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REVIEW

Fat deposition and accumulation in the damaged and inflamed skeletal muscle: cellular and molecular players

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Abstract The skeletal muscle has the capacity to repair damage by the activation and differentiation of fiber sub-laminar satellite cells. Regeneration impairment due to reduced satellite cells number and/or functional capacity leads to fiber substitution with ectopic tissues including fat and fibrous tissue and to the loss of muscle functions. Muscle mesenchymal cells that in physiological conditions sustain or directly contribute to regeneration differentiate in adipocytes in patients with persistent damage and inflammation of the skeletal muscle. These cells comprise the fibro-adipogenic precursors, the PW1-expressing cells and some interstitial cells associated with vessels (pericytes, mesoangioblasts and myoendothelial cells). Resident fibroblasts that are responsible for collagen deposition and extracellular matrix remodeling during regeneration yield fibrotic tissue and can differentiate into adipose cells. Some authors have also proposed that satellite cells themselves could transdifferentiate into adipocytes, although recent results by lineage tracing techniques seem to put this theory to discussion. This review summarizes findings about muscle resident mesenchymal cell differentiation in adipocytes and recapitulates the molecular mediators involved in intramuscular adipose tissue deposition.

Keywords Skeletal muscle · Adipogenesis · Fibro-adipogenic precursor · Mesenchymal cells · Fat · Nitric oxide · Satellite cells

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Abbreviations

SC	Satellite cells
MRF	Myogenic regulatory factors
MHC	Myosin heavy chain
MEF	Myogenic enhancer factors
EMC	Extracellular matrix components
MMP	Matrix metalloproteinases
PPARγ2	Peroxisome proliferator-activated receptor γ2
UCP1	Uncoupling protein 1
DMD	Duchenne muscular dystrophy
MRI	Magnetic resonance imaging
sca-1	Stem cell antigen 1
PDGFR	Platelet-derived growth factor receptor
FAP	Fibro-adipogenic precursors
TGFβ1	Transforming growth factor beta
SP	Side population
PIC	PW1-expressing cells
NG2	Neural/glial antigen 2
PG	Proteoglycans
miRNAs	MicroRNAs

HDAC	Histone deacetylases
WNT	Wingless-type mouse mammary tumor virus integration site family
BMP	Bone morphogenetic proteins
IGF1	Insulin-like growth factor 1
IL	Interleukin
NO	Nitric oxide
NOS	Nitric oxide synthase
WT	Wild type
TSA	Tricostatin A
αSMA	α-smooth muscle actin

Satellite cells and skeletal muscle regeneration

Skeletal muscle physiologically responds to fiber degeneration with a complex and highly coordinated regenerative process. This leads to the repair of damaged tissue or to the establishment of new fibers that progressively substitute those damaged, restoring original integrity [1]. Satellite cells (SC) are localized between the sarcolemma and the basal lamina of healthy fibers in a resting, quiescent state; they are the protagonist of this regenerative process [2]. Following damage, they activate myogenic program, proliferate and differentiate into myoblasts that are able to fuse with themselves or with other fibers [3, 4]. The state of quiescence is characterized by a reversibly arrested G0 phase and expression of the paired-box protein Pax7 [5]. Activated, cell cycling SC express myogenic regulatory factors (MRF) (Myf-5, MyoD, and MRF4) [4, 6]. The down-regulation of Pax7 and the expression of the myogenic factor myogenin characterized the final commitment of these cells to myogenic differentiation [7, 8]. Terminally differentiated cells form multinucleated structures and express muscle proteins such as myosin heavy chain (MHC) [8]. During this process, a subset of SC progeny does not down-regulate Pax7 and returns to the quiescent state during the process of self-renewal [9].

Expression of Pax7 marks SC [5]. In the absence of Pax7, SC can be detected, but their maintenance and proliferation are defective: in mutant mice, postnatal growth and regeneration are severely compromised with progressive loss of SC after birth mainly because of apoptosis [10, 11]. Pax3, the Pax7 paralog, is transcribed in these cells at least in some anatomical districts [12], but it does not exert any anti-apoptotic role [11, 13].

