle mass, acting in competition with the Myostatin pathway (Sartori et al., 2013; Winbanks et al., 2013). The hypertrophic phenotype caused by myostatin inhibition is characterized by an upregulation of BMP signalling, which promotes muscle growth. Myostatin and BMPs activate different set of R-SMADs (2/3 and 1/5/8 respectively), which compete for Co-SMAD4 binding. Mstn mutation decreases SMAD2/3 phosphorylation allowing BMP-activated R-SMADs to bind Co-SMAD4 and to elicit their positive muscle mass regulation. Conversely, blockage of BMP activity via over-expression of Noggin reverted the hypertrophic phenotype of Myostatin null mice (Sartori et al., 2013).

4.3. The role of TGFβ signalling pathway in skeletal muscle

Early studies on TGFβ pathway functions in muscle tissue

In the last decades of the 20th century multiple studies described, mainly *in vitro*, various TGFβ functions in myogenic cells, strongly suggesting a pivotal role of TGFβ pathway in skeletal muscle development. First, administration of TGFβ proteins at different concentrations and in different combinations with other growth factors on cultured MuSCs derived from different mammals, led to a reduction of muscle cell proliferation rate (Allen and Boxhorn, 1989; Cook et al., 1993). TGFβ activation was also reported to have a negative impact on myoblast differentiation. Although mutations in TGFβR2 with consequent impairment of canonical TGFβ pathway led to inhibition of myoblast differentiation (Filvaroff et al., 1994), numerous reports clearly delineated a negative role for TGFβ during muscle cell differentiation (Allen and Boxhorn, 1989; Angelis et al., 1994; Florini et al., 1986; Massague et al., 1986). Activation of the TGFβ signalling in rat or chick-derived myoblasts led to a strong inhibition of their differentiation process, blocking myotube formation (Allen and Boxhorn, 1989; Massague et al., 1986). Moreover, while the treatment of limb bud organs with TGFβ protein inhibited myoblast differentiation, the administration of a TGFβ neutralizing antibody resulted in a premature appearance of large myotubes (Angelis et al., 1994).

To exert its negative regulation, TGFβ cascade alters the gene expression program underlying myogenic differentiation. It was soon described that TGFβ-induced signalling is able to functionally repress the activity of MRFs, such as MyoD and Myogenin. In particular, TGFβ cascade inhibits Myogenin activity without affecting its DNA-binding capabilities (Brennan et al., 1991). Upon TGFβ activation, Myogenin protein is still able to accumulate into the nucleus, however it does not initiate myogenic differentiation. These results suggest that TGFβ-mediated regulation of Myogenin acts through a mechanism distal to DNA sequence recognition by Myogenin. Interestingly, the negative effects of TGFβ are aimed at the bHLH region of the MRFs, but not at bHLH regions of other protein classes (Martin et al., 1992). This observation indicates that TGFβ-mediated repression is specific for myogenic bHLH proteins and, since their DNA-binding ability is not altered, other intracellular proteins are involved. Furthermore, TGFβ does not only reduced the transcriptional activity of MRFs, but also downregulates their expression, as in the case of MyoD (Vaidya et al., 1989).

The molecular basis of TGFβ-induced inhibition of myogenic differentiation are complex. SMAD3 plays a crucial role by physically interfering with MyoD protein. This interaction masks the bHLH domain of MyoD barring the access of the myogenic transcription factor to the DNA with a consequent impairment of its activity (Liu et al., 2001). Additionally, SMAD3 binds to another myogenic differentiation inducer termed MEF2, but in this case the association prevents MEF2 interaction with its co-activator GRIP-1, thus blocking MEF2-mediated gene activation (Liu et al., 2004). SMAD2 has also been reported to interact with MEF2. Indeed, SMAD2 is found in myotube nuclei complexed with MEF2, but not in myoblasts, where SMAD2 remains mostly cytoplasmic and MEF2 is less expressed. However, the precise role of this cooperation remains not fully understood, since SMAD2-MEF2 interaction promotes MEF2-mediated myogenic gene expression (Quinn et al., 2001).

The widespread role of TGFβ cascade in skeletal muscle *in vivo*

Although the role of TGFβ cascade *in vitro* is now well-established, the effect of canonical TGFβ activation in muscle regeneration *in vivo* has been poorly studied. First, indications of an *in vivo* role for TGFβ have been inferred by studies on Fibrillin-1-null mice (Cohn et al., 2007). As previously described, Fibrillin-1 is a structural component of the ECM able to bind the latent form of TGFβ ligands, preserving its latency. Genetic removal of Fibrillin-1 in mice results in an increased TGFβ signalling activity and a consequent impairment of muscle regeneration. Conversely, inhibition of TGFβ pathway via neutralizing antibody or Losartan administration (TGFβ antagonist) rescues the tissue repair process in Fibrillin-1-null mice.
Recently, an interplay between TGFβ and Wnt/β-catenin pathway has been implicated in the maintenance of satellite cell quiescence (Aloysius et al., 2018). Specifically, Lef1 (transcription factor that mediates Wnt/β-catenin responses) cooperates with SMAD3 in inactivated MuSCs, whereas collaborates with β-catenin in activated MuSCs. This partner switch resulted essential for a proper self-renewal rate of MuSC ex vivo, supporting a potential role for TGFβ in quiescent state maintenance.

A specific role of TGFβ pathway in satellite cell activation has been proposed by Carlson and colleagues in aged muscle (Carlson et al., 2008). Old muscle stem cells are characterized by an excessive accumulation of phosphorylated-SMAD3 due to an elevated TGFβ signalling activity. This unbalanced signalization has repercussions on satellite cell activation, as TGFβ inhibits cell-cycle progression by activating CDK inhibitors.

The pivotal role of TGFβ signalling is also confirmed by studies on Smad3-null mice, in which lack of this intracellular mediator results in altered myogenic differentiation both *in vitro* and *in vivo* (Ge et al., 2012). Smad3-null primary myoblasts showed decreased proliferation rate, impaired differentiation and defective fusion, confirming the essential role of TGFβ in myoblast functionality. Smad3 deficiency *in vivo* leads to a clear impairment of the muscle regeneration process recapitulating the results obtained *in vitro*. MuSC numbers were strongly decreased after injury indicating defective MuSC self-renewal capability. Number and size of regenerated myofibres was remarkably reduced in SMAD3-null injured TA muscles compared to the control, as well as the overall muscle weight.

Besides all these muscle cell-related effects, removal of Smad3 also led to severe alterations of other cell population functionality and processes. Indeed, Smad3-null muscle were characterized by an impaired inflammatory response, decreased mitochondrial biogenesis and reduced fibrotic deposition. Altogether, these results describe the crucial role of TGFβ pathway during muscle regeneration, regulating tissue repair at multiple levels, processes and cell types. As anticipated above, TGFβ pathway is in fact involved in the regulation of several processes, such as fibrosis and inflammatory response. Both processes occur and are essential during skeletal muscle regeneration, supporting the idea of broad functions of TGFβ during adult myogenesis.

TGFβ signalling is the major coordinator of fibrotic deposition as it promotes, together with other signalling molecules, the production of ECM components. The increased ECM component production is observed under physiological situations, like muscle healing, in which is essential for a correct myofibre formation (Gosselin et al., 2004). However excessive TGFβ-driven ECM deposition is related to fibrosis in pathological contexts, such as Duchenne Muscular Dystrophy (DMD) (Bernasconi et al., 1999; Kharraz et al., 2014).
In addition, during muscle regeneration TGFβ is produced and secreted by many cell types, such as infiltrating immune, inflammatory, mesenchymal and tissue-specific cells (Wynn, 2008). TGFβ1 is a well-established chemoattractant for neutrophils, which in turn recruit macrophages (Reibman et al., 1991). After, a subpopulation of proinflammatory macrophages releases cytokines (also TGFβ) to promote inflammatory response and are also responsible for the phagocytosis of muscle debris.

IV. Goal of the Project

As presented in the introduction, adult myogenesis is a complex process that requires a finely tuned balance between extrinsic cues and activation of intrinsic transcriptional cascades. MuSCs express a large variety of membrane receptors and, upon injury, several ligands are secreted and released by muscle-resident cells, damaged myofibres and disrupted ECM. Multiple signalling pathways like BMP, Notch, Wnt have all been reported to participate in adult myogenesis regulation (Brack et al., 2008; Conboy and Rando, 2002; Rudolf et al., 2016; Sartori et al., 2013), as well as the growth factors HGF (hepatocyte growth factor) and FGF (fibroblast growth factor) (Gal-Levi et al., 1998; Shea et al., 2010).

Specifically, in the recent years, our lab focused on understanding the role of Wnt signalling during muscle development and regeneration. Our team recently published that an adequate level of canonical Wnt signalling (Wnt/β-catenin cascade) is fundamental for controlling muscle progenitor function during muscle regeneration (Rudolf et al., 2016). In this study, MuSC-specific β-catenin loss-of-function and gain-of-function mutations *in vivo* resulted in altered tissue regeneration. MusC descendants deprived of canonical Wnt signalling differentiated less efficiently, while constitutively active β-catenin lead to a premature differentiation.

The critical role of Wnt signalling during adult myogenesis was further confirmed by a following study from our group, in which Lacour and colleagues described the function of the canonical Wnt enhancer R-spondin1 (Rspo1) (Lacour et al., 2017). Genetical ablation of Rspo1 gene in mice significantly affected myogenic progenitor cell differentiation both *in vitro* and *in vivo*. In contrast, Rspo1-null myoblasts generated larger syncytia with more nuclei compared to wild-type cells. The authors then proposed that Rspo1 negatively regulates muscle cell fusion through canonical Wnt signalling. Interestingly, Wnt/β-catenin cascade has generally been reported as a promoter of myoblast differentiation and fusion (Hulin et al., 2016; Zhuang et al., 2014), hence it was counter-intuitive to suggest that canonical Wnt signalling could inhibit cell fusion.

We linked this suggested mechanism to the previous work by Rudolf and colleagues, whom identified that canonical Wnt pathway activates TGFβ signalling (Rudolf et al., 2016). In fact, as extensively described in the introduction (see III.4.3. The role of TGFβ signalling pathway in skeletal muscle), TGFβ cascade is a well-established negative regulator of muscle tissue formation and regeneration. However, although it has been shown that TGFβ pathway has broad effects on MuSC quiescence and activation and on myoblast proliferation and differentiation, the impact of TGFβ cascade specifically in muscle cell fusion has never been investigated. We then suggested that while Wnt/β-catenin pathway promotes the transition from proliferating myoblast to differentiated myocyte, it also stimulates autocrine TGFβ signalling to limit the rate of myocyte fusion.

For these reasons, the main objective of my PhD project was to study the role of TGFβ signalling pathway during muscle regeneration *in vivo* and myoblast differentiation *in vitro*, with a special regard to its impact on muscle cell fusion.

RESULTS

The results obtained during my PhD are summarized in the article reported hereafter, which has been published on the open access preprint database BioRxiv (Feb. 21, 2019; doi: http://dx.doi.org/10.1101/557009) and is currently in revision on *Nature Communication* Journal.

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TGFβ signaling curbs cell fusion and muscle regeneration

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Summary

Fusion of muscle progenitor cells is necessary for skeletal muscle development and repair. Cell fusion is a multistep process involving cell migration, adhesion, membrane remodeling and actin-nucleation pathways to generate multinucleated myotubes. While the cellular and molecular mechanisms promoting muscle cell fusion have been intensely investigated in recent years, molecular brakes restraining cell–cell fusion events to control syncytia formation have remained elusive. Here, we show that transforming growth factor beta (TGFβ) signaling is active in adult muscle cells throughout the fusion process and reduce muscle cell fusion independently of the differentiation step. In contrast, inhibition of TGFβ signaling enhances cell fusion and promotes branching between myotubes, a mechanism we find is conserved across mice and human. Pharmacological modulation of the pathway *in vivo* perturbs muscle regeneration after injury. Exogenous addition of TGFβ protein results in a loss of muscle function while inhibition of the TGFβ pathway induces the formation of giant myofibres. Transcriptome analyses and functional assays revealed that TGFβ acts on actin dynamics to reduce cell spreading through modulation of actin-based protrusions. Together our results reveal a signaling pathway that limits mammalian myoblast fusion and add a new level of understanding to the molecular regulation of myogenesis.

Text

Introduction

The adult skeletal muscle cell is a syncytial myofibre that contains hundreds of myonuclei. Formation and regeneration of the myofibre requires fusion of mononuclear progenitors (myoblasts) to form multinucleated myotubes. Located in a niche around the myofibres are quiescent muscle stem cells [1], called satellite cells, which can activate and proliferate to give rise to adult myoblasts competent to fuse with each other and with myofibres [2]. As such, cell fusion plays essential roles in the adult, allowing physiological muscle hypertrophy [3,4] and muscle regeneration following injury [5,6].

When induced to fuse, adult myoblasts exit the cell cycle, commit to terminal differentiation and migrate toward each other [7]. They then adhere through membrane integrins [8] and cadherins [9]. The later stages of fusion are controlled by the muscle-specific protein Myomaker [10] and peptide Myomerger [11,12,13] (also known as Minion, Myomixer). Together, Myomaker and Myomerger reconstitutes cell fusion. Recent studies demonstrated that muscle cell fusion is promoted by actin-based structures [14] generating protrusive forces [15] and membrane stress then coalescence [16]. The fusion process must be tightly controlled to safeguard that fusogenic myoblasts do not form aberrant hypertrophic syncytia or fuse with non-muscle cells. However, while it is known that muscle cell fusion can be prevented by tetraspanins at the cell membrane [17], no signaling pathway that can limit this process and prevent unscheduled cell fusion has been identified.

Canonical Wnt/β-catenin signaling is a crucial regulator of satellite cells and adult muscle regeneration [18,19] . Interestingly, β-catenin activation in muscle precursor cells induces the expression of TGFβ ligands and receptors [20]. TGFβ signaling has been shown, mainly *in vitro*, to negatively regulate myoblast differentiation through functional repression of the myogenic regulatory factors Myod1 [21] and Myogenin [22]. However, TGFβ signaling has broader function in muscle cells, including quiescence [23] and activation [24], through the impact of TGFβ in syncytia formation has never been investigated. This gap in our knowledge mainly comes from the fact that the primary effects of TGFβ over-expression in skeletal muscles are the development of endomysial fibrosis, due to its role as a potent growth factor for connective tissue cells [25,26]. Here, we asked whether TGFβ signaling might have a role in myoblast fusion.

Results

TGFβ ligands bind to TGFBR2 that will recruit a type I receptor dimer. The receptor complex will then phosphorylate SMAD2 and SMAD3 that will accumulate in the nucleus where they act as transcription factors [27]. To evaluate TGFβ isoforms expression by adult muscle progenitor cells, we purified limb muscle satellite cells and grew them *in vitro* as primary myoblasts. We observed that Tgfb1 and Tgfb2 expression levels were high in proliferating cells, and diminished following induction of differentiation, while Tgfb3 expression pattern showed an opposite trend (Fig. 1a). Of note, the expression levels of the TGFβ receptors (Tgfbr1, also known as Alk5; and Tgfbr2) did not significantly change during the course of *in vitro* myogenesis (Fig. 1b). We next investigated the state of TGFβ signaling in primary myoblasts, differentiated myocytes and multinucleated myotubes. We observed that the expression level of the TGFβ/SMAD2/3 target gene Smad7 diminished during myogenic progression (Fig. 1c). While immunolocalization of phosphorylated-SMAD2/3 proteins showed that the canonical TGFβ pathway is active at all studied stages (Fig. 1e), quantitative western blotting experiments demonstrated that the intensity of TGFβ signaling decreases during muscle cell differentiation but is not abrogated in multinucleated cells (Fig. 1d). Previous work has established that TGFβ ligands are secreted during muscle tissue repair [28]. Gene expression analysis of regenerating *Tibialis Anterior* (TA) muscles demonstrated that the 3 TGFβ isoforms are dynamically expressed following injury (Fig. 1e) and peak between 3- and 5-days post-injury (d.p.i.). Likewise, we detected the expression of phosphorylated SMAD3 proteins in nuclei both inside and outside the regenerating myofibres at 4 d.p.i (Fig. 1f). We thus aimed to investigate the role of TGFβ in the fusion process.

Since all 3 TGFβ ligands are expressed by cultured satellite cells, we evaluated the impact of recombinant proteins stimulations on adult myogenesis (Fig. S1a). After 72 hours of differentiation, muscle cells aggregated to form multinucleated myotubes, while addition of recombinant TGFβ proteins forced muscle cells to remain mostly mononucleated (Fig. S1b). Quantification of *Myh3* gene expression, which codes for the embryonic myosin heavy chain isoform, further indicated that the cells in TGFβ-treated cultures were in a less mature state than control cultures (Fig. S1c). However, quantification of the percentage of differentiated nuclei expressing pan-MYOSIN HEAVY CHAIN proteins revealed that the vast majority (>90%) of myoblasts did undergo differentiation in all conditions (Fig. S1d), suggesting that TGFβ signaling does not primarily block muscle cell differentiation (Fig. S1e).

To test this hypothesis, we adapted the protocol used by Latroche and colleagues to uncouple differentiation and fusion of primary muscle cells [29]. In this experimental set up, primary myoblasts were differentiated for 2 days at low density that does not allow contact between cells. The cells were then split and re-plated at a high density and cultured for an additional two days to evaluate muscle cell fusion (Fig. 2a). Following re-plating, almost all muscle cells were terminally differentiated and expressed MYOGENIN (>94%) (Fig. 2b). We thus evaluated the effect of TGFβ proteins stimulations in this experimental setting, *i.e.* on mononucleated differentiated muscle cells (myocytes) and observed that all 3 TGFβ isoforms strongly inhibited cell fusion (Fig. 2c) despite the muscle cells progressing down the differentiation pathway (Fig. 2d; ~100% $MyHC^{+}$). Activation of the TGFβ pathway reduced the fusion index (Fig. 2e) and completely blocked the formation of large myotubes (Fig. 2f), thus demonstrating that TGFβ signaling limits muscle cell fusion independently of myogenic differentiation. Importantly, addition of TGFβ proteins to adult muscle progenitor cells did not alter their proliferation (Fig. S2a), nor did it induce programmed cell death (Fig. S2b), and it did not alter their motility (Fig. S2c).

We next asked if inhibition of TGFβ signaling in fusing myocites could enhance the formation of multinucleated myotubes. To this aim, we selected ITD-1, a highly selective TGFβ inhibitor which triggers proteasomal degradation of TGFBR2 [30]. ITD-1 clears TGFBR2 from the cell surface and selectively inhibits intracellular signaling. ITD-1 treatment of primary myocytes resulted in reduced expression of TGFβ target genes (Fig. 3a) and blocked the phosphorylation of nuclear SMAD2/3 proteins induced by TGFβ1 treatment (Fig. 3b). Importantly, treatment of differentiated mononucleated muscle cells re-plated at high density (as in Fig. 2a) with ITD-1 enhanced the fusion process (Fig. 3c). As such, ITD-1-treated cultures showed higher fusion index (Fig. 3e) and were composed of myotubes containing more nuclei (Fig. 3d), of larger diameter (Fig. 3f) and characterized by an aberrant branched shape (Fig. 3c, 3g). Taken together our data demonstrate that inhibition of TGFβ receptor function boosts fusion and suggest that the levels of TGFβ signaling must be tightly controlled to ensure proper syncytia formation.

To test if TGFβ signaling controls fusion cell-autonomously, we expanded primary myoblasts from satellite cells expressing either H2B-GFP or membrane tdTomato (pseudocoloured in blue). Both primary cell types were pre-differentiated at low density, but only the GFP-expressing myocytes were treated with either TGFβ1 or ITD-1 and their F-actin content stained with SiR-Actin (pseudocoloured in red) (Fig. 4a). Cells were then mixed, re-plated at high density, and fusion events were imaged live (Fig. 4b). We observed that fusion of GFP-labeled myonuclei into tdTomato myotubes was controlled by the intrinsic state of TGFβ signaling in the fusing cells (Fig. 4c). As such the incidence of heterologous fusion was controlled by TGFβ signaling (Fig. 4d). Interestingly, we noticed that multinucleated myotubes could fuse together and that TGFβ signaling regulates the frequency of myotube-to-myotube fusion (Fig. 4e) (Supp. Movies). These results suggest that TGFβ acts cell-autonomously to limit the fusion between muscle cells, and to prevent fusion between syncytia.