MRF gene family encodes for nuclear proteins with a conserved basic helix–loop–helix domain responsible for dimerization, DNA binding and the establishment of myogenic lineage as well as the control of terminal differentiation. These proteins drive the myogenic program when ectopically expressed from a constitutive promoter in non-myogenic cell [14]. Mice with homozygous deletion of *MyoD* or *Myf-5* have, however, fairly normal muscles indicating overlapping functions for these MRF [15, 16]. On the contrary, myogenesis is severely disrupted in double mutant mice that do not express *MyoD* and *Myf-5*, and *Myogenin* is not transcribed [17]. This may be explained considering that expression of MyoD depends on Pax7, whereas in this postnatal context expression of Myf-5 is Pax independent [11]. Similarly, *MRF4* null mice have a normal muscle phenotype even if they are characterized by myogenin overexpression [18]. MRF myogenin has a unique function in the transition from myoblast to a fully differentiated myotube [19, 20].

In vitro studies demonstrate that, once expressed, MRF translocate to the nucleus and bind to DNA by heterodimerization with non-myogenic proteins encoded by the E1A and HEB genes [21, 22]. Their activity is indeed finely regulated: MRF are also subjected to negative control at the post-transcriptional level by direct interaction with repressors that block their binding to DNA or by indirect mechanisms (phosphorylation/dephosphorylation, acetylation, ubiquitination). Expression of Id protein induced by growth factors sequestrates the MRF dimers blocking their activity [23], and a similar control is exerted by cell cycle regulators [24, 25] and other repressors (MyoR, Mist1, ZEB, I-mfa) [26–29]. Protein kinases A and C [30, 31], cell cycle kinases [32, 33] and mitogen-activated protein kinases regulate MRF via phosphorylation that inhibits or promotes their activity [33, 34]. MyoD coprecipitates with co-activators that have acetyltransferase activity, suggesting that gene acetylation is an additional regulation mechanism [35]. Finally, MRF and in particular MyoD and myogenin have short half-lives and their degradation is regulated by the ubiquitin system [33].

A second class of transcription factors that controls myogenesis is the myogenic enhancer factor (MEF)-2 family, which transcriptionally activates various muscle-specific genes including creatine kinase and MHC, desmin and MRF [36, 37]. Although these factors are not muscle specific, mRNA splicing regulates their muscular expression [38]. Similarly to MRF, they initiate myogenic program when overexpressed in non-muscle cells [37]. Many studies have underlined a regulatory network between MFR and MEF: their DNA-binding sites are located in close proximity and overexpression of MRF induces MEF [39, 40].

These complex and highly regulated mechanisms of SC's activation and differentiation are necessary for regeneration, since the muscle cannot restore its original integrity in the absence of SC [41, 42]. Nevertheless, the regenerative process involves additional cells. Immune cells, firstly dominated by neutrophils and subsequently by macrophages, rapidly infiltrate tissue upon damage. They

remove debris, but also drive SC myogenesis [43, 44]. In the regeneration contest, endothelial/vascular cells are then responsible for the vasculature remodeling and supply of energy to newly formed fibers [45]. Angiogenesis and myogenesis proceed simultaneously and endothelial cells regulate SC's activation [46]. Interstitial fibroblasts exert another important role in regeneration. They proliferate upon fiber damage and synthesize collagen and other extracellular matrix (ECM) components to provide a scaffold that supports SC migration and new fiber formation. ECM presents growth factors as well as other signaling molecules to fibers [47]. The subsequent degradation of ECM, driven by proteases (including matrix metalloproteinases, MMP), is required for normal tissue repair [48].

During the last decade, other muscle cells of mesenchymal origin that contribute to regeneration have been also identified: they reside in the interstitium between fibers eventually associated with vessels. They, in physiological conditions, sustain SC activation and differentiation and/or directly differentiate in myoblasts and form new fibers [49–51].

Muscle regeneration versus fat tissue deposition

Despite the regeneration ability of healthy skeletal muscle, extensive and widespread fiber destruction is a common feature of various diseases in which the trigger cannot be eliminated, such as skeletal muscular dystrophies. Genetic defects of different dystrophin complex proteins that lead to sarcolemma fragilities during contraction are the basis of these diseases; persistent injury jeopardizes the ability of the tissue to heal and progressively leads to fiber substitution with ectopic tissues such as bone, fibrotic tissue and fat [52, 53].