To determine the human relevance of the hyper-fusion phenotype we observed with mouse cells, we evaluated the influence of TGFβ pathway inhibition on human muscle cell differentiation in a 3D culture format [31] (Fig. 5a). By quantifying the tissue remodeling and compaction process (Fig. 5b, 5c), we found that inhibition of TGFBR1 function by the small molecule SB-431542 did not change the thickness of human muscle microtissues (hMMTs) at early differentiation timepoints (Days 0-4), but that as the hMMTs further matured, there was a bifurcation and TGFBR1 inhibitor-treated hMMTs were significantly thicker than control hMMTs. In this system, hMMT thickening was due to an increase in the width of individual fibres (Fig. 5d, 5e). Notably, SB-431542-treated human muscle fibres contained more nuclei than control fibres, confirming that increased muscle cell fusion as the underlying cellular mechanism (Fig. 5f). By treating hMMTs with acetylcholine to induce tissue contraction and capturing short videos to visualize the magnitude of vertical rubber post deflections, we found that TGFBR1 inhibition renders the hMMTs stronger than their control counterpart Fig. 5g, 5h). Together these data demonstrate that TGFβ signaling regulates human cell fusion.

To evaluate the impact of TGFβ signaling in muscle cell fusion *in vivo*, we injured TA muscle of adult mice and injected either TGFβ1 protein or ITD-1 compound, at 3 d.p.i., the time-point when fusion begins (Fig. 6a). Evaluation of regenerating tissues at 7 d.p.i. revealed that both

treatments lead to striking changes in myofibres size and morphology (Fig. 6b). TGFβ1 addition resulted in a robust decrease in nuclear number in newly-formed myofibres compared with controls (Fig. 6e), resulting in a dramatic drop in fibre cross-sectional area (CSA) (Fig. 6c, 6d). In contrast ITD-1 induced a large increase in myonuclear accretion (Fig. 6e) resulting in the formation of larger fibres compared to controls (Fig. 6c, 6d). To further elucidate if modulating TGFβ signaling *in vivo* affects regenerated muscle tissue structure and function, we performed TA muscle injury, followed by 3 successive injections of TGFβ1 or ITD-1, and evaluated the regenerated muscles 2 weeks after injury (Fig. 6f). In this setup, the effects of modifying TGFβ signaling were more pronounced (Fig. 6g). Activation of TGFβ signaling induced the formation of very small myofibres while ITD-1 treatment generated giant myofibres (Fig. 6h, 6i). We next performed *in situ* force measurement of regenerated TA muscles. As suggested by the severe myofibre atrophy observed in TGFβ1-injected muscles, ectopic activation of TGFβ signaling during tissue regeneration lead to a strong reduction of muscle specific force (Fig. 6j). Despite being composed of larger myofibres, compared to control regenerated muscles, ITD-1-treated muscles did not show any improvement in force generation. These observations indicate that TGFβ signaling determine the numbers of fusion events occurring during tissue regeneration *in vivo*.

To identify the genetic networks regulated by TGFβ signaling in muscle cells, we performed transcriptome analysis of primary myocytes differentiated for 24 hours and stimulated with either TGFβ1 or ITD-1 for another 24 hours (Fig. 7a). We first observed that the relative expression levels of myogenic transcription factors (Pax7; Myod1; Myogenin) were not significantly changed, confirming that the modulation of TGFβ signaling does not act on myogenesis in these experimental conditions. Recently identified fusion master regulatory factors (Myomaker and Myomixer) were also unaffected by TGFβ signaling (Fig. 7b). We then used Ingenuity Pathway Analysis (IPA) to reveal the pathways affected by TGFβ signaling. Interestingly, we found that "Actin Cytoskeleton" was among the top-regulated pathways (Fig. 7c). This is significant, since actin remodeling and the formation of finger-like actin protrusions are essential for myoblast fusion [32]. IPA further revealed changes in the transcription of numerous genes implicated in actin dynamics following TGFβ treatment (Fig. S3a). Visualization of the F-ACTIN network and measure of the local orientation of actin filaments

in differentiated muscle cells showed that the level of TGFβ signaling negatively correlates with cytoskeleton reorganization (Fig. 7d) and elongated cell shape with an effect on cell spreading (Fig. 7e) and the coherency of actin filament alignment (Fig. 7f). To validate the pivotal role of Actin remodeling during fusion, we treated pre-differentiated mononucleated muscle cells at high density (as in Fig. 2a) with Latrunculin, a toxin able to block F-actin polymerization (Fig. 7g). As expected, inhibition of Actin polymerization significantly decreased myoblast fusion. However, Latrunculin administration reduced fusion also in the presence of ITD-1, thus indicating that TGFβ pathway acts upstream of Actin remodeling (Fig. 7h, 7i, 7j).

To better understand actin remodeling during muscle cell fusion, we performed live-imaging experiments to visualize accumulation of F-ACTIN foci in invasive podosome-like structures at the sites of fusion between cells [33]. By mixing cells stained with SiR-Actin and unstained cells, we observed dynamic actin remodeling in untreated cells (Fig. S3b, Top) and cells stimulated with ITD-1 (Fig. S3b, Bottom), while TGFβ1 stimulation prevented the formation of actin-rich invasive structures and promoted the maintenance of a rounded cell shape (Fig. S3b, middle). Lastly, we asked whether TGFβ-driven effect on fusion is conserved in a non-muscle context. To do so, we took advantage of a fibroblast dox-inducible cell fusion reconstitution system [34]. Myomaker- and Myomerger-transduced 10T1/2 fibroblasts were seeded and stimulated with dox to induce fusion (Fig. S4a). TGFβ1 recombinant protein was administrated at multiple time points (from day 0 to day 3), but none of the different setting led to a reduction of fusion, compared to the untreated fibroblasts (Fig. S4b, S4c). These results show that TGFβ1 protein is unable to inhibit the fusion process in a non-muscle cell type and that its signaling cascade acts independently from Myomaker and Myomixer, confirming the evidences obtained from the transcriptome analysis (Fig. 7b). Finally, our data suggest that TGFβ acts upstream of Myomaker/Myomerger function, which represent the final step of fusion.

Discussion

The data presented here identify an unexpected negative role for TGFβ in the fusion of adult myocytes to form myotubes. TGFβ signaling has previously been shown to play a major role in the skeletal muscle morphogenesis. Throughout development, TGFβ ligands are expressed mostly by connective tissue cells and in close proximity to growing muscle tissue [35]. While it is known that connective tissue cells provide a pre-pattern for limb muscle patterning [36] and control the amount of myofibres within the developing muscle masses [37], we propose that TGFβ is the main signal limiting muscle cell fusion.

During adult tissue repair TGFβ1 and TGFβ3 are mainly expressed by inflammatory macrophages invading the regenerating muscle tissue [38,39] while TGFβ2 is secreted by activated satellite cells and differentiating myotubes [38]. This sequential expression of ligands by different cell types may be instrumental in preventing premature fusion between transient amplifying myoblasts and then in avoiding fusion events between syncytia and prevent the formation of aberrant branched myotubes. Indeed, we speculate that it is the lack of other cell types that stunts the maturation of myotubes in 3D human skeletal muscle microtissue. It is only upon introducing a missing signal to induce myotube-to-myotube fusion do we release a developmental brake preventing the next phase of tissue maturation.

In the context of disease, myofibres with branches are found in muscular dystrophy [40]. They present morphological malformations, as well as alterations in calcium signaling [41], and arise from asynchronous myofibre remodeling. Aberrant fusion events driven by chronic elevation of TGF-β signaling in muscle pathologies [42], associated with impaired regeneration may contribute to disease progression and the severity of disease phenotypes. Knowledge of the signaling pathways regulating muscle cell fusion may help design therapeutic strategies to decrease myofibre branching in dystrophic patients.

Our *in vivo* experiments indicate that TGFβ signaling must be tightly regulated in muscle progenitor cells during tissue repair. Treatment of regenerating muscle tissues with TGFβ1 protein strongly blocks muscle progenitor cell fusion, and the impair the function of the

regenerated tissue. Interestingly, ITD-1 administration lead to the formation of giant myofibres containing more nuclei. However, while functional, the regenerated muscle did not generate higher force compared to mock-treated regenerated tissues. While appealing, it remains to be demonstrated if enhancing cell fusion might improve regenerated muscle function. As such, "bigger" does not always means "stronger", and this is exemplified by our previous analysis showing that lack of Rspo1 results in the formation of larger myofibres containing supernumerary nuclei following regeneration [6]. Further to that point, the end goal of coordinated muscle tissue regeneration is to restore a functional tissue architecture and mechanical properties in accordance with the other components of the musculoskeletal system.

Work in *Drosophila melanogaster* previously demonstrated that actin polymerization drives muscle cell fusion [32]. Our demonstration that TGFβ stimulation breaks down actin architecture links extracellular cues to cell mechanics. We show that the state of TGFβ signaling in pre-fusing cells controls their shape, and the formation of actin-based protrusions which are necessary for fusion of mammalian cells [43]. Importantly, TGFβ signaling may regulates multiple points of the fusion pathway through actin nucleation. Our result also demonstrate that blockade of actin polymerization blunts the over-fusion phenotype induced by inhibition of TGFβ signaling. Interestingly, TGFβ stimulation of Myomaker- and Myomerger-expressing fibroblast enhanced fusion of these cells. This can be explained by the fact that TGFβ induces cytoskeletal reorganization and promote F-actin polymerization in fibroblasts [44,45] in an opposite way as we observed in muscle cells. Future work should investigate why TGFβ signaling has different effect on actin dynamics, depending of the cell identity.

In the present state of our knowledge, most of the fusion-promoting factors have been discovered through *in vivo studies in the fly embryo. As such, the concept of* the fusogenic synapse; the site where an attacking fusing cell propels an actin-rich membrane protrusion towards a receiving cells, is poorly characterized in mammalian cells [46]. Here, we identify numerous actin-related transcripts, of which the expression is regulated by TGFβ signaling, that may be integral parts of the molecular fusion machinery. Further work should thus be dedicated to the study of the TGFβ-regulated genes, and their specific roles in actin dynamics to better our understanding of how muscle cell membranes are brought together for fusion.

In conclusion, the elucidation of TGFβ signaling as a brake for myoblast fusion opens new avenues to study this fundamental cellular process at a molecular level and to understand how fusion is perturbed in neuromuscular diseases.

Main Figures

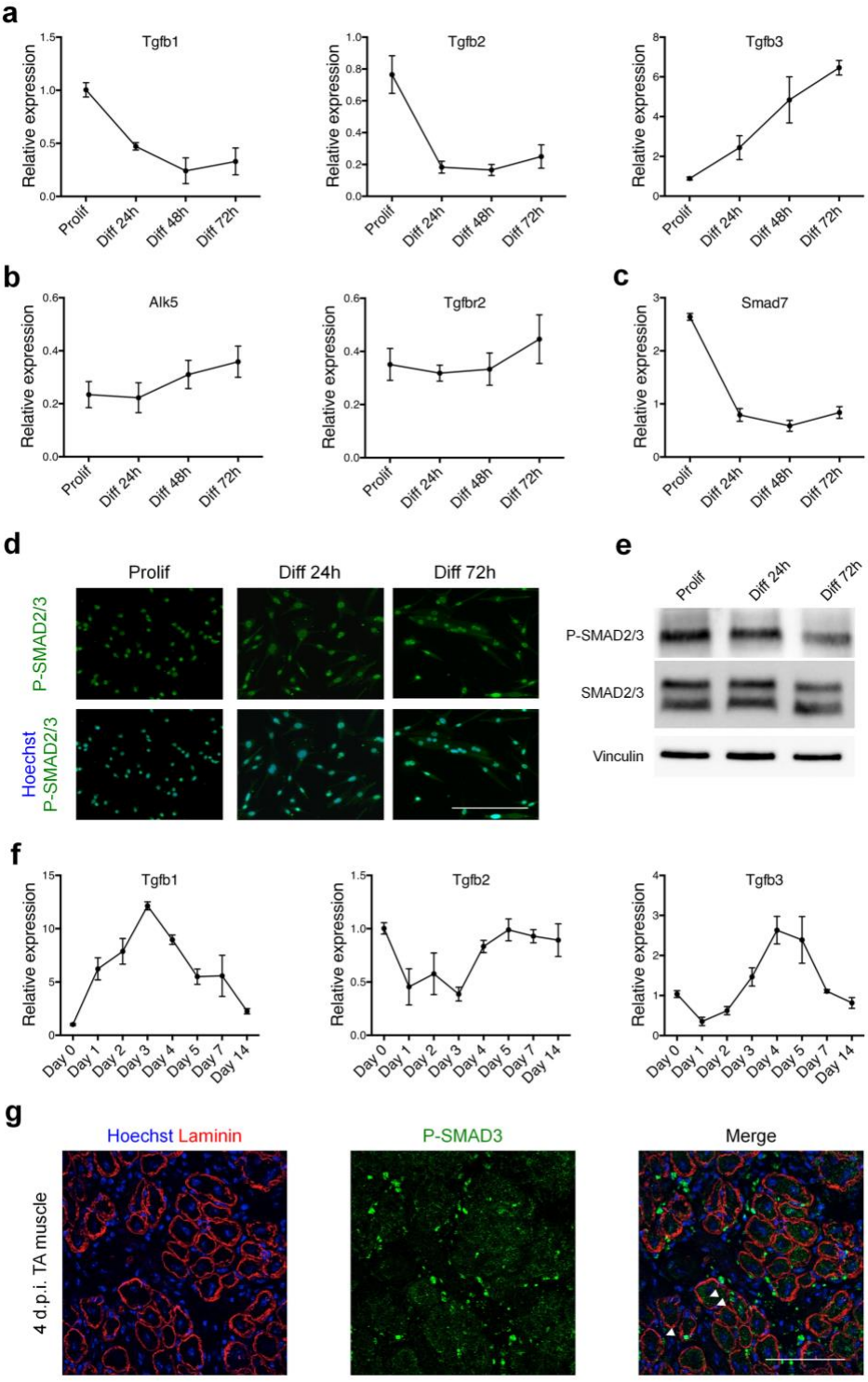


Figure 1 | **TGFβ signaling pathway remains active during myoblast differentiation.**
a, qRT-PCR analysis of Tgfb1, 2 and 3 transcripts expression during *in vitro* differentiation of primary muscle cells shows different profiles. **b,** qRT-PCR analysis of Alk5 and Tgfbr2 transcripts expression describes a constant expression of the receptors during primary muscle cells differentiation. **c,** qRT-PCR analysis of the TGFβ target gene Smad7 transcript expression reveals a decreased activity of the pathway alongside *in vitro* primary muscle cell differentiation. **d,** Phospho-SMAD2/3 immunofluorescent staining of proliferating, differentiating and differentiated primary myoblasts reveals a constant and basal activation of the pathway. **e,** Phospho-SMAD2/3 and SMAD2/3 western-blot analysis of proliferating, differentiating and differentiated primary myoblasts confirms a decrease in SMAD2/3 phosphorylation during differentiation. **f,** qRT-PCR analysis of Tgfb1, 2 and 3 transcripts expression during muscle tissue regeneration induced by CTX injection shows specific expression profiles. **g,** Immunofluorescent staining for phospho-SMAD3 on 4-days regenerating TA muscles cryosections confirms the presence of active TGFβ signaling in the tissue. Arrowheads indicate phospho-SMAD3^{+} nuclei within myofibres. Scale bars: **e,** 200µm. **g,** 200µm. Data are presented as mean ± s.e.m. from at least three independent experiments.

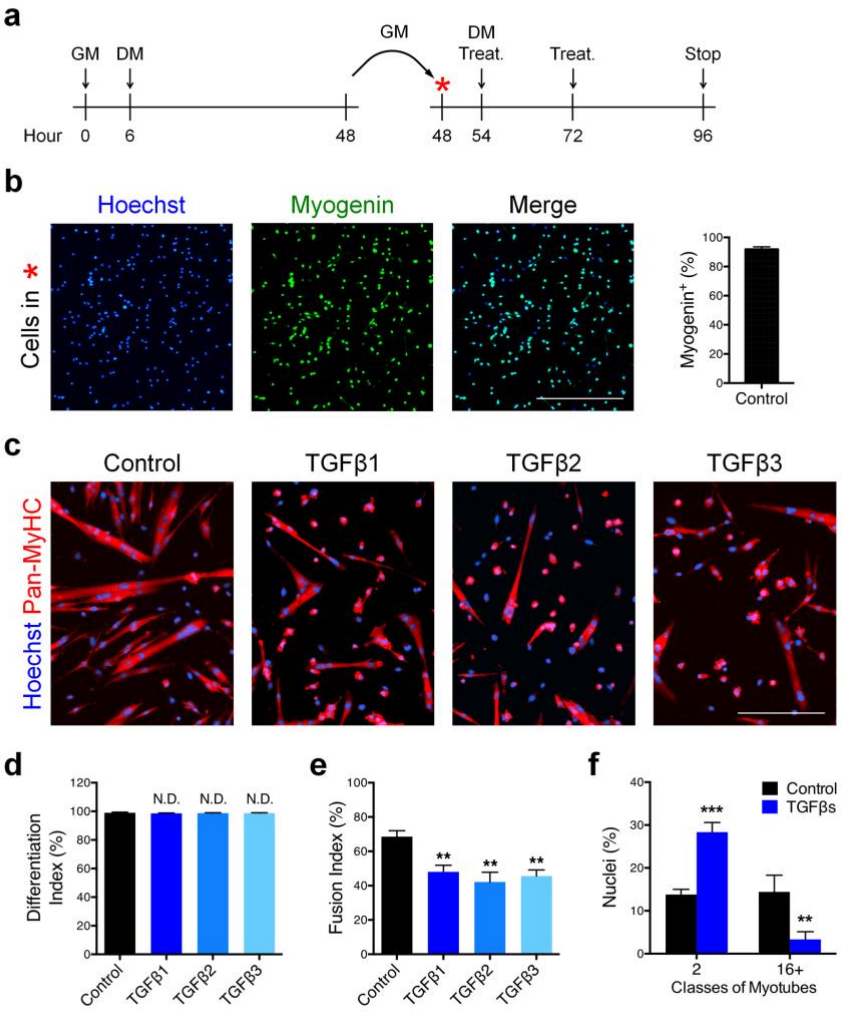


Figure 2 | **TGFβ signaling limits cell fusion. a,** Experimental scheme. Primary myoblasts seeded at low density (5,000 cells/cm2) were differentiated for two days, split and re-plated at high density (75,000 cells/cm2) and cultured for two more days. **b,** Immunofluorescent staining for MYOGENIN of primary myocytes pre-differentiated for 48h and re-plated at high density, confirms that more than 90% of cells express Myogenin. **c,** Immunofluorescent staining for the Myosin Heavy Chain isoforms (Pan-MyHC) of re-plated primary myocytes cultured for 48 hours. **d,** Percentage of Pan-MyHC-expressing cells of re-plated myotubes shows that cells were differentiated in all conditions. **e,** Fusion index of re-plated myotubes reveals that TGFβ stimulation inhibits fusion. **f,** Percentage of nuclei in the smallest and largest myotube classes. TGFβ-treated myotubes are characterized by less nuclei per myotube. Scale bars: **b,** 400µm **c,** 200µm. Data are presented as mean ± s.e.m. from at least three independent experiments. ***P*<0.01, ****P*<0.001, N.D.=Not significant, compared with Control (Unpaired two-tailed Student's t-test).

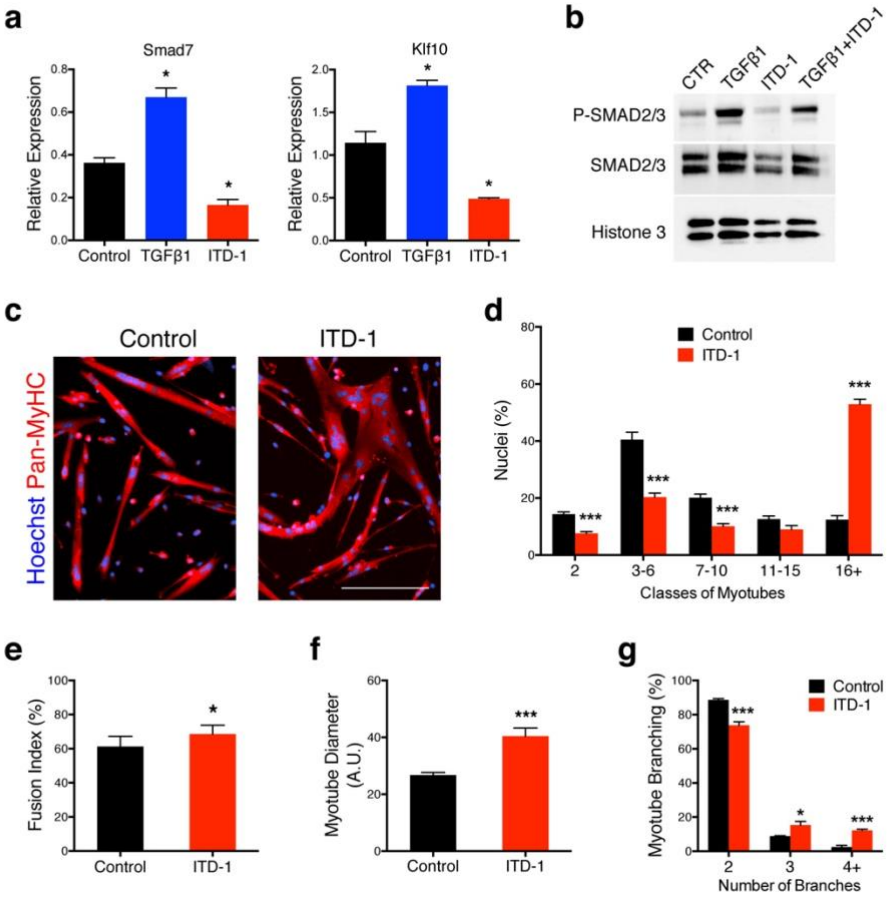
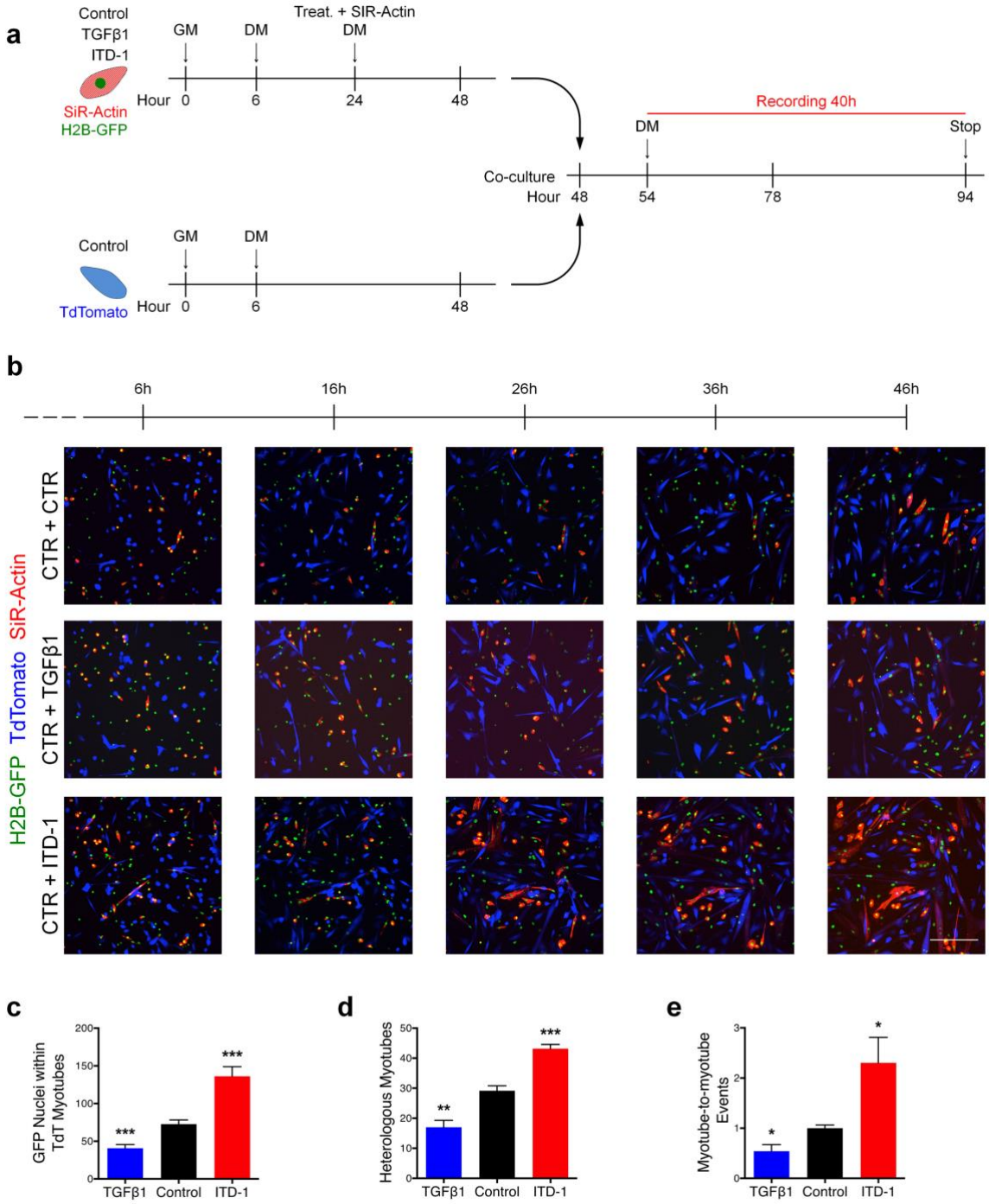


Figure 3 | **Inhibition of TGFBR2 function in differentiated muscle cell enhance fusion. a,** qRT-PCR analysis of TGFβ target genes transcript expression in primary myocytes treated with TGFB1 protein or ITD-1 compound proves that Smad7 and Klf10 are over-expressed when the signaling pathway is activated and inhibited when TGFβ cascade is blocked. **b,** Nuclear phospho-SMAD2/3 and SMAD2/3 western blot analysis of primary myoblast treated with TGFB1 protein, ITD-1 compound or both combined. The intracellular mediators SMAD2/3 are phosphorylated upon TGFβ stimulation, while ITD-1 is able to reduce their phosphorylation. **c,** Immunofluorescent staining for Pan-MyHC of re-plated myocytes cultured for 48 hours. **d,** Aggregation index of re-plated myocytes shows that ITD-1 treatment leads to the formation of myotubes with higher numbers of nuclei compared to the control. **e,** Fusion index of re-plated myocytes confirms the enhanced fusion when TGFβ cascade is inhibited. Diameter of re-plated myotubes (**f**) and of the distribution of branched-myotubes (**g**) of re-plated cells highlight aberrant morphology of syncitia treated with ITD-1. Scale bars: **c,** 200µm. Data are presented as mean ± s.e.m. from at least three independent experiments. **P*<0.05, ****P*<0.001, compared with Control (Unpaired two-tailed Student's t-test).


a
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TGFβ1
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DM
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Recording 40h
DM
Stop
Co-culture
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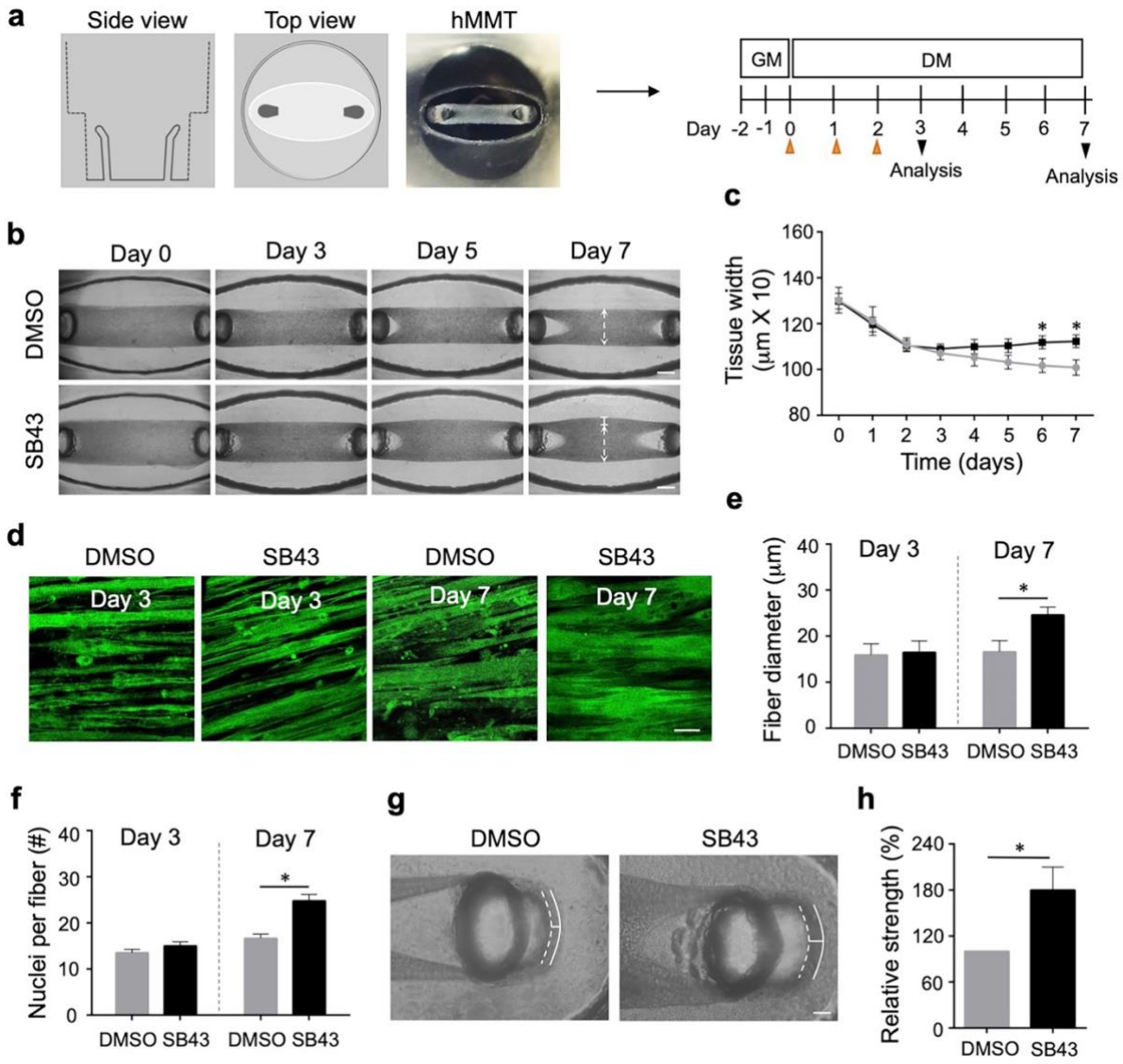
Figure 4 | **Live-imaging of myoblast fusion.**
a, Experimental scheme. H2B-GFP primary myoblasts were seeded at low density (5000 cells per cm^2), treated with TGFβ1 protein or ITD-1 compound, stained with SiR-Actin and differentiated for 2 days. Membrane-TdTomato primary myoblasts seeded at low density (5000 cells per cm^2) and were differentiated for two days. Both populations were split and co-cultured (50/50) at high density (75000 cells/cm^2) and cultured for two more days. In the last 40 hours, cells were recorded live by confocal microscopy. **b,** Live-imaging frames of co-cultured pre-differentiated myocytes confirm the phenotype previously observed. TGFβ activation inhibits fusion, while ITD-1 enhance fusion. **c,** Quantification of H2B-GFP nuclei within TdTomato myotubes. **d,** Quantification of heterologous myotubes (double positive for SiR-Actin and TdTomato). **e,** Quantification of Myotube-to-Myotube events. ITD-1 treatment allows more myotube-to-myotube events compared to the control. Scale bars: **b,** 200µm. Data are presented as mean ± s.e.m. from at least three independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001, compared with Control (Unpaired two-tailed Student's t-test).


a
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hMMT
GM
DM
Day -2 -1 0 1 2 3 4 5 6 7
Analysis
Analysis
b
Day 0
Day 3
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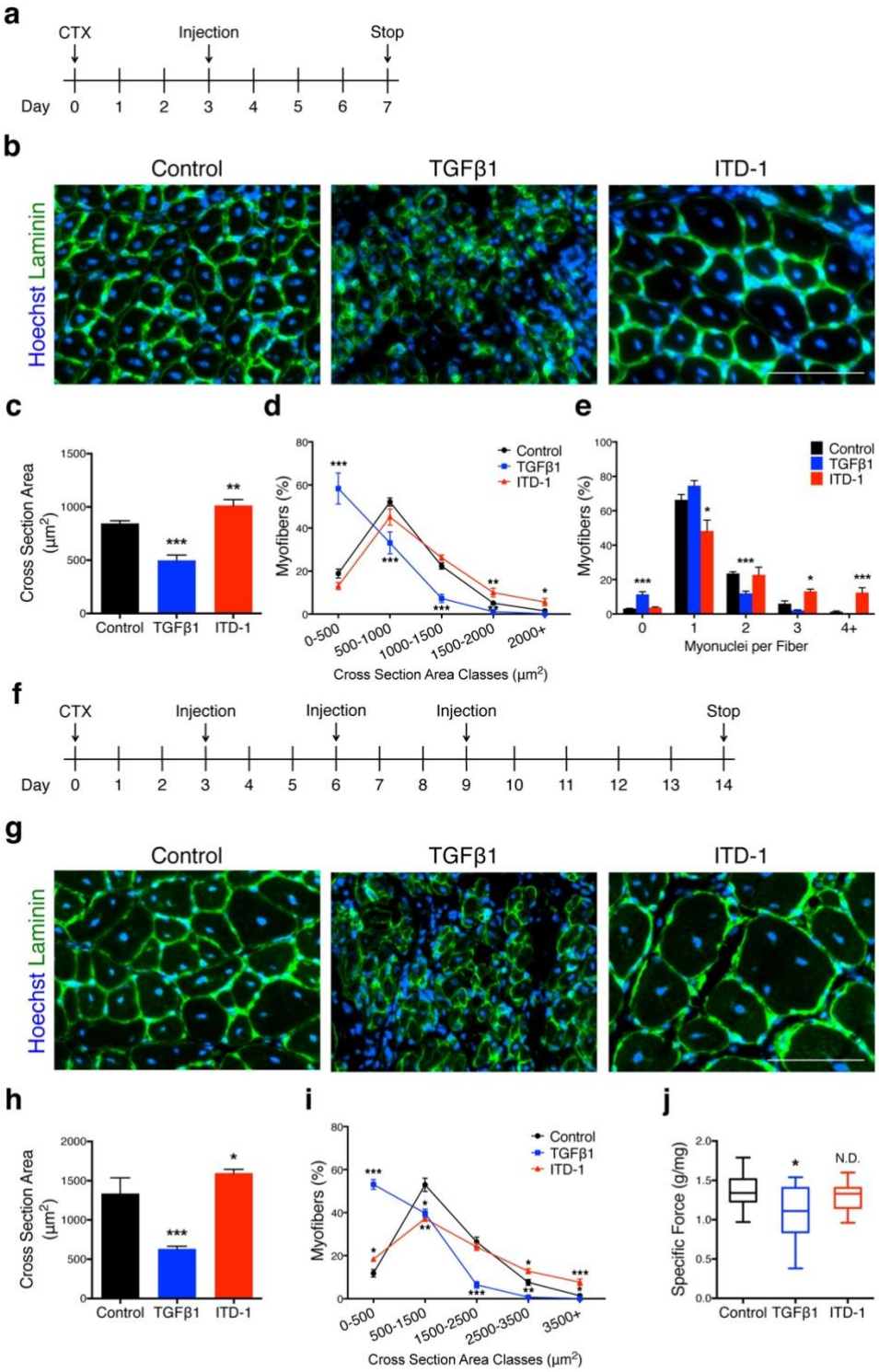
Day 7
DMSO
SB43
c
Tissue width (μm X 10)
Time (days)
d
DMSO
SB43
DMSO
SB43
Day 3
Day 3
Day 7
Day 7
e
Fiber diameter (μm)
Day 3
Day 7
DMSO SB43 DMSO SB43
f
Nuclei per fiber (#)
Day 3
Day 7
DMSO SB43 DMSO SB43
g
DMSO
SB43
h
Relative strength (%)
DMSO SB43

Figure 5 | TGFβ inhibition induces human myotube fusion in 3D culture resulting in increased microtissue strength.

a, Schematic representation (left) and timeline (right) of 3D human muscle cell experimental approach utilized in (b-h). Briefly, immortalized human myoblasts are suspended in a fibrin / reconstituted basement membrane protein scaffold and seeded into the bottom of a custom rubber 96-well plate culture device. A side view depicts the vertical posts across which the cells remodel the protein scaffold, align, and fuse to form a 3D human muscle microtissue (hMMT). For the first two days of culture (Day -1, Day -2), tissues are maintained in growth media (GM). On Day 0, GM is removed from wells and replaced with differentiation media (DM). TGFBR1 inhibitor SB-431542 (SB43, 10 μM) was included in the DM on Day 0 – Day 2 (orange arrowheads) of culture. **b,** Representative bright-field images of 3D hMMT culture over the time-course of differentiation treated with SB43 as compared to DMSO-treated control. White arrows demarcate the region of tissues that are assessed in (c). **c,** Line graph quantifying hMMT width over the time-course of differentiation in DMSO (grey line) or SB43 (black line) conditions. **d,** Representative confocal slices of hMMT cultures immunostained for sarcomeric α-actinin (green) on Days 3 and 7 of culture. **e-f,** Bar graph quantifying muscle fibre diameter **(e)** and average number of nuclei per fibre **(f)** at Days 3 and 7 of culture. **g,** Representative brightfield images of hMMTs. Micro-post position before (solid white line) and after (dashed white line) acetylcholine stimulation is represented. **h,** Bar graph quantifying relative strength of SB43-treated hMMTs compared to DMSO-treated hMMTs. Scale bars: **b,** 500μm, **d,** 50μm, **g,** 100μm. n = 3 biological replicates with at least 2 hMMTs replicates per experiment. A minimum of 30 microscopic images per culture condition was analyzed. Data are presented as mean ± s.e.m.. * P< 0.05 compared with Control (Unpaired two-tailed Student's t-test).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CTX
Injection
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TGFβ1
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Cross Section Area (μm²)
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d
Myofibers (%)
Control
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Cross Section Area Classes (μm²)
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0-500 500-1500 1500-2500 2500-3500 3500+
Cross Section Area Classes (μm²)
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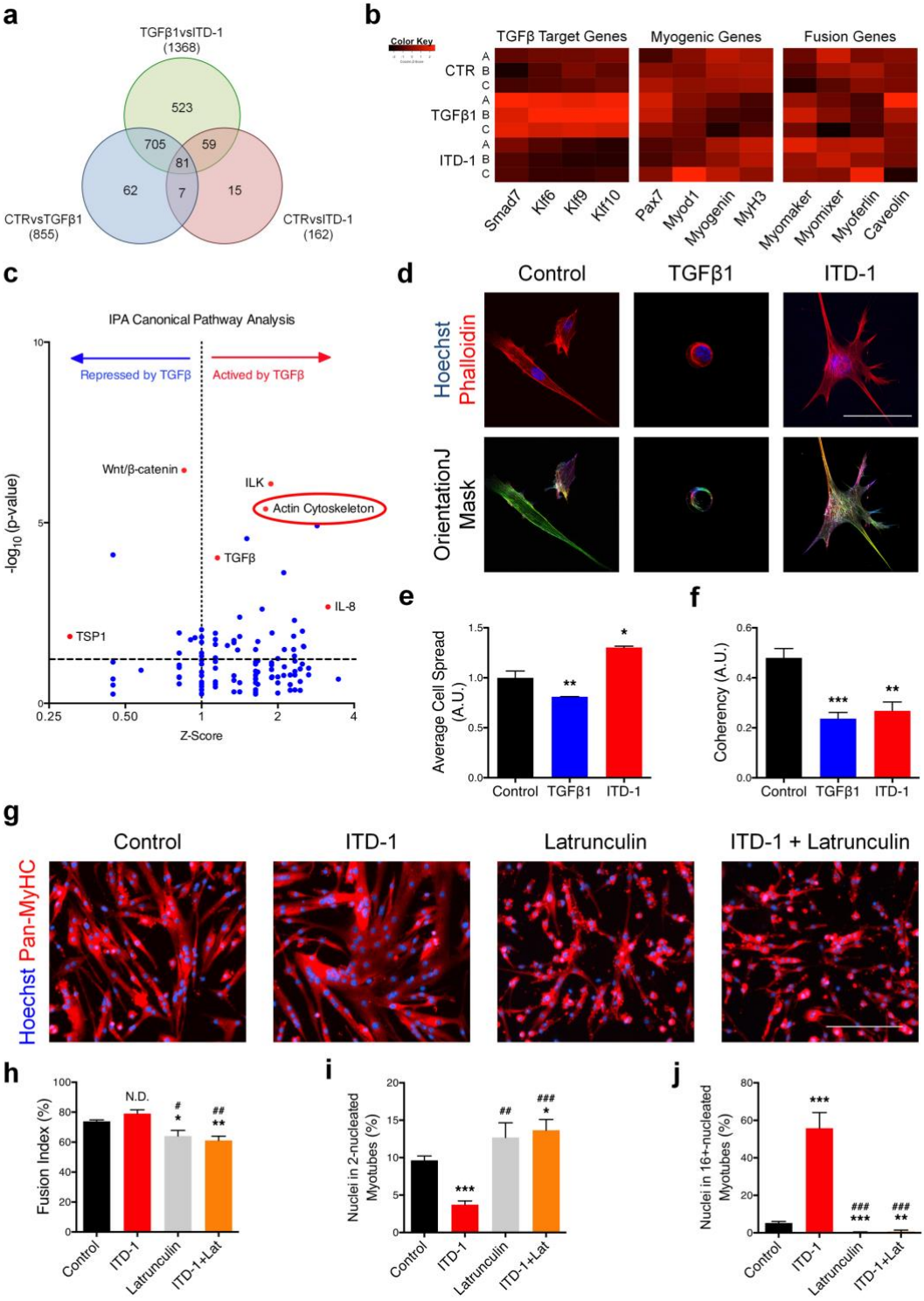
Control TGFβ1 ITD-1

Figure 6 | **TGFβ signaling regulates muscle cell fusion *in vivo*. a,** Experimental scheme. Adult murine *tibialis anterior* (TA) muscles were subjected to CTX injury and regenerating tissues were injected intramuscularly with either TGFβ1 proteins or ITD-1 compound 3 days after damage. **b,** Immunofluorescent staining for LAMININ of 7-days regenerating TA muscles. **c,** Quantification of myofibre size (cross sectional area, CSA). While the injection of TGFβ strongly reduces the fibres size, ITD-1 administration increases the CSA. **d,** Distribution of myofibre CSA. **e,** Distribution of myonuclei per fibre shows that the inhibition of TGFβ cascade leads to the formation of multi nucleated myofibres, while TGFβ activation reduces the number of myonuclei per fibres. **f,** Experimental scheme. Adult murine TA muscles were subjected to CTX injury and regenerating tissues were injected with either TGFβ proteins or ITD-1 compound 3, 6 and 9 days after damage. 14 days after injury, force measurements were performed, and TA muscles collected. **g,** Immunofluorescent staining for LAMININ of 14-days regenerating TA muscles. **h,** Quantification of myofibre size confirms the phenotypes observed at 7d.p.i.. **i,** Distribution of myofibre CSA. **j,** Specific force measurement of regenerating muscles. While TGFβ1-treated muscles are weaker compare to the control, ITD-1 injected muscles show no differences. Scale bars: **b, g,** 100μm. Data are presented as mean ± s.e.m. from at least three independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001, N.D.=Not significant, compared with Control (Unpaired two-tailed Student's t-test). Control represents mock-treated contralateral tibialis anterior muscle.


a
TGFβ1vsITD-1
(1368)
523
705
59
81
62
7
15
CTRvsTGFβ1
(855)
CTRvsITD-1
(162)
b
Color Key
TGFβ Target Genes
Myogenic Genes
Fusion Genes
CTR
TGFβ1
ITD-1
A
B
C
Smad7
Klf6
Klf9
Klf10
Pax7
Myod1
Myogenin
MyH3
Myomaker
Myomixer
Myoferlin
Caveolin
c
IPA Canonical Pathway Analysis
Repressed by TGFβ
Actived by TGFβ
-log10 (p-value)
Wnt/β-catenin
ILK
Actin Cytoskeleton
TGFβ
IL-8
TSP1
Z-Score
d
Control
TGFβ1
ITD-1
Hoechst
Phalloidin
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e
Average Cell Spread (A.U.)
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Control
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Latrunculin
ITD-1 + Latrunculin
Hoechst Pan-MyHC
h
Fusion Index (%)
N.D.
Control
ITD-1
Latrunculin
ITD-1+Lat
i
Nuclei in 2-nucleated Myotubes (%)
Control
ITD-1
Latrunculin
ITD-1+Lat
j
Nuclei in 16+-nucleated Myotubes (%)
Control
ITD-1
Latrunculin
ITD-1+Lat

Figure 7 | **Fusogenic actin remodeling is controlled by TGFβ signaling.** Transcriptomic analysis was performed on differentiated myocytes treated with either TGFBI or ITD1. **a,** Venn Diagram showing differentially expressed gene overlap across the three conditions. **b,** Heatmaps of TGFβ Target genes, myogenic genes and fusion genes. **c,** Volcano Plot showing the Ingenuity Pathway Analysis (IPA). Among the top modulated pathways, Actin Signaling Pathway is highlighted. **d,** Phalloidin staining of 1-day differentiated myocytes. These pictures were analyzed with OrientationJ (ImageJ Plug-in) to obtain a color-coded orientation mask. **e,** Average cell spread quantification. TGFβ1 treatment reduces cell size; ITD-1 promotes cell spreading. **f,** Quantification of orientation coherency of the Actin fibres. Both treatments reduce coherency compare to the control. **g,** Immunofluorescent staining for all the Mysosin Heavy Chain isoforms (Pan-MyHC) of re-plated primary myotubes cultured for 48 hours with ITD-1, Latrunculin, or both. **h,** Fusion index of re-plated myotubes shows that Latrunculin significantly reduces the parameter when administrated. **i,** Percentage of nuclei in the smallest myotube classes. ITD-1-treated myotubes are characterized by less nuclei per myotube, while Latrunculin increases the percentage of nuclei in small myotubes when administrated alone or together with ITD-1. **j,** Percentage of nuclei in the biggest myotube classes. ITD-1 strongly increases the number of nuclei in big myotubes, but Latrunculin blunts this effect, reducing the percentage. Scale bars: **d,** 40µm. **g,** 200µm. Data are presented as mean ± s.e.m. from at least three independent experiments. Coherency was calculated from at least 150 cells per condition. **P*<0.05, ***P*<0.01, ****P*<0.001, compared with Control. #*P*<0.05, ##*P*<0.01, ###*P*<0.001, compared with ITD-1 (Unpaired two-tailed Student's t-test).

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End Notes

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Author Contributions: F.L.G conceived and designed the study. F.G. performed most of the experiments. A.T. performed western blotting experiments. L.G. performed RT-qPCR experiments. F.L.G performed cell culture experiments. B.C. conceived the live imaging experiments. A.D., M.E., and P.M.G. designed, performed, and interpreted human muscle cell culture experiments. D.G.G., and D.P.M. designed, performed, and interpreted the fibroblast experiment. F.G. and F.L.G. wrote the manuscript. All authors reviewed the manuscript.

Competing interests: The authors declare no competing interests.

Supplementary Information:

4 figures

2 tables

3 movies

Supplementary Information:

Methods

Mice

Wild-type mice used in this project were 2 to 5 months-old C57Bl6/N mice purchased from Janvier Laboratories. Experiments were performed at the Centre d'Expérimentation Fonctionnelle (UMS28) Animal Facility following the European regulations for animal care and handling. Experimental animal protocols were performed in accordance with the guidelines of the French Veterinary Department and approved by the Sorbonne Université Ethical Committee for Animal Experimentation. Cardiotoxin (CTX) injection in Tibialis Anterior (TA) muscle and hindlimb muscle dissection were performed following the protocol described in [47].

Skeletal Muscle Injury

Mice were anaesthetized by intraperitoneal injection of Ketamin at 0,1mg per gram body weight and Xylazin at 0,01mg per gram body weight diluted in saline solution. 30 ul of CTX (12 mM in saline, Latoxan) was injected into hindlimb TA muscles to induce injury, and mice were euthanized 0, 1, 2, 3, 4, 5, 7 or 14 days afterward. Recombinant mouse TGFβ1 (R&D Systems) was diluted in saline and 250 ng (25 ul) was injected into the TA every injection. ITD-1 compound was diluted in saline and 2000 ng (25 ul) was injected into the TA every injection. Muscles were freshly frozen in OCT Embedding Matrix compound (CellPath) and cut transversally at 10 um with a Leica cryostat.

In situ Physiological assay

Tibialis anterior muscles were evaluated by the measurement of in situ isometric muscle contraction in response to nerve stimulation. Mice were anaesthetized intraperitoneal injection of Ketamin at 0,1mg per gram body weight and Xylazin at 0,01mg per gram body weight diluted in saline solution. Feet were fixed with clamps to a platform and knees were immobilized using stainless steel pins. The distal tendons of muscles were attached to an isometric transducer (Harvard Bioscience) using a silk ligature. The sciatic nerves were proximally crushed and distally stimulated by bipolar silver electrode using supramaximal square wave pulses of 0.1ms duration. All data provided by the isometric transducer were

recorded and analysed on a microcomputer, using PowerLab system (4SP, AD Instruments). All isometric measurements were made at an initial length L0 (length at which maximal tension was obtained during the tetanus). Responses to tetanic stimulation (pulse frequency from 75 to 143Hz) were successively recorded. Maximal tetanic force was determined. Muscle masses were measured to calculate specific force.

Murine Cell Cultures

Skeletal muscle-derived primary myoblasts were isolated from wild-type mice using the Satellite Cell Isolation Kit MACS protocol (Miltenyi Biotec). Briefly, hindlimb muscles were dissected out, placed in a sterile Petri dish and minced to a pulp with curved scissor. The pulp was then incubated in a CollagenaseB/DispaseII/$CaCl^2$ solution at 37°C for 40 minutes with two trituration steps. The enzymes are then blocked by addition of Fetal Bovine Serum (FBS) and the muscle extract was treated with Red Blood Cell Lysis Solution to remove erythrocytes. After this step, magnetic labeling is performed by adding Buffer (5% BSA, 2mM EDTA, PBS) and Satellite Cell Isolation Kit (a mixture of antibodies specific for non-satellite cells conjugated with magnetic beads). Cell suspension is then poured into the column in the magnetic field. Unlabeled cells (satellite cells) flow through the column while magnetically labeled cells are retained within the column.

Satellite cells were resuspended in growth medium (Ham's F10 with 20% FBS, 1% penicillin/streptomycin and 2.5ng/ml of bFGF) and plated into a collagen-coated 60mm Petri dish. Cells were maintained in growth medium until cells reached 80% confluence. To induce myogenic differentiation and fusion, myoblasts were plated at different concentrations depending on the experimental design (5000, 20000 or 75000 cells/cm^2) onto matrigel coated plates in growth medium. Once adherent, cells were incubated in differentiation medium (DMEM with 2% Horse serum and 1% penicillin/streptomycin) for up to 3 days. For recombinant protein treatments, we used TGFβ1 (eBioscience), TGFβ2 (Biotechne) and TGFβ3 (Biotechne) administrated at a final concentration of 20ng/ml. ITD-1 (Tocris) compound was administrated at 5mM.

PDMS mold fabrication for 3D human muscle microtissues (hMMT)

To generate 3D human muscle microtissues (hMMTs) we employed a second-generation micro-molded device in a 96-well format made of polydimethylsiloxane (PDMS;

monomer/cross-linker ratio = 15: 1) in a single simple molding process. At the bottom of each well of the 96-well microfabricated device an oval shaped pool was designed with a vertical flexible PDMS post on each side of it. PDMS culture plates were sterilized via an autoclave. Just prior to use, wells were further sterilized by an overnight incubation with a 5% pluronic acid solution (100µL/well) at 4°C, which also served to create a non-adhesive surface to support tissue self-organization.

Human skeletal muscle microtissue (hMMT) culture

The 3D hMMTs were generated using human immortalized myoblast lines obtained from V. Mouly (AB1167 from fascia lata muscle of a healthy 20-year old male, AB1190 from paravertebral muscle of a healthy 16-year old male, and KM155 from thigh muscle of a healthy 25-year old male) [48]. Immortalized myoblasts were cultured in Skeletal Muscle Cell Basal Medium with Skeletal Muscle Cell Growth Medium Supplement Mix (PromoCell) supplemented with 20% FBS and 1% P/S. Myoblasts were harvested by trypsinization and resuspended (1.5 x 10^5 cells/tissue or 1.0 x 10^7 cells/mL) in a hydrogel mixture consisting of fibrinogen (4 mg/mL, 40% v/v; Sigma) and Geltrex (20% v/v; Thermo Fisher Scientific) in DMEM (40% v/v) in the absence of thrombin. Then, 0.2 unit of thrombin (Sigma) per each mg of fibrinogen was added just before seeding the cell-hydrogel mixture into the wells and left for 5 min in an incubator at 37°C to allow optimal fibrin gel formation. Subsequently, 200 µL of growth medium consisted of Skeletal Muscle Cell Growth Medium lacking supplement mix (Skeletal Muscle Cell Basal Medium; PromoCell) supplemented with 20% FBS, 1% P/S and 1.5 mg/mL 6-aminocaproic acid (ACA; Sigma) was added to each well. The hMMTs were cultured in the growth medium for two days, and then the medium was replaced to differentiation medium (DMEM supplemented with 2% horse serum, 1% P/S, and 10 µg/mL human recombinant insulin) containing 2 mg/mL ACA to induce differentiation. SB43 (Sigma) was added at the final concentration of 10 µM into the differentiation medium of hMMTs for Day 0 to Day 2 of differentiation, while an equivalent volume of DMSO was added to control samples. On Day 3 of differentiation, a final full media exchange was performed to remove SB43 or DMSO. Half of the culture medium was replaced every other day for the remaining differentiation period.

3D tissue compaction and tissue remodelling analysis

The effect of SB-43 treatment on hMMT compaction was evaluated by measuring tissues diameter as an indication for tissue self-organization and remodelling over differentiation period. Phase contrast 4x magnification images were captured over time using an inverted microscope (Olympus) to analyze 3D tissue compaction. In each image four width measurements were done across the length of the tissue using ImageJ software and the average diameter was calculated. The data was shown as absolute diameter change over time and the result was compared between SB43 treated and DMSO treated control tissues.

hMMT Myofibre width and nuclear index analyses

Myofibre width was measured using 40x magnification stack images of SB43 and DMSO treated hMMTs at Day 3 and 7 day of the differentiation period. Analysis of SAA immunostained images of 3D muscle tissues was facilitated by use of NIH ImageJ software. We analyzed a total of three to five images per tissue, to determine the diameter of each muscle fibre. Myofibres were only qualified for fibre diameter analysis if they were visible across the length of the stacked image. In this work, myotubes were defined as multinucleated cells comprising at least three fused nuclei. Three width measurements were done across the length of each qualified fibre to ensure that the thickest and the thinnest parts were included in the measurements, and subsequently the average fibre diameter per condition was calculated. To determine the average number of nuclei per fibres, we quantified the total number of Hoechst+ nuclei contained within each SAA+ muscle fibre in each hMMT culture condition.

hMMT relative force quantification

To evaluate the effect of TGFβ inhibition on the function of hMMTs, we evaluated hMMTs contraction in response to acetylcholine (ACh; Sigma) stimulation and tissue contraction. Briefly, ACh solution (in DMEM) was directly added (1mM final concentration) into the wells containing hMMTs after 7 days differentiation. We then captured short phase-contrast videos at 10x magnification to visualize the movement of the movement of the flexible PDMS posts. Post displacement was quantified using ImageJ software. Relative hMMTs strength data was evaluated by normalizing the post displacement of SB43 treated tissues to DMSO treated control tissues.

Myomaker and Myomerger-expressing 10T1/2 fibroblasts

Myomaker and Myomerger-expressing fibroblasts were used and described previously (Leikina et al., 2018). Myomaker and Myomerger transduced 10T1/2 fibroblasts were seeded in 8 -chamber Ibidi slides with a 3 x 10^3 cell density per well (Day 0). 8 hours of post-seeding (Day 0), Myomerger expression was induced by treating the cells with dox containing culture medium (1 μg/mL) and replaced every 24 hours. Each experimental chamber was treated with human TGFβ1 recombinant protein (20 ng/mL; ebioscience) as specified. 4 days after seeding, cells were fixed, and fusion was evaluated by analyzing the number of nuclei in GFP^+ cells. The experiment was performed three times in duplicate and at least 3 images per well was quantified.

RNA Extraction and Quantitative Real Time PCR (RT-qPCR)

Total RNA was isolated from cultured cells and TA muscles using TRIzol Reagent (Thermo Fisher) or Direct-zol RNA Kit (Zymo Research) according to the manufacturer's protocol. TA muscle tissue was destroyed using the MagNa Lyser System (Roche). RNA concentration was evaluated with Nanodrop. After subsequent DNAse treatment, cDNA was generated using High Capacity Reverse Transcription Kit (Life Technologies). cDNA was then used for quantitative PCR (qPCR) done with LightCycler 480 SYBR Green Master Mix (Roche) and run in LC480 for 40 cycles. Primers are reported in **Table 1**. All samples were duplicated and transcripts levels were normalized for a housekeeping gene relative abundance (TBP, transcription regulator).

Microarray and bioinformatics

The RNA from primary myoblasts were isolated using Direct-zol RNA Kit (Zymo Research) according to the manufacturer's protocol. After validation of the RNA quality with Bioanalyzer 2100 (using Agilent RNA6000 nano chip kit), 100ng of total RNA is reverse transcribed following the GeneChip® WT Plus Reagent Kit (Affymetrix). Briefly, the resulting double strand cDNA is used for *in vitro* transcription with T7 RNA polymerase (all these steps are included in the WT cDNA synthesis and amplification kit of Affymetrix). After purification according to Affymetrix protocol, 5.5ug of Sens Target DNA are fragmented and biotin labeled. After control of fragmentation using Bioanalyzer 2100, cDNA is then hybridized to GeneChip® MouseGene2.0ST (Affymetrix) at 45°C for 17 hours.

After overnight hybridization, chips are washed on the fluidic station FS450 following specific protocols (Affymetrix) and scanned using the GCS3000 7G. The scanned images are then analyzed with Expression Console software (Affymetrix) to obtain raw data (cel files) and metrics for Quality Controls. Data were normalized using RMA algorithm in Bioconductor with the custom CDF vs 22. Statistical analyses were carried out with the use of Partek® GS. First, variations in gene expression were analyzed using unsupervised hierarchical clustering and PCA to assess data from technical bias and outlier samples. To find differentially expressed genes, we applied a one-way ANOVA for each gene. Then, we used unadjusted p-value and fold changes to filter and select differentially expressed genes. In TGFβ1 or ITD-1-treated myocytes, the genes were selected with $p<0,05$ significance and at least 50% difference. Gene networks and canonical pathways representing key genes were identified using the curated Ingenuity Pathways Analysis ® (IPA) database.

Muscle Histology and Immunofluorescence

Cell cultures were fixed with 4% paraformaldehyde in PBS for 15 minutes, washed 3 times with PBS and permeabilized with 0.25% Triton-X in PBS for 10 minutes. Blocking step was performed using 4% Bovine Serum Albumin (BSA) for 45 minutes at room temperature. Cultures were then incubated with primary antibodies diluted in blocking solution (4% BSA) overnight at 4°C. After a quick wash with 0.1% Np40 in PBS, samples were washed with PBS twice. Cells were incubated with secondary antibodies for 45 minutes at room temperature. Secondary antibodies used were anti-Mouse IgG or anti-Rabbit IgG coupled with Alexa Fluor 488 or 546 dyes, from Life Technologies. F-Actin staining was performed using FITC-conjugated Phalloidin (Sigma) or SiR-Actin (Spiro Chrome). Following antibody staining, cultures were washed 3 times with PBS and nuclei were stained with Hoechst, before being analyzed with the EVOS FL Cell Imaging System microscope (Life Technologies) or with a Nikon Ti2 microscope equipped with a motorized stage and a Yokogawa CSU-W1 spinning disk head coupled with a Prime 95 sCMOS camera (Photometrics). hMMT samples were fixed with 4% formaldehyde (Alfa Aesar) for 15 min at room temperature (RT). Following three washes with PBS, samples were permeabilized and blocked with a blocking solution containing 10% goat serum (Life Technologies), 0.3% Triton (BioShop) in PBS for 30 min at RT. Samples were then incubated with a mouse anti sarcomeric α-actinin antibody (Sigma) diluted 1:800 in the blocking solution overnight at 4°C. After three washes with PBS, samples were then incubated

with goat anti-mouse IgG conjugated with Alexa-Fluor 488 secondary antibody (1:500; Invitrogen) and Hoechst 33342 (1:1000; Invitrogen) diluted in the blocking solution for 60 min at RT. Multiple confocal stacks through each tissue were captured at multiple randomized locations using an Olympus IX83 inverted confocal microscope equipped with FV-10 software.

Live Imaging

Fluorescent-labeled myoblast cultures were pre-differentiated 48 hours at a low density (5000 cells/cm^2) onto matrigel coated plates and and re-plated in Nunc Lab-Tek Chamber Slide system at a high density (75000 cells/cm^2) and cultured for about two more days. Cells were recorded for the last 40 hours of differentiation using a Nikon Ti2 microscope equipped with a motorized stage and a Yokogawa CSU-W1 spinning disk head coupled with a Prime 95 sCMOS camera (Photometrics). Specifically, for each condition and replica 4 fields at 20x magnification were recorded every 10 minutes.

Western Blot

Cells were lysed with RIPA buffer (Sigma-Aldrich) supplemented with Protease and Phosphatase Inhibitor (Thermo Fisher). If needed, cytoplasmic and nuclear proteins were separated using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher). Protein quantification was performed using BCA Protein Assay Kit (Thermo Fisher) and after were denatured and reduced incubating the samples with 2X Laemmli Sample Buffer (Santa Crus BioTechnology) for 30 minutes at room temperature. Equal amounts of proteins were loaded in SDS-PAGE gel NuPAGE™ 4-12% Bis-Tris Protein Gels (Thermo fisher) along with molecular weight marker. Load 10µg of total protein from cell lysate and transferred on nitrocellulose membranes (Bio-Rad Laboratories, Inc.). After blocking in 5% milk or BSA and 0.1% Tween-20/TBS, membranes were incubated with primary antibodies (Table 2) overnight and then with HRP-conjugated secondary antibodies for 1 hour. Specific signals were detected with a chemiluminescence system (GE Healthcare).

BrdU Assay

Cell cultures were grown at 20000 cells/cm^2 density in growth medium on collagen-coated plates. Cells were treated with TGFβ isoforms for 1 day and then incubated with BrdU for 40 minutes before fixing them with 4% PFA for 5 minutes at RT. After a brief wash in PBS, cells

were denaturated with 2M HCl for 30 minutes at 37°C. To neutralize the acid, 6 consecutive washes in PBS of 5 minutes each are performed. Cells were blocked with 2% Goat Serum, 0.2% Tween20 PBS for 30 minutes at 37°C. Cells were incubated with primary antibody **(Table 2)** for 2 hours at RT and then washed 3 times in PBS. Secondary antibody (anti-Rat IgG conjugated with Alexa Flour 546 from Life Technologies) was added and incubated for 45 minutes at RT. Before microscope observation, three washes with PBS and Hoechst staining were performed.

TUNEL Assay

Cell cultures were grown at 20000 cells/cm^2 density on collagen-coated plates and treated with TGFβ proteins for 1 day both in proliferating and in differentiating conditions. Cells were fixed in 4% PFA at RT for 20 minutes, washed three times with PBS and permeabilized with 0.1% TritonX-100, 0.1% Sodium Citrate PBS for 2 minutes on ice. After three washes in PBS, cultures were treated according to the protocol of In Situ Cell Death Detection Kit (Roche). Before observation, three washes with PBS and Hoechst staining were performed.

Scratch-Wound Assay

Primary myoblasts were plated at 30000 cells per cm^2 density on collagen-coated plates. When cells reached 80% confluence, we scratched the monolayer of cells in a straight line, washed with PBS few times and incubated the cells in growth medium with TGFβ1. Cells were stained by NucBlue Live ReadyProbes Reagent (Thermo Fisher) to ensure that all cells were removed within the scratch. After 24 hours, myoblasts were fixed with 4% PFA and the number of cells in the scar was counted manually.

Statistical Analysis

A minimum of 3 biological replicates was performed for the presented experiments. Error bars are standard errors. Statistical significance was assessed by the Student's t-test, using Microsoft Excel® and GraphPad Prism 5®. Differences were considered statistically significant at the $p<0.05$ level. For each sample, 4 images were taken with a 4X, 10X or 20x magnification depending on the experimental design. Cell quantification and analysis was performed using ImageJ®. Phalloidin staining (F-actin) images of single cells were analysed for filament coherency using the OrientationJ plugin for ImageJ [49].

Primer	Sequence
Tbp_Fwd	CCCCACAACTCTTCCATTCT
Tbp_Rev	GCAGGAGTGATAGGGGTCAT
Smad7_Fwd	GGCCGGATCTCAGGCATTC
Smad7_Rev	TTGGGTATCTGGAGTAAGGAGG
Klf10_Fwd	GTGACCGTCGGTTTATGAGGA
Klf10_Rev	AGCTTCTTGGTCGATAGGTGG
Myh3_Fwd	AAGGCCAAAAAGGCCATC
Myh3_Rev	TCTTCTGCTCCCCTTCCA
Tgfb1_Fwd	CTCCCGTGGCTTCTAGTGC
Tgfb1_Rev	GCCTTAGTTTGGACAGGATCTG
Tgfb2_Fwd	ATCGTCCGCTTTGATGTCTC
Tgfb2_Rev	GCTGGGTGGGAGATGTTAAG
Tgfb3_Fwd	AGGATCACCACAACCCACAC
Tgfb3_Rev	ATAAAGGGGGCGTACACAGC
Alk5_Fwd	TTATGAGAGAATGCTGGTATG
Alk5_Rev	AAGAGAGCAGAGTTCCCACGG
Tgfbr2_Fwd	CGGATGTGGAAATGGAAGCC
Tgfbr2_Rev	TGTCGCAAGTGGACAGTCTC

Table 1: RT-qPCR primers used in this study

Antibody target	Dilution	Supplier	Reference
Myogenin	1:25	Santa-Cruz Biotech.	Sc-52903
eMyHC	1:100	Santa-Cruz Biotech.	Sc-53091
Pan-MyHC	1:10	DSHB	MF-20
BrdU	1:100	Abcam	Ab6326
Vinculin	1:1000	Abcam	Ab18058
Histone3	1:1000	Cell Signaling Tech.	4499
SMAD2/3	1:5000	Cell Signaling Tech.	3102
Phospho-SMAD2/3	1:5000 (WB) 1:200 (IF)	Cell Signaling Tech.	8828
Phospho-SMAD3	1:200	Abcam	Ab52903

Table 2: Antibodies used in this study

Supplementary Figures:

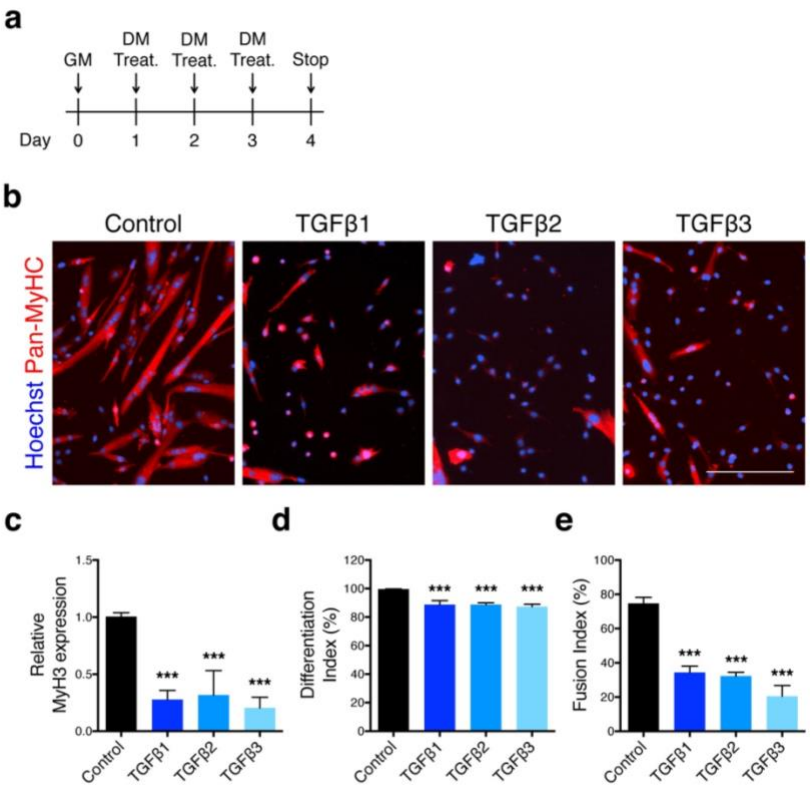

Figure S1 | TGFβ signaling effects on *in vitro* myogenic differentiation.
a, Experimental scheme. Primary myoblasts were induced to differentiate in medium containing TGFβ recombinant proteins. **b,** Immunofluorescent staining for Pan-MyHC of 3-days differentiated myotubes. **c,** qRT-PCR analysis for Myh3 (Embryonic Myosin Heavy Chain) transcript expression by of 3-days differentiated primary myoblasts indicates that stimulation of the pathway downregulates its expression compared to the control. **d,** Percentage of Pan-MyHC-expressing cells of 3-days differentiated primary myoblasts. **e,** Fusion index of 3-days differentiated primary myoblasts shows that TGFβ stimulation inhibits fusion. Scale bars: **c,** 1000µm. Data are presented as mean ± s.e.m. from at least three independent experiments. N.D.=Not significant, compared with Control (Unpaired two-tailed Student's t-test).

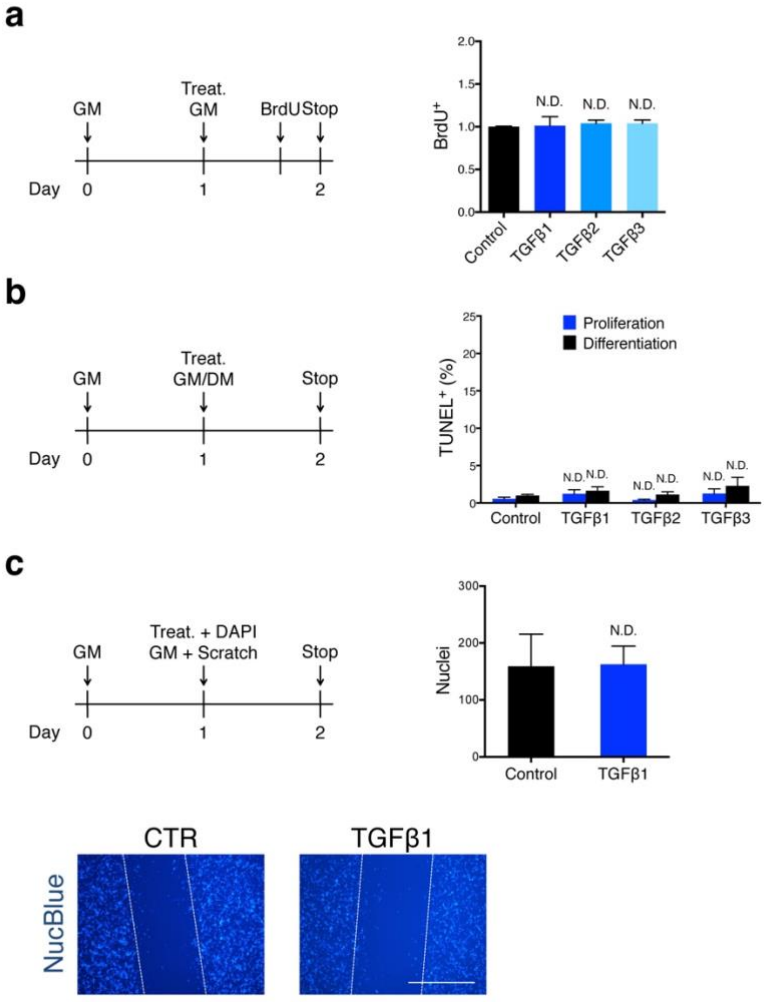

Figure S2 | **TGFβ signaling does not affect myoblast proliferation, death and motility.**
a, Primary myoblasts were treated with TGFβ1, 2 or 3 for 24h and incubated with BrdU for the last 40 minutes before fixation. Quantification of $BrdU^+$ cells shows no differences. **b,** Primary myoblasts were treated with TGFβ1, 2 or 3 for 24h in proliferating or differentiating conditions. $TUNEL^+$ cells were quantified, and no particular death rates were detected. **c,** Primary myoblasts were treated with TGFβ1, 2 or 3 for 24h in proliferating condition. When treated, cell layer was scratched and washed with PBS. Scratch-wound images were taken after 24 hours of treatment. Cells were stained with NucBlue. The quantification of nuclei within the scratch-wound reveals that motility is not affected. Scale bars: **b,** 200µm. Data are presented as mean ± s.e.m. from at least three independent experiments. ***P<0.001, compared with Control (Unpaired two-tailed Student's t-test).

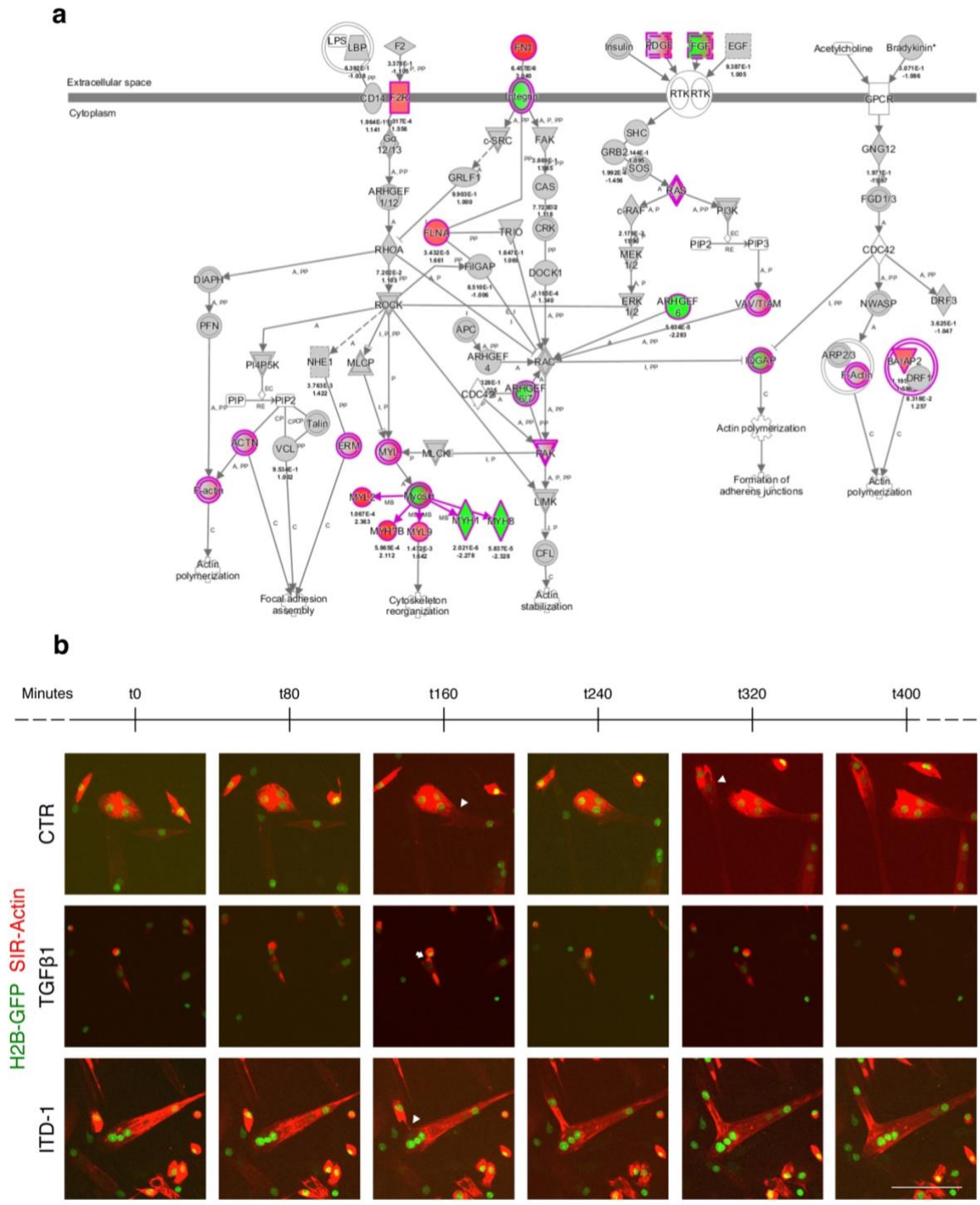

Figure S3 | Effects of TGFβ cascade on Actin signaling pathway.
a, Representative scheme of the Actin signaling pathway genes modulated by TGFβ cascade. **b,** Live-imaging frames of pre-differentiated myocytes expressing H2B-GFP and stained with SiR-Actin. Arrowheads indicate fusion events, arrow depicts cell-cell interaction. In control condition, fusion occurs linearly, while ITD-1 treatment allows perpendicular fusion. On the other hand, TGFβ1 allows cell-cell interactions, but blocks fusion. Scale bars: **b,** 200μm. Data are presented as mean ± s.e.m. from at least three independent experiments.

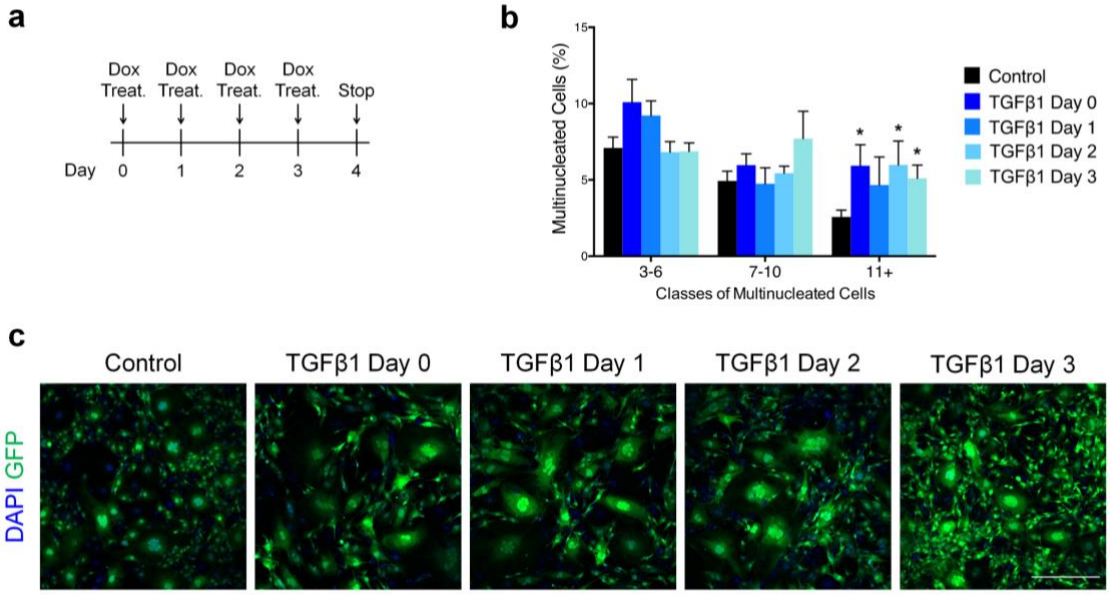

Figure S4 | TGFβ signaling exerts its effect independently from Myomaker and Myomerger. **a,** Experimental scheme. A dox-Inducible Myomaker- and Myomerger-expressing fibroblasts were used to test TGFβ1 effect on cell-cell fusion. Dox was administrated at day 0 and refreshed every day, while TGFβ1 either at day 0, 1, 2 or 3. **b,** Aggregation index of 4-days Myomaker and Myomerger-expressing fibroblasts showing no significant reduction of the fusion process when TGFβ1 is administrated compared to the control. **c,** GFP-myomaker-infected fibroblasts, transduced with dox-inducible myomerger, were visualized with fluorescent microscopy. TGFβ stimulation does not reduce the fusion process. Scale bars: **c,** 400µm. Data are presented as mean ± s.e.m. from at least three independent experiments. **P*<0.05 compared with Control (Unpaired two-tailed Student's t-test).

DISCUSSION

Together, the results obtained during my PhD delineate a specific role for TGFβ signalling cascade in the fusion of adult myoblasts to form myotubes. Combining both *in vitro* and *in vivo* approaches we described how muscle cell fusion is "kept in check" during muscle tissue repair. After having confirmed the negative role of TGFβ in myoblast differentiation, we specifically studied the effect of TGFβ activation and inhibition during syncytium formation, by carefully excluding any impact in proliferation, differentiation, cell death and motility. While TGFβ stimulation strongly reduces the fusion process, its inhibition leads to the generation of large syncytia *in vitro* and giant myofibres *in vivo*. Importantly, this enhanced fusion resulted aberrant as ITD-1-treated myotubes displayed multiple branches and abnormal shape, whereas *in vivo*, over-sized myofibres did not appeared to be stronger compared to mock-treated contralateral leg. Altogether, these experiments suggest that TGFβ signalling is an essential signal limiting muscle cell fusion and must be tightly regulated during myoblast fusion and muscle regeneration.

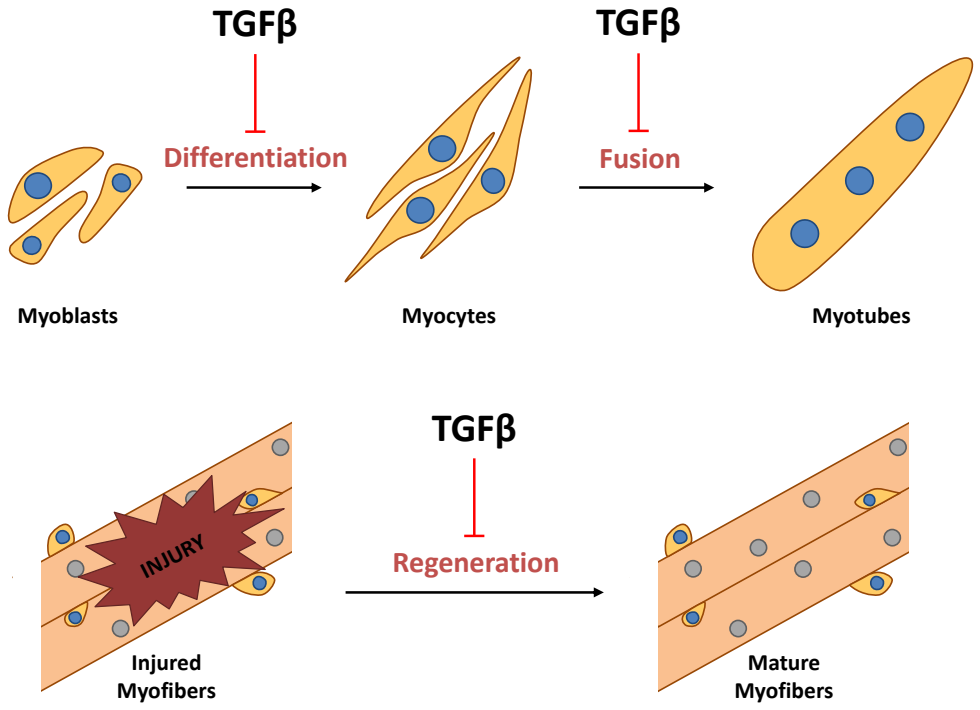

Figure 15. TGFβ Signalling regulates Muscle Cell Fusion. Besides the established negative role of TGFβ cascade on myogenic differentiation, TGFβ specifically regulates myoblast fusion. Moreover, TGFβ cascade plays a key role also during muscle regeneration *in vivo*, where a correct balance of the signalling pathway is essential to obtain a correct tissue repair process.

1. TGFβ signalling pathway state in skeletal muscle tissue

Although the potent role of TGFβ signalling has been a major area of interest in the last decades, its expression profile and cellular source have been poorly characterized. In order to gain insights into TGFβ dynamics in muscle cell and tissue repair, we first analysed the expression profiles of the three TGFβ isoform transcripts during myoblast differentiation and muscle regeneration. In both experiments, the three isoforms showed different expression profiles. During *in vitro* myogenesis, both TGFβ1 and 2 expression levels diminish with myoblast differentiation, while TGFβ3 expression augments as the cells differentiate. During the course of *in vivo* regeneration TGFβ1 expression is strongly induced at 3 days post-injury, TGFβ3 expression peaks later and finally TGFβ2 expression diminishes during the early phases of regeneration.

These divergent expression profiles appear complementary, providing during these time frames the expression of at least one of the TGFβ isoform. Indeed, during *itro*myogenesis, although the intensity of TGFβ cascade decreases as cell differentiate, we noticed a constant basal state activity of the pathway all through the three days of differentiation. Consequently, it is tempting to speculate that this sequential expression of ligands could be instrumental in preventing premature and unscheduled fusion events. Unfortunately, validating this hypothesis *in vivo* results technically impractical due to the complexity of the muscle system. However, in *in vitro* myoblast differentiation it would be interesting to silence tgfb3 gene (tgfb3 isoform expression is increasing during myoblast differentiation) and test if its endogenous expression alone is enough and sufficient to curb the fusion process.

Importantly, the contribution to these different expression profiles *in vivo* however remains obscure with this approach. Various TGFβ sources have been described in the skeletal muscle context: TGFβ1 and TGFβ3 are mainly expressed by pro-inflammatory macrophages during regeneration (Arnold et al., 2007; McLennan and Koishi, 1997), while TGFβ2 by activated satellite cells and myotubes (McLennan and Koishi, 1997). However, clarifying the complete TGFβ transcripts cellular sources in muscle tissue, as well as their protein localization, would be a resource to the community with therapeutic relevance for regenerative medicine for muscle. To this aim, combination of new technologies such as RNAScope® in situ hybridization (ACDBio) and Imaging Mass Cytometry™ (IMC™, FLUIDIGM) could represent an important

advancement in answering this intriguing question. These techniques are respectively able to characterize RNA and protein localization in situ on muscle section, and thus they could provide essential and definitive insights into TGFβ source and isoform localization in the resting skeletal muscle, and their dynamic adjustment and responses during tissue repair.

2. The complex regulation of the fusion process

Here, we identify a negative role for TGFβ in myoblast fusion. TGFβ recombinant protein administration during "regular" myoblast differentiation strongly reduced myotube formation. We then uncoupled muscle cell differentiation and fusion by treating pre-differentiated myocytes re-plated at high-density. In this experimental set up, even though all the cells were committed Myogenin+ myocytes, stimulation of TGFβ pathway was still reducing muscle cell fusion, suggesting that TGFβ acts on fusion independently from previous differentiating steps. Importantly, activation of TGFβ pathway did not alter proliferation rates or premature cell death in myoblasts, validating that the observed phenotype was not biased by prior events.

However, as intensively described in the introduction, fusion is a complex process composed of multiple steps, such as migration, cell-cell contact, adhesion, actin-remodelling and ultimately membrane merging. In our study, scratch-wound assay confirmed that neither the activation nor the inhibition of TGFβ cascade was affecting myoblast motility. Accordingly, in our live-imaging experiments myoblasts were clearly able to migrate and come in contact with surrounding myoblasts in all three conditions (Control, TGFβ1 and ITD-1). However, although we specifically documented cell-cell contact events between myoblast treated with TGFβ1 protein that did not result in actual cell fusion, a precise quantification of these events together with the frequency of cell-cell contacts would constitute a further evidence that the effect observed is fusion-specific and not depended on prior steps.

Cell-cell adhesion molecules play an essential role during myoblast fusion. In our transcriptomic analysis none of the main adhesion proteins involved in muscle cell fusion, such as Nephrin, Tetraspanins, M- and N-cadherins, were significantly affected by TGFβ modulation, thus we did not pursue this line of research. However, future studies of these adhesion molecules at a protein level are required to definitively exclude their involvement in TGFβ-mediated fusion regulation.

On the other hand, Ingenuity Pathway Analysis revealed that "Actin Cytoskeleton Signalling" is one of the most regulated pathways by TGFβ cascade. Due to the established role of Actin cytoskeleton in fusion we specifically analysed the F-Actin remodelling in myocytes stimulated with either TGFβ1 or ITD-1, finding out a significant disorganization of the cytoskeleton induced by both treatments. The critical nature of F-actin was further confirmed by the strong blockage of fusion mediated by Latrunculin administration. Interestingly, Latrunculin was able to reduce fusion also in the presence of ITD-1, thus indicating that TGFβ pathway acts upstream of Actin remodelling. However, the precise mechanism by which TGFβ cascade regulates Actin filament organization remains unknown. Microarray analysis identified several actin-related genes modulated by the TGFβ signalling, therefore, further studies on these potential candidates will need to be undertaken to gain a greater understanding on the molecular mechanism by which TGFβ cascade and Actin Cytoskeleton regulates muscle cell fusion.

Lastly, as described in the introduction, membrane fusion is governed by Myomaker and Myomerger. Experiments carried out by Doug Millay's group using a Dox-inducible Myomaker- and Myomerger-expressing fibroblast system revealed that TGFβ-driven effect on fusion is not conserved in non-muscle context and suggested that TGFβ cascade acts independently from Myomaker and Myomerger. This result supports our evidences obtained from the transcriptome analysis in which none of the two membrane proteins expression levels were significantly altered.

3. The broad impact of TGFβ cascade in skeletal muscle

In accordance with the literature, our results *in vivo* showed and confirmed the essential role of TGFβ pathway in skeletal muscle tissue. However, our compound-based approach cannot exclude that TGFβ or ITD-1 injection altered other cell populations and processes. In fact, our intramuscular injections impact most of the muscle-resident cells, as virtually all the cells display TGFβ receptors.

Thus, to gain a greater understanding on the TGFβ impact on muscle tissue, further research should consider also other cell populations with particular regard to cell types known to be impacted by this pathway, such as macrophages, endothelial cells, FAPs. A great advancement could be provided by single-cell transcriptomic analysis or single-cell mass cytometry; technologies that allow a stronger characterization of the cells affected by our treatments. Combining these high-depth analyses offers the opportunity to visualize dynamic changes in cell population relative abundances and the activation of the signalling pathway at the single-cell level (by using p-SMAD antibodies in a mass cytometry panel, or visualizing TGFβ target genes by single-cell RNA-seq).

Moreover, to overcome the broad impact of TGFβ on multiple cell types and focus on MuSCs, a genetic approach represents an appropriate solution. Conditional MuSC-specific gain-of-function or loss-of-function mutations of TGFβ-related genes will allow further detailed studies of the regeneration process. Genetic activation of TGFβ pathway can be achieved via multiple strategies, such as generation of constitutive active TGFβR1 (Gao et al., 2015), Smad7 loss-of-function mutation (Zhou et al., 2018) or Tgfb1 (2 or 3) gene knock-in (Muraoka-Cook et al., 2004). However, all these approaches are not completely specific. In fact, genetic modification of TGFβR1 or Smad7 (inhibitor of TGFβR1) will lead to an unavoidable alteration of other convergent TGFβ pathways, like Nodal, Activin and Myostatin, while TGFβ1 knock-in will result in an increased secretion of the TGFβ1 ligand that will affect also surrounding cells. Of note, this technical limitation derives from the fact that the only exclusive target for the prototypical TGFβ pathway is TGFβR2, which, being constitutive active, cannot be over-activated. On the contrary, genetic ablation of TGFβR2 represents a very specific tool for the inhibition of TGFβ signalling cascade. Conditional MuSC-specific loss-of-function mutation of TGFβR2 will allow to validate whether the inhibition of TGFβ pathway in MuSCs alone is

enough and sufficient to drive the hyper-fusion phenotype we obtained via ITD-1 injection. Additionally, specific knockout of TGFβR2 in muscle fibres could provide more insights into the role of TGFβ cascade in the "receiving" fusogenic cell.

4. Excessive fusion is not beneficial: "bigger" is not "stronger"

To gain a better understanding about the physiological state of TGFβ1 or ITD-1 treated muscles, we measured the force of the TA muscles. As expected, the strong negative impact on regeneration caused by intramuscular injection of TGFβ1 led to a significant reduction of the generated force. However, while ITD-1-treated muscles were composed of larger myofibers containing more myonuclei, the overall tissue did not result stronger compared to the mock-treated regenerated tissue. Altogether, these results confirm the importance of a proper balance of TGFβ signalling during regeneration, but also call into questions whether enhancing cell fusion improves muscle force generation.

First, we have to take into account that in our experimental setup, we measured muscle force at a single and relatively short time point following injury, thus it remains to be demonstrated whether inhibition of TGFβ for longer periods might result in increased force generation. However, achieving long-term TGFβ inhibition in skeletal muscle is problematic, as excessive intramuscular injections of ITD-1 imply undesirable damage of the TA and stress for the animal. For these reasons, to test this hypothesis genetic tools appear more appropriate, where MuSC-specific TGFβR2-null mice could be used to evaluate the physiological state of mutant muscles on long periods.

Nonetheless, the counterintuitive lack of increase in force exhibited by ITD-1-treated muscles can be explained in multiple ways. While surprising, it is not the first case reported; as mentioned in the introduction, Myostatin-null mouse is characterized by a remarked hypertrophy with no improvements in force (Amthor et al., 2007). Similarly, regenerated muscles of Rspo1-null mouse are composed of larger myofibres but do not show a commensurate increase of force compared to regenerated wild-types (Lacour et al., 2017). Importantly, Myostatin-null fibre enlargement is not accompanied by a proportionated increase in myonuclei, and this altered nuclei to cytoplasm volume ratio has been described

as pathological and leads to functional impairment (Matsakas et al., 2013). On the other hand, Rspo1-deficient and ITD-1-treated muscles show a commensurate increase in nuclear content. These results suggest that, even though enhanced fusion is still able to generate functional fibres, to obtain a physiological and beneficial effect on muscle force increasing the fibre size is not sufficient. Indeed, contractile properties of muscle do not rely solely on fibres, in fact, also tendons and connective tissue, as well as myofibril architecture, necessitate to growth accordingly to finally generate a stronger muscle.

Another potential explanation came from our results obtained *in vitro*, where we observed that inhibition of TGFβ cascade during myoblast differentiation and fusion leads to the formation of aberrant and branched myotubes. Whether this is indeed the case also *in vivo* is an important question for future research. In fact, branched myofibres in muscle pathologies are linked to a loss of muscle force. Thus, if confirmed also in ITD-1-treated muscles, could at least in part explain the lack of increase in force. Although we observed some aberrant fibre-into-fibre structures in ITD-1 injected muscles (data not shown), further analyses are required to validate this hypothesis, such as longitudinal muscle section or single fibre isolation.

5. Therapeutic applications and concluding remarks

Given the widespread activity of TGFβ cascade, it is not surprising that alteration of this signalling pathway is detrimental to human health. As such, mutations in genes coding for components of TGFβ pathway have been associated with several human pathologies, many concerning fibrotic disorders.

As previously described, TGFβ is a master regulator of both physiological and pathological fibrosis and the major coordinator of extracellular matrix deposition in skeletal muscle (Bernasconi et al., 1999; Yamazaki et al., 1994). In addition, elevated TGFβ signalling has been reported to promote the acquisition of fibrogenic traits in MuSCs (Pessina et al., 2015).

Similarly, the mouse model for Duchenne Muscular Dystrophy (also known as the mdx mouse) shows a chronic upregulation of TGFβ activity, which in turn alters muscle progenitor cell fate, promoting an alternate fibrotic identity (Biressi et al., 2014). Interestingly, although

characterized by an elevated TGFβ signalling activity, the mdx mouse model displays a remarkable hypertrophy, due to multiple rounds or degeneration/regeneration cycles. Moreover, Duchenne Muscular Dystrophy, as well as many other dystrophies, is characterized by branched myofibers, with morphological malformations and altered functionality (Lovering et al., 2009; Pichavant and Pavlath, 2014). These conflicting observations put emphasis on the importance of understanding whether the signalling pathways that regulate fusion are altered in diseased MuSCs. Future work should help to identify new mechanisms explaining muscle frailty in muscle dystrophies, and potentially new therapeutic targets since these pathways may be pharmacologically controlled.

Consequently, advancing our knowledge of signalling cascade regulating muscle cell fusion and myofibre and identifying whether these molecules are overexpressed or downregulated in dystrophic muscle will give insights on the mechanisms aggravating these pathologies, and also may open to strategies for muscle balance improvement through the formation of new myofibers.

ANNEX

Reported hereafter the revision “Wnt Signaling in Skeletal Muscle Development and Regeneration” written by Fabien Le Grand and me.

CHAPTER FIVE

Wnt Signaling in Skeletal Muscle Development and Regeneration

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Abstract

Wnt is a family of signaling molecules involved in embryogenesis, adult tissue repair, and cancer. They activate canonical and noncanonical Wnt signaling cascades in target cells. Several studies, within the last decades, showed that several Wnt ligands are involved in myogenesis and both canonical and noncanonical Wnt pathways regulate muscle formation and the maintenance of adult tissue homeostasis. In this review, we provide a comprehensive overview of the roles of Wnt signaling during muscle development and an updated description of Wnt functions during muscle repair. Lastly, we discuss the crosstalk between Wnt and TGFβ signaling pathways in skeletal muscle.

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1. WNT SIGNALING PATHWAYS

Wnt proteins are secreted growth factors belonging to a conserved family of cysteine-rich glycoproteins. The signaling pathways mediated by these ligands are essential to a large variety of biological processes, ranging from organ formation during embryogenesis to adult stem cell homeostasis.[1,2] The human genome harbors 19 Wnt genes, each with a different expression pattern and playing a different role.[3] Depending on the involvement of β-catenin, Wnt signaling can be classified as either canonical (β-catenin-dependent) or noncanonical (β-catenin-independent).

The canonical Wnt cascade begins with the binding of Wnt proteins to specific 7-transmembrane domain receptors belonging to the Frizzled (Fzd) family and to the low-density lipoprotein receptor-related protein (LRP5/6) acting as a coreceptor.[4] In unstimulated conditions, β-catenin is phosphorylated by a cytoplasmic destruction complex formed by Axin, Adenomatous Polyposis Coli (APC), Casein Kinase I (CKI) and Glycogen Synthase Kinase 3β (GSK3β). Phosphorylated β-catenin is then ubiquinated and degraded by the proteasome. Upon Wnt-Fzd-LRP5/6 interaction, Axin is recruited to the phosphorylated tail of LRP5/6 by the scaffold protein Dishevelled (Dvl). This relocalization disassembles the destruction complex leading to the inhibition of β-catenin ubiquitination. Under these conditions, β-catenin accumulates within the cytoplasm and translocates to the nucleus, where it associates to the transcription factors, TCF and LEF, to activate the transcription of target genes.

As mentioned earlier, Wnt ligands can also activate the noncanonical pathways, which are called Planar Cell Polarity (Wnt/PCP) pathway and calcium-dependent (Wnt/Ca^{2+}) pathway. In the Wnt/PCP, Wnt ligands binding to Fzd receptors activate the small GTPase proteins, Rho and Rac, which in turn activate Rho-associated protein kinase (ROCK) and c-Jun N-terminal kinases (JNKs). These two kinases trigger a phosphorylation cascade that ultimately results in cytoskeletal reorganization and/or in activation of target genes. In turn, in the Wnt/Ca^{2+}, the Wnt-Fzd interaction induces the production of inositol triphosphate (IP3) and diacylglycerol (DAG) by the phospholipase C (PLC). Accumulation of IP3 triggers the release of intracellular calcium (Ca^{2+}), which in turn activates calcineurin (CaN), Ca^{2+} and calmodulin-dependent kinase (CAMKII), and protein kinase C (PKC). As a result, the NFAT, CREB, and NF-κB transcriptional regulators are activated and induce the expression of target genes.

2. SKELETAL MUSCLE DEVELOPMENT

Skeletal muscle is a highly plastic and complex tissue. The muscles are fixed to the bones by the tendons and have the function to move the body in space under the control of the central nervous system. They are constituted of individual components known as muscle fibers (myofibers), cylindrical multinucleated cells containing contracting myofibrils. Myogenesis, the process of muscle formation, is the result of a well-orchestrated coordination of transcriptional cascades and signaling pathways that spatio-temporally direct cell proliferation and differentiation, migration, and morphological changes.[5] In vertebrates, the skeletal muscles of the body and limbs derive from somites, which are segments of the paraxial mesoderm (Fig. 1A). More precisely, myogenesis initiates in the dermomyotome, a transient embryonic structure localized in the dorsal part of the somite. In the dermomyotome, two regions can be distinguished: the epaxial domain, which gives rise to the back muscle, and the hypaxial domain, from which originates the body wall muscles, the diaphragm, and the limb muscles. During embryonic and fetal development, myogenesis occurs in different sequential phases. In the first phase, postmitotic dermomyotome border cells loose their epithelial organization and delaminate underneath the remaining dorsal dermomyotome (Fig. 1A). Here, myogenic progenitors (myoblasts) differentiate to form the myotome, a structure composed of aligned myocytes and myotubes that serve as a scaffold for the following waves of myogenesis.[6,7] In the second phase, called primary myogenesis, proliferating muscle progenitors migrate from the central region of the dermomyotome and differentiate and fuse with the first myofibers thereby giving rise to skeletal muscles (Fig. 1A).[8] Myogenesis in the limbs will follow a similar process with a delay in developmental time as muscle progenitor cells first have to migrate from the ventral lip of the dermomyotome to the limb bud anlagen where they will form the primary muscle fibers. Secondary myogenesis occurs during fetal growth, when proliferating muscle progenitors continuously generate fetal myoblasts that fuse to form secondary myofibers around this scaffold of first generation of muscle fibers.

Dermomyotomal cells express Pax3, a paired-box transcription factor essential for the induction of skeletal myogenesis.[9] Before delamination, $Pax3^{+}$ cells initiate the expression of the myogenic regulatory factors (MRFs), which are a family of bHLH transcription factors that orchestrates the myogenic lineage. The first MRF to be activated is Myf5 that in turn promotes the expression of the MRF Myod1. Together, they activate the last

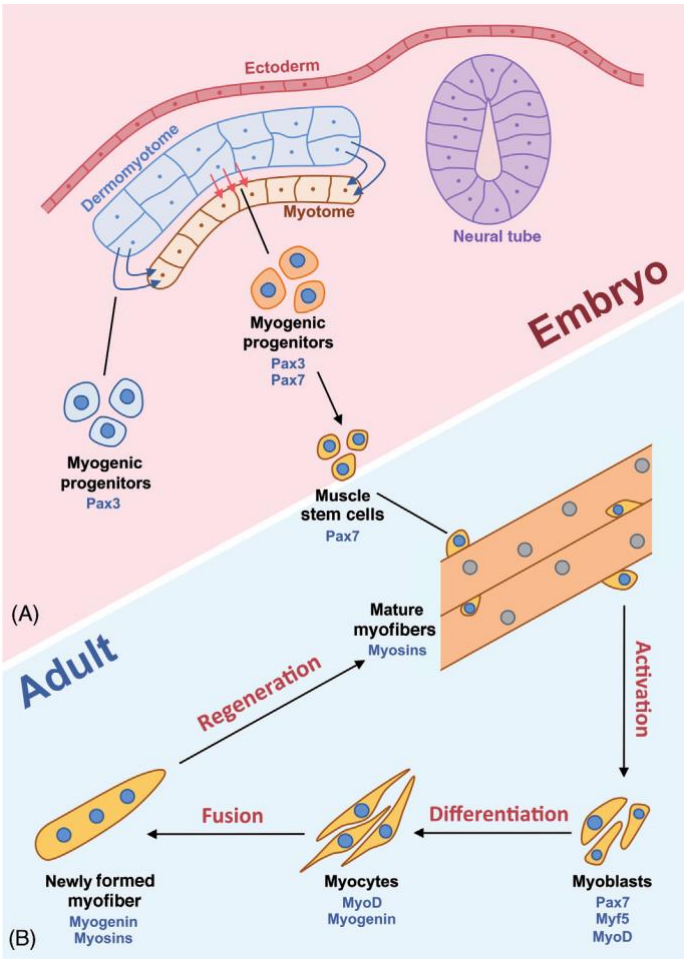

Fig. 1 Schematic representation of embryonic myogenesis and muscle regeneration. (A) Muscle formation is initiated in the dermomyotome and can be divided in sequential phases. During the first part, $Pax3^{+}$ cells delaminate from the extremities of the dermomyotome (blue arrows) and form the myotome. In the second phase (primary myogenesis), Pax3/Pax7 positive cells migrate from the central region of the dermomyotome to complete skeletal muscle formation (red arrows). At the end of fetal development, a group of Pax3/Pax7 positive cells give rise to adult muscle stem cells. (B) Satellite cells are located under the basal lamina that surrounds myofibers. After injury, satellite cells exit from quiescent and get activated giving rise to myoblasts. Myoblasts differentiate into myocytes, which in turn fuse to form new myofibers leading to muscle regeneration.

MRF Myogenin and this event is correlated with irreversible cell cycle exit and the activation of terminal differentiation. Another important factor during muscle formation is Pax7, a paralog of Pax3. Whereas Pax3 is activated in all myogenic progenitor cells, Pax7 expression is restricted to the central domain of the dermomyotome and its function is limited to primary, secondary, and adult myogenesis.[10] At the end of fetal development, $Pax3^{+}$/$Pax7^{+}$ myogenic progenitors locate underneath the basal lamina of myofibers, thereby giving rise to adult muscle stem cells (see Section 4).[11]

3. WNT SIGNALING IN SKELETAL MUSCLE DEVELOPMENT

As morphogens, Wnt proteins are involved in many embryonic processes, including somitogenesis, dermomyotome specification, and myogenesis (Fig. 2). Oscillations in canonical Wnt signaling in the presomitic mesoderm play a pivotal role in embryonic segmentation and somitogenesis ("Clock and Wavefront" mechanism) (Fig. 2A).[12] Consequently, correct activation of the Wnt signaling pathways is essential during the various steps of muscle formation. For instance, it has been shown that Wnt1 and Wnt3a are necessary for the induction of the dermomyotome (Fig. 2B).[13] By surgical ablation or by injection of protein-secreting cells in chick embryos, Marcelle and colleagues proved that the neural tube expresses Wnt1 and Wnt3a and that these two secreted factors mediate the formation of the dermomyotome.[13] Indeed, in Wnt1/Wnt3a mutant mouse embryos, the dermomyotome is reduced in size and lacks the median compartment, as shown by the reduction in the size of Pax3 and Myf5 expression domains.[14] In addition, implantation of Wnt1- and Wnt3a-secreting cells in chick embryos results in the expansion of the Pax3 expression domain and in the enlargement of the dermomyotome.[15] More recently, Hutcheson and colleagues demonstrated that canonical Wnt signaling is required for correct dermomyotome and myotome formation, as targeted β-catenin gene recombination in the Pax3 lineage abolishes the generation of embryonic myoblasts and results in a blockade of embryonic myogenesis.[16] Wnt proteins have also been shown to play a role in the maintenance of the epithelial organization of the dermomyotome.[17] Wnt6-mediated canonical cascade activation is required for the preservation of the epithelial structure of somites, while the absence of Wnt6 leads to the de-epithelialization (thus delamination) of the dermomyotome.[17]

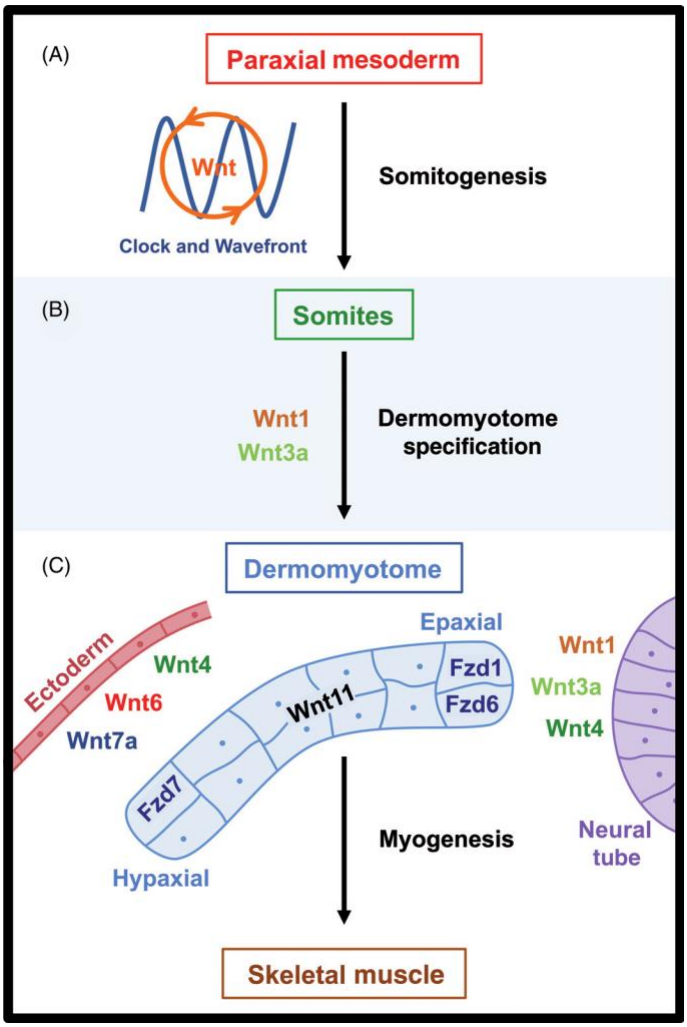

Fig. 2 Wnt functions from the paraxial mesoderm to skeletal muscle tissue. (A) Somites derive from the paraxial mesoderm and Wnt signaling plays a pivotal role in the "Clock and Wavefront" mechanism, a well-established model of somitogenesis. (B) After somite formation, Wnt1 and Wnt3a drive dermomyotome specification in the dorsal region of the somites. (C) Dermomyotome receives signals from the surrounding embryonic structures. Precisely, neural tube secretes Wnt1, Wnt3a, and Wnt4, while ectoderm expresses Wnt4, Wnt6, and Wnt7a. Dermomyotome epaxial region expresses Fzd1 and Fzd6, while in the hypaxial domain Fzd7 is present. Dermomyotome cells are characterized by Wnt11 expression. Together, all these Wnt ligands and receptors take part to embryonic myogenesis, leading to skeletal muscle formation.

The capacity of Wnt signaling to induce dermomyotome specification is strongly related to its ability to regulate the expression of Pax3, Pax7, and MRF genes. In explant cultures of chick segmental plate and somites, it has been shown that Wnt3a dramatically increases the levels of the two paired-box transcription factors and strongly enhances explant cell proliferation.[18] Subsequent in vivo experiments based on either Wnt electroporation or on engraftment of Wnt-secreting cells in chick embryos confirmed these results, demonstrating that Wnt1, Wnt3a, Wnt4, and Wnt6 regulate and maintain Pax3 and Pax7 expression in myogenic precursor cells, therefore controlling the number of dermomyotomal $Pax3^+/Pax7^+$ progenitors.[18–20]

As mentioned earlier, Wnt proteins are also able to modulate the expression of MRFs, thus playing an essential role during embryonic myogenesis (Fig. 2C).[21] Early studies showed that Wnt1 induces muscle formation through the preferential activation of Myf5, while Wnt6 or Wnt7a preferentially promote MyoD expression, which can occur independently from Myf5.[22] The identification of functional TCF/LEF binding sites in the Myf5 enhancers revealed that its Wnt1-dependent expression is mediated by the canonical Wnt cascade.[23] Conversely, Wnt7a promotes MyoD expression through the PKC-dependent noncanonical pathway, as proved by MyoD downregulation in the presence of PKC inhibitors in explant cultures.[24] Furthermore, it has been reported that Wnt1 and Wnt7a proteins can stimulate protein kinase A (PKA) signaling and CREB-mediated transcription of Pax3, Myf5, and MyoD in the somite, providing evidence for the involvement of multiple pathways.[25] Interestingly, a recent study demonstrated that migrating neural crest cells trigger β-catenin signaling and myogenesis in epithelial dermomyotome progenitor cells.[26] Mechanistically, Notch is activated in somitic cells by Delta-1 (Dll1)-expressing neural crest cells, and this leads to an epithelial to mesenchymal transition that allows the recruitment of β-catenin from the membrane independently of Wnt ligands. Once translocated into the nucleus, β-catenin acts as a transcriptional cofactor to activate Myf5 expression.[26] Most importantly, activation of the canonical Wnt cascade in dermomyotomal cells leads to the expression of Wnt11 in the medial border of the somites. Wnt11, then acts as a directional cue to orient myocyte elongation through the noncanonical Wnt/PCP pathway.[27] As such, the sequential action of the WNT/PCP and the WNT/β-catenin pathways is necessary for the formation of functional embryonic muscle fibers.

Myogenesis thus relies on a finely orchestrated spatial expression of Wnt proteins and Fzd receptors (Fig. 2C). Analysis of the expression pattern of

Wnt and Fzd isoforms in mouse or chick embryos revealed that Wnt1, Wnt3, and Wnt4 are specifically expressed in the dorsal neural tube,[28,29] while Wnt4, Wnt6, and Wnt7a are secreted from the dorsal ectoderm.[22,30] Fzd isoforms are also specifically distributed in the somites where they mediate different functions. Fzd1 and Fzd6 are expressed in the epaxial domain of the somite, where they promote Myf5 activation through the canonical Wnt cascade.[23] Whereas in the hypaxial region the receptor Fzd7 is present, which interacts with Wnt7a to activate MyoD expression.[31]

In summary, multiple Wnt ligands and receptors are expressed in different domains during embryonic myogenesis and all of them, together with many other signaling pathways, are part of a complex regulatory network that modulates and drives the formation of skeletal muscle.

4. SKELETAL MUSCLE REGENERATION

Adult skeletal muscle has a remarkable capacity to regenerate. Muscle regeneration (or adult myogenesis) relies on satellite cells (MuSCs). This adult muscle stem cell population is necessary for skeletal muscle growth and reconstruction following injury (Fig. 1B).[32] Satellite cells are engendered from fetal muscle progenitor cells at the end of embryonic development. They are characterized by the expression of Pax7 and no longer express Pax3.[33] Satellite cells are present in healthy muscle as mitotically quiescent cells[34] located in a niche between the basal lamina and the myofibers sarcolemma. In response to myofiber damage, satellite cells leave quiescence, become activated and proliferate to give rise to a transient amplifying population expressing Myf5 and MyoD (Fig. 1B). At this stage they are referred to as adult myoblasts. Akin to embryonic myogenesis, these myogenic precursors once they exit the cell cycle will express Myogenin and fuse with the damaged myofibers or with each other to form new myotubes. Then, additional myoblasts incorporate into the nascent myotubes to form new myofibers (Fig. 1B).[35] Generally, 3–4 weeks after injury, skeletal muscle returns to a normal morphological and histological condition and are characterized in mice by centrally located nuclei.[36]

In common with all adult stem cells, a hallmark of the muscle satellite cells is their ability to self-renew.[37] During regeneration, a correct balance between self-renewal and differentiation is essential. Symmetric divisions allow the maintenance of the satellite cell pool, while asymmetric expansion leads to the generation of myogenic progenitor cells.[38]

5. WNT SIGNALING IN SKELETAL MUSCLE REGENERATION

Unlike embryonic myogenesis, in which Wnt signaling is highly active, in mature skeletal muscle this pathway is poorly activated[39] (see Section 6). In contrast, early studies showed that some Wnts are expressed in freshly isolated myofibers and in uninjured muscles.[40,41] Even though Wnt expression in uninjured adult muscle is still unclear, its activation during muscle regeneration is well established (Fig. 3).[40–43] After injury, many Wnt

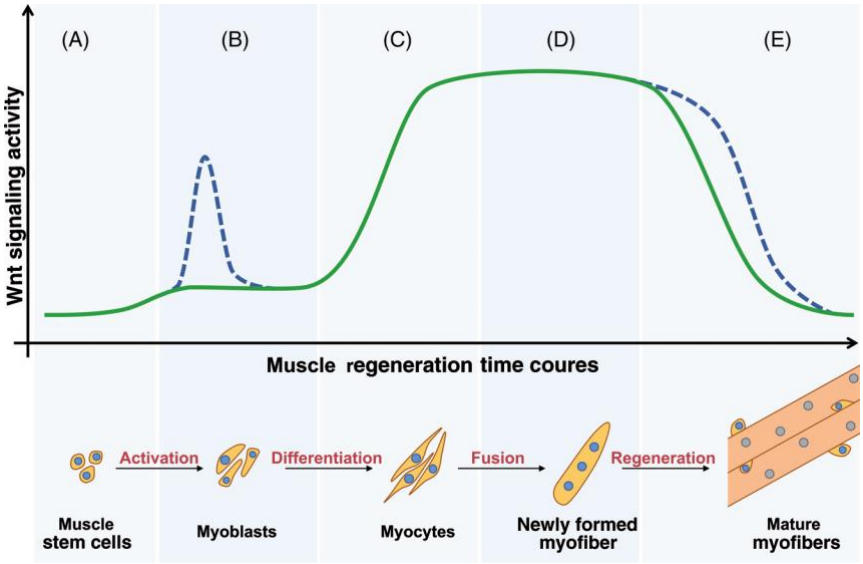

Fig. 3 Wnt signaling activity during muscle regeneration. (A) Wnt signaling (green line) is inactive in quiescent satellite cells and Adenomatous Polyposis Coli protein is essential to limit this signaling and to allow proper cell cycle entry.[43] (B) After satellite cell activation, Wnt7a (blue dashed line) stimulates symmetric division of satellite cells through the Planar Cell Polarity pathway,[44] while Axin1 inhibits Wnt/β-catenin cascade to maintain muscle progenitor proliferation.[46] (C) Canonical Wnt signaling inhibits Notch pathway causing the satellite cell transition from proliferation phase to differentiation phase.[42] (D) Wnt signaling promotes myoblast differentiation and fusion by inducing myogenic gene expression via Barx2 activation,[47] stimulating Follistatin,[48] inhibiting myostatin[49] and translocating the negative regulator Setdb1 into the cytoplasm.[50] (E) In mature myofibers Wnt7a regulates Akt/mTOR pathway increasing muscle size and strength.[51] Lastly, Wnt activity returns at low levels.[39] Overall, a timely regulation of Wnt/β-catenin signaling is essential for muscle regeneration.[45]

ligands (such as Wnt3a, Wnt5a, Wnt5b, Wnt7a, and Wnt7b) are expressed and secreted from multiple sources but in particular myofibers and activated satellite cells. Both canonical and noncanonical pathways have been implicated in muscle repair and accumulating evidences indicate that Wnt signaling is more particularly involved in the regulation of satellite cell differentiation and self-renewal.[42,44,45]

One of the first indications for a potential role of canonical Wnt signaling in adult muscle cell differentiation was suggested by the in vitro studies on immortalized cell lines, in which the Wnt1/β-catenin cascade and/or GSK3 inhibition were able to enhance myogenic differentiation.[52,53]

The involvement of the canonical Wnt cascade during satellite cell differentiation was further confirmed in mouse.[42] In particular, a switch from high Notch signaling levels to high Wnt/β-catenin signaling levels is essential for the transition of satellite cell descendants from proliferation to differentiation (Fig. 3C).[42] Indeed, exogenous activation of the canonical pathway in regenerating muscles stimulates muscle lineage progression in proliferating myoblasts and leads to premature differentiation (accelerated commitment of proliferating cells to postmitotic myocytes). Therefore a correct timing of Wnt signaling activation is fundamental to obtain correct tissue repair. In agreement with this notion, recent studies have shown that maintenance of low β-catenin activity is crucial during the early phases of muscle regeneration (Fig. 3B).[43,46] In vivo β-catenin overactivation via APC genetic depletion, specifically in satellite cells, results in complete failure of muscle tissue repair. In this context, we have shown that the massive upregulation of β-catenin signaling in APC-null satellite cells abolishes cell cycle entry and results in programmed cell death. Thus APC activity is essential to dampen Wnt signaling and to allow satellite cells to exit quiescence and proliferate (Fig. 3A).[43] Furthermore, siRNA-mediated knockdown of Axin1 in satellite cells leads to reduced proliferation and precocious myotube formation in vitro, indicating that Axin1, by limiting Wnt signaling, acts to maintain satellite cell expansion and prevent premature differentiation.[46] Lastly, our lab recently demonstrated that an adequate level of Wnt/β-catenin signaling is fundamental for controlling muscle progenitor function during muscle regeneration (Fig. 3).[45] In this study, satellite cell-specific β-catenin loss-of-function and gain-of-function mutations resulted in the alteration of the regeneration process in vivo. Satellite cell descendants deprived of canonical Wnt signaling differentiate less efficiently (reduced generation of Myogenin^{+} cells), while constitutively active Wnt/β-catenin cascade leads to precocious growth arrest and premature differentiation. As a

result, muscle regeneration is impaired in both conditional mutant mice. In conclusion, these studies suggest that a correct timing of activation together with a proper level of canonical Wnt pathway is crucial during muscle regeneration (Fig. 3).

At the molecular level, how Wnt signaling regulates muscle repair remains to be fully elucidated. Early studies on immortalized cell lines proposed that canonical Wnt signaling regulates MRF activity during myogenic differentiation,[54,55] but more recent papers have suggested novel and alternative mechanisms through which Wnt/β-catenin pathway controls muscle regeneration (Fig. 3D).[47,50] In differentiating myoblasts, Wnt signaling has been shown to be involved in the inhibition of Pax7 protein expression (negative regulator of differentiation) and in the activation and stabilization of Barx2, a homeobox protein that promotes myogenic differentiation gene expression via its association with MRFs. Barx2 also positively regulates Axin2 activity, suggesting the presence of a negative feedback loop modulating the Wnt cascade (Fig. 3D).[47] Finally, Beyer and colleagues showed that the histone lysine methyltransferase Setdb1 is a potent inhibitor of terminal differentiation and its activity is Wnt/β-catenin-dependent. Indeed, canonical Wnt signaling regulates satellite cell fate by driving the nuclear export of this chromatin modifier and this subcellular relocalization allows myoblast differentiation (Fig. 3D).[50] It should be noted that the promotion of myogenic differentiation driven by Wnt is also regulated by BCL9, an important mediator of β-catenin nuclear localization.[56] Loss of BCL9 (and its homologous BCL9-2) reduces canonical Wnt signaling activation and consequently impairs myogenic progenitor differentiation both in vitro and in vivo. Together, these results confirm not only the essential role of the Wnt/β-catenin cascade during muscle regeneration, but also the complexity of the regulatory network underlying tissue repair.

As mentioned earlier, noncanonical Wnt pathways are involved in adult myogenesis. In regenerating muscles, Wnt7a is expressed by newly formed regenerated myofibers and acts through the PCP pathway to regulate satellite cell self-renewal (Fig. 3B).[44] Specifically, Wnt7a interacts with its receptor Fzd7 and causes the redistribution of Vangl2 protein (an essential regulator of PCP pathway) on opposite poles of dividing satellite cells. This polarization induces satellite cell symmetric expansion and thus regulates self-renewed satellite cell numbers at the end of tissue regeneration. In addition, a recent work proposed the involvement of fibronectin (FN) and its receptor Syndecan-4 (Sdc4) in this process, expanding our knowledge about the molecular mechanism underlying satellite cell pool regulation.[57]

Sdc4 is a plasma membrane proteoglycan expressed by satellite cells and acts as Fzd7 coreceptor.[58] Following their activation, satellite cells express FN, which binds the Fzd7/Sdc4 receptor complex to potentiate Wnt7a signaling and thus promoting satellite cell symmetrical expansion. Wnt7a-mediated PCP pathway participates also to the modulation of satellite cell and myoblast motility.[59] By impacting cytoskeletal organization through activation of the small GTPase Rac1, Wnt7a enhances muscle stem cell migration and markedly improves cell engraftment in vivo. Moreover, Wnt7a has been implicated in the regulation of myofiber size and strength independently of the PCP pathway (Fig. 3E).[51] In postmitotic myotubes and myofibers, Wnt7a is able to activate the AKT/mTOR pathway leading to muscle hypertrophy and improved force generation. Taken together, these studies suggest that Wnt7a could have a potential therapeutic application. Further work is required to investigate the potential of Wnt7a as a biological protein that could be used to stimulate intrinsic repair in muscle-wasting diseases by coupling enhancement of satellite cell self-renewal and myofiber hypertrophy.

A family of canonical Wnt signaling activators called R-spondins (Rspo1, 2, 3, and 4) has recently been implicated in the regulation of adult myogenesis. In the myogenic cell line C2C12, R-spondin-dependent activation of the canonical Wnt cascade leads to the stimulation of myoblast differentiation and fusion.[60] Rspo2 induces the Wnt/β-catenin pathway, which in turn positively modulates the expression of the TGFβ antagonist Follistatin (Fst) (see Section 7).[61] In vivo, *Rspo1-null* mice exhibit a transient delay in the first phases of regeneration suggesting that Rspo1 is required during muscle repair.[62] Indeed, Rspo1 promotes myogenic progenitor cell differentiation through the Wnt/β-catenin cascade, but it also dampens the noncanonical PCP pathway specifically in differentiating myocytes. As a result of a perturbed equilibrium between these two Wnt signaling cascades, Rspo1-deficient myoblasts fuse with a better efficiency both in vitro and in vivo. Together, these data indicate that Rspo1 acts on both Wnt pathways and thus controls the balance between canonical and noncanonical signaling in differentiating muscle cells.

In conclusion, we are just beginning to understand the pivotal role of Wnt signaling during muscle regeneration. However, many questions, such as the spatio-temporal expression in uninjured myofibers, the precise molecular mechanism, and the molecular pathways that control the interplay between canonical and noncanonical Wnt signaling, remain unsolved.

6. CONTROL OF MYOFIBER TYPING BY WNT SIGNALING

Adult skeletal muscles in vertebrates are composed of different types of muscle fibers with distinct contraction speeds and metabolic profiles ranging from slow/oxidative to fast/glycolytic. There are four fiber types in mammalian muscle, one slow (type 1) and three fast (types 2A, 2X and 2B), each characterized by the expression of one specific myosin heavy chain isoform (MyHC). The Wnt signaling cascades have been shown to control the genesis of this fiber-type heterogeneity during development.[16,63–65]

The first indications of this role were reported in 2003, when Anakwe and colleagues showed that Wnt ligands control muscle fiber typing during avian wing development.[63] They showed that Wnt5 stimulation increases the number of slow and reduces the number of fast myofibers respectively, while Wnt11 promotes fast-type fiber formation. Another Wnt ligand involved in fiber-type specification is Wnt4, which has been shown to increase the number of fast MyHC fibers in the chick limb bud.[65] In contrast, Hutcheson and colleagues clearly demonstrated that β-catenin intrinsically regulates the formation of slow myofibers during fetal mouse development. Consequently, β-catenin gene recombination in Pax7-expressing muscle progenitor cells led to a decreased number of myofibers expressing slow MyHC, while most fetal myofibers expressed type 1 MyHC following targeted β-catenin gain-of-function.[16] Finally, a recent paper has shown that the downstream component of Wnt signaling pathway, TCF4, controls myofiber type by intrinsically regulating MyHC isoform expression in myogenic progenitors.[64] Moreover, ablation of TCF4-positive fibroblasts in fetal mouse myogenesis leads to a reduction of slow myofiber numbers. Together, these results indicate that Wnt signaling plays a role in fiber type determination, and also the presence of intrinsic and extrinsic mechanisms that drive myofiber diversification during development.

Intriguingly, Wnt signaling pathways have not been precisely linked to adult myofiber typing maintenance in adulthood. As such, distinct canonical Wnt activation domains have been proposed in healthy adult myofibers. By using a TCF-LacZ reporter mouse, Wnt signaling was detected exclusively in slow myofibers,[39] while the usage of Axin2-LacZ reporter mice showed that the Wnt cascade was active in fast myofibers only.[66] These conflicting results might be due to the different sensibilities of the two reporter alleles;

however, further studies are required to accurately define the Wnt signaling state in uninjured adult myofibers.

7. THE CROSSTALK BETWEEN WNT AND TGFβ SIGNALING IN SKELETAL MUSCLE

The transforming growth factor beta (TGFβ) superfamily is comprised of over 30 secreted factors, including TGFβ isoforms (TGFβ1, 2, and 3), bone morphogenetic proteins (BMPs), activins and myostatin. This cytokine family is ubiquitously expressed in different tissues and it plays a fundamental role in embryonic and adult myogenesis. The strongest regulator of skeletal muscle phenotype is myostatin, a potent inhibitor of muscle tissue growth and mass.[67] Other members of the TGFβ family are also essential in driving embryonic myogenesis. For instance, TGFβ1 has been shown to have an inhibitory effect on myoblast differentiation,[68] while BMP signaling is a positive regulator of muscle mass.[69] As such, myostatin negatively regulates myoblast proliferation and differentiation[70] and TGFβ1 inhibits the myogenic differentiation program at a gene expression level.[71] In contrast, modulation of TGFβ signaling using genetic approaches results in the alteration of satellite cell self-renewal and myoblast differentiation.[72,73] Taken together, these conflicting results indicate that the TGFβ signaling pathway has generally a negative role on muscle regeneration, but is still essential.

Even though the roles of Wnt and TGFβ signaling pathways during embryonic development have been intensively studied, the analysis of a potential interaction between these two pathways in embryonic myogenesis has not been fully addressed. Kuroda and colleagues showed that, during muscle development, the canonical Wnt cascade promotes slow myofiber formation by activating BMP4 expression. Nevertheless, further studies are required to properly dissect the interplay between Wnt and TGFβ cascades in the embryo.[39]

In contrast, multiple interactions between the two pathways have been described during adult myogenesis. In the C2C12 cell line, it has been reported that Wnt4 antagonizes myostatin-mediated phosphorylation of Smad2, thus promoting myoblast proliferation and differentiation.[65] Subsequent experiments confirmed this evidence and showed that Wnt4 overexpression in satellite cell-derived primary myoblasts enhances muscle cell fusion and myotube size. By acting through canonical signaling, Wnt4 limits myostatin function leading to Pax7-downregulation and MRF-

upregulation (Fig. 3D).[49] An early study using C2C12 showed that myostatin modulates myoblast proliferation state by repressing Wnt4 expression.[74] Together, these experiments suggest a potential dynamic and reciprocal interplay between both pathways, but further studies are required to explore this hypothesis.[74] Wnt4 has also been described to interact with BMP signaling, but in this case in a β-catenin-independent manner. Indeed, noncanonical Wnt4 signaling can potentiate the BMP cascade leading to a negative regulation of myogenic differentiation.[75] Therefore additional analyses are needed to elucidate this dual role of canonical and noncanonical Wnt4 activation. Moreover, recent studies have shown that the Wnt/β-catenin cascade stimulates the expression of the TGFβ antagonist Fst, providing novel evidence of Wnt-TGFβ interaction in skeletal muscle cells.[48,61] Fst is known to neutralize several TGFβ members and it can positively regulate myogenic differentiation in a Wnt-dependent manner both in vitro and in vivo. In particular, Wnt signaling triggers the expression of Myogenin, which in turn binds to a regulatory E-Box element upstream of the Fst gene, leading to Fst expression and premature differentiation (Fig. 3D).[48] In addition, Rspo2 has also been reported to promote Fst transcription through the activation of the canonical Wnt pathway.[61] However, the mechanism by which Fst exerts its positive regulation on muscle stem cell differentiation remains unclear.

Finally, transcriptome analysis of primary myoblast cultures has revealed that TGFβ2 and 3 transcription are under the control of canonical Wnt signaling.[45] In fact, in regenerating muscles Wnt/β-catenin cascade activates TGFβ signaling and this step is crucial for proper muscle repair. As mentioned earlier, in vivo β-catenin conditional gene disruption in satellite cells results in perturbed myogenic differentiation, but a wild-type phenotype can be restored via exogenous TGFβ2 injection in injured mutant muscles, suggesting a positive role for TGFβ2 on myoblast differentiation. As the TGFβ pathway has generally been described as a negative regulator of adult myogenesis and is a well-established pro-fibrotic factor, this study calls into question the negative role of TGFβ signaling in muscle regeneration. At this point, an interesting hypothesis is that TGFβ, downstream of canonical Wnt signaling, may be required to instruct cell cycle arrest in proliferating muscle cells, as a prerequisite for terminal differentiation.

Together, these multiple interactions between Wnt and TGFβ signaling during adult myogenesis represent a clear indication that an elaborate interplay between these two pathways exists and is essential for the correct regeneration of muscle tissue.

8. WNT SIGNALING IMPLICATIONS IN MUSCULAR DISEASE AND TREATMENT

In the light of their pivotal role in myogenesis, Wnt proteins represent promising compounds for therapeutic purposes in muscle-related pathologies, such as cachexia or muscular dystrophies. These diseases are characterized by muscle weakening and wasting, and molecules such as Wnt7a that are able to improve muscle size and strength are potential candidates for the treatment of these specific conditions. Indeed, Wnt7a represents a current focus for therapeutic use, but the high hydrophobicity that characterizes all Wnt ligands hinders its production and application.[76] Recently, von Maltzahn and colleagues generated a truncated Wnt7a protein, which lacks the palmitoylation site and thus has reduced hydrophobicity.[77] This Wnt7a variant retained full biological functions in vivo and has an enhanced dispersion compared to the full-length protein, leading to muscle hypertrophy. This finding represents a significant advance for the development of Wnt7a-based therapeutic approaches.

Moreover, mis-regulation of canonical Wnt pathway has been described in both aged and dystrophic muscles. Modifications of the systemic environment that occurs during aging increases Wnt signaling activity in myogenic precursors, leading to impairment in muscle regeneration and increased fibrosis.[78] Similarly, the Duchenne Muscular Dystrophy mouse model (also known as the *mdx* mouse) shows a chronic upregulation of Wnt pathways in skeletal muscles.[79] In particular, the elevated Wnt activity in dystrophic muscles drives TGFβ2 activation, which in turn alters muscle progenitor cell fate promoting an alternate fibrotic identity. Although it has recently been shown that Wnt-dependent TGFβ2 activation in regenerating muscle is an essential step for proper muscle repair,[45] TGFβ2 upregulation in the context of the *mdx* mouse compromises the regenerative potential of the satellite cells and stimulates fibrogenic gene expression.[79] Taken together, these conflicting results confirm the crucial role of the Wnt/TGFβ crosstalk in skeletal muscle, but additional studies are needed to fully understand the precise function of the Wnt-TGFβ axis in adult myogenesis. Considering the negative effects of enhanced Wnt activity in aged and dystrophic muscles, downregulation of Wnt signaling could ameliorate these pathophysiological conditions. In support of this hypothesis, exogenous inhibition of the Wnt cascade through Dickkopf-1 (DKK1, Wnt antagonist) in aged regenerating muscles and in dystrophic *mdx* muscles results in reduced fibrosis.[78,80] For

these reasons, in vivo modulation of Wnt signaling in pathological contexts represents a potential therapeutic strategy for the improvement of aged muscle and for the treatment of muscular dystrophies.

9. CONCLUDING REMARKS AND OUTSTANDING QUESTIONS

The multiple studies performed during the last decades clearly indicate the pivotal role of Wnt signaling in both embryonic and adult myogenesis. During muscle formation, many Wnt ligands and receptors have been described to have an impact on muscle progenitor cells and, although some mechanisms remain unclear, their roles and functions are currently well established. However, as embryonic myogenesis relies on a complex network of signaling pathways, it would be interesting to analyze potential crosstalk between these different pathways during embryogenesis (e.g. TGFβ signaling pathway), to further expand our knowledge on muscle development.

The role of Wnt signaling in adult myogenesis starts to be elucidated; however, many questions remain unresolved. For instance, conflicting Wnt expression domains have been proposed in mature healthy muscle fibers[39,66] and a clarification of these contrasting results could represent an important step to understand the Wnt signaling in uninjured myofibers and its role in the maintenance of adult tissue homeostasis in physiological conditions. Furthermore, the novel regulatory mechanisms proposed by recent papers revealed a complicated system underlying the myogenic modulation driven by Wnt signaling.[47,50] Hence, further studies are required to completely dissect the extensive molecular mechanism through which Wnt ligands control adult myogenesis. Lastly, the exquisite balance between canonical and noncanonical Wnt signaling represents an important point of interest. Based on the current knowledge, canonical and noncanonical pathways are complementary and exert different functions. Wnt/β-catenin cascade appears to be mainly involved in the regulation of satellite cell commitment and terminal differentiation,[42,43,45] while the noncanonical Wnt7a pathway controls satellite cell self-renewal and migration, and myofiber size and strength.[44,51,59] However, our understanding concerning the precise mechanism that regulates canonical and noncanonical cascades during muscle regeneration remains poor.

Satellite cell self-renewal is an intriguing and barely understood mechanism. Our observations and those of others concur to the fact that after

muscle regeneration, the satellite cell niche reforms to the exact extent as that prior to regeneration.[81] We previously identified the noncanonical Wnt7a-PCP signaling as a pathway controlling satellite cell self-renewal, via control of stem cell division orientation.[44] On the other hand, the impact of canonical Wnt signaling on the return of satellites to the niche has not been clearly investigated. It is an ongoing question in multiple research fields whether both canonical and noncanonical Wnt pathways act sequentially, in parallel, or compete to regulate stem cell self-renewal. Consequently, we propose two hypotheses: first, it is possible that both Wnt signaling pathways compete, and that Wnt/β-catenin inhibits Wnt/PCP; second, Wnt/β-catenin signaling may feed into the Wnt/PCP cascade by regulating the transcription of PCP components. In vivo epistasis experiments and in vitro transcriptomic analyses may validate one of these hypotheses in the future.

One of the key properties that distinguish adult stem cells from their differentiated progenies is their ability to remain in the quiescent state for an extensive period of time. In contrast to various stem cell populations in fast-turnover tissues, the satellite cells are a population of primarily quiescent adult stem cells in adult skeletal muscle. Interestingly, canonical Wnt signaling pathways have been previously shown to control hematopoietic stem cell quiescence[82] and to be negatively regulated in quiescent hair follicle stem cells.[83] Our studies demonstrate that upregulation of canonical Wnt signaling in satellite cells following APC gene disruption prevented the mutant cells to enter the cell cycle in activating conditions.[43] Thus suggesting that β-catenin signaling may promote quiescence. In contrast, analysis of mice with targeted β-catenin gene disruption in satellite cells only showed that the number of Pax7 cells remained constant one month following the induction of Cre/Lox activity in adult mice.[45] However, more precise analyses, such as evaluation of the cell cycle status of mutant satellite cells, should be performed to clearly demonstrate an implication of canonical Wnt signaling in satellite cell quiescence. Moreover, intramuscular injection of recombinant Wnt7a protein to uninjured skeletal muscle promoted satellite cell amplification, suggesting that noncanonical Wnt ligand may be a satellite cell "activating" factor. Unfortunately, it is also possible that the needle injury was sufficient to send a stress signal throughout the muscle tissue that would trigger the satellite cell to leave quiescence. Hence, the development of new experimental setups, such as transgenic over-expression of Wnt7a, or targeted Fzd7 gene disruption is now needed to evaluate if noncanonical Wnt-PCP signaling plays a role in breaking satellite cell quiescence.

Another important question that will have to be resolved in the next years is what is/are the source(s) of Wnt proteins in skeletal muscle, either in the healthy adult skeletal muscle or during regeneration? Earlier studies have mainly used RT-PCR analysis of cultured muscle cells or bulk muscle tissue. While providing the blueprint for the expression pattern of the Wnt family members, positional information (a.k.a which cell type) was not gathered and temporal information was only patchy. Furthermore, it is now clear that satellite cells need to receive clues from neighboring cells, such as macrophages[84] or fibro-adipogenic progenitors[85] in order to function correctly. In this new era of single-cell analyses, we hope to soon be able to access single-cell RNA-sequencing and single-cell proteomic datasets of multiple muscle-resident cell types that will allow us to construct a precise mapping of the sources of Wnt signaling in skeletal muscle.

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