The muscle fat includes both acellular lipid droplets within the fibers and interstitial adipocytes characterized by drops of triglycerides and cholesterol ester (that can be stained by O-red oil) and by the expression of peroxisome proliferator-activated receptor $\gamma 2$ (PPAR$\gamma 2$), perilipin, leptin, adiponectin and fatty acid-binding protein 4 [54]. Intra- and interfiber fat is usually white, but brown adipocytes have also been identified [55]. These cells that express uncoupled protein 1 (UCP1) and are characterized by uncoupled respiration could be useful when skeletal muscles fail to produce heat during physical exercise [56].

Intramuscular fat is a characteristic of muscles with impaired SC function as demonstrated in injured mutant mice with ablation of Pax7, myogenin or MyoD [13, 15, 19]. It is also a histopathological characteristic of muscle dystrophies [57, 58], inflammatory myopathies [59], sarcopenia due to disuse or nerve injury as well as aging [60–65], obesity, diabetes and other metabolic diseases [66–68].

In Duchenne muscular dystrophy (DMD), the most severe and prevalent among dystrophies, intramuscular fat can reach as much as 50 % of muscle mass in young boys [69]. Evaluation of muscular fat content by magnetic resonance imaging (MRI) represents, for DMD as well as for inflammatory myopathies, a biomarker of disease progression or activity. It also provides a potential outcome measure for the assessment of treatment efficacy in clinical trials [69–71]. In DMD, older boys show higher muscle fatty infiltration as measured by MRI; their characteristic pattern is the presence of fat mainly in the *gluteus* and the *adductor magnus*. Semimembranous muscles, biceps and *rectus femoris*, are fatty infiltrated too, while *gracilis* and *sartorius* muscles are usually spared [72]. Images show both intermuscular and intramuscular adipose depositions and seem to correlate [72]. MRI analyses of DMD muscles reveal that muscles showing clear signs of inflammation and edema are not always infiltrated by adipose tissue. In contrast, other muscles undergo marked fatty deposition in the absence of a prominent inflammatory reaction [73].

To investigate muscle adipose tissue deposition in experimental mouse model, glycerol injection is most commonly used [74], but adipogenesis can be also detected after cardiotoxin injection or freeze injury, depending on the genetic background of mice [75–77]. This indicates that activation of cells responsible for fat deposition could be a hallmark of regeneration processes regardless of the trigger, and that there is a strict connection between SC and adipocyte precursors. It seems important to take into consideration that some cytokines released by adipocyte precursors can positively regulate myogenic cell proliferation and differentiation [78], and ablation of muscle adipogenic cells impairs regeneration [79, 80]. This suggests that the intramuscular pre-adipocytes are per se important for muscle homeostasis and underlines the importance of investigating the factors that influence the choice between regeneration and ectopic tissue deposition.

Different cells of mesenchymal origin, including SC themselves, appear to be able to differentiate into adipocytes when cultured in vitro in the presence of adipogenic-inducing factor (including insulin, dexamethasone and 3-isobutyl-1-methylxanthine) [81] and, therefore, contribute to adipose tissue deposition in vivo. This review focuses on muscle resident cells responsible for intrafiber adipogenesis in damaged/inflamed muscle including recent findings about SC transdifferentiation obtained relying on innovative methods for cell isolation and lineage tracking. We also analyze molecular regulators of muscle adipogenic precursors in muscle and consider the meaning of adipose cells in the pathophysiology of the skeletal muscle. Finally, since fatty degeneration occurs and often, but not always, associates with skeletal muscle fibrosis in chronic

neuromuscular diseases, we discuss differences and resemblances between the two events.

Skeletal muscle resident cells are sources of intramuscular adipose tissue deposition after damage

Many cells contribute to ectopic intrafiber adipose tissue formation, both resident in the skeletal muscle and originating from adipose tissue or bone marrow. Figure 1 depicts muscle resident cells involved in regeneration or in fibrosis and fatty degeneration tissue formation (SC, endothelium, interstitial cells and fibro-adipogenic precursors, matrix fibroblasts) and their interactions during the two events.

SC transdifferentiation

SC exist as heterogenic populations of precursor cells committed to the myogenic lineage and are endowed with "stem cell" properties, such as self-renewal. Upon damage, SC re-enter the cell cycle and, after some rounds of division, the majority of them proceed along the myogenic pathway. Some cells, however, down-regulate myogenic-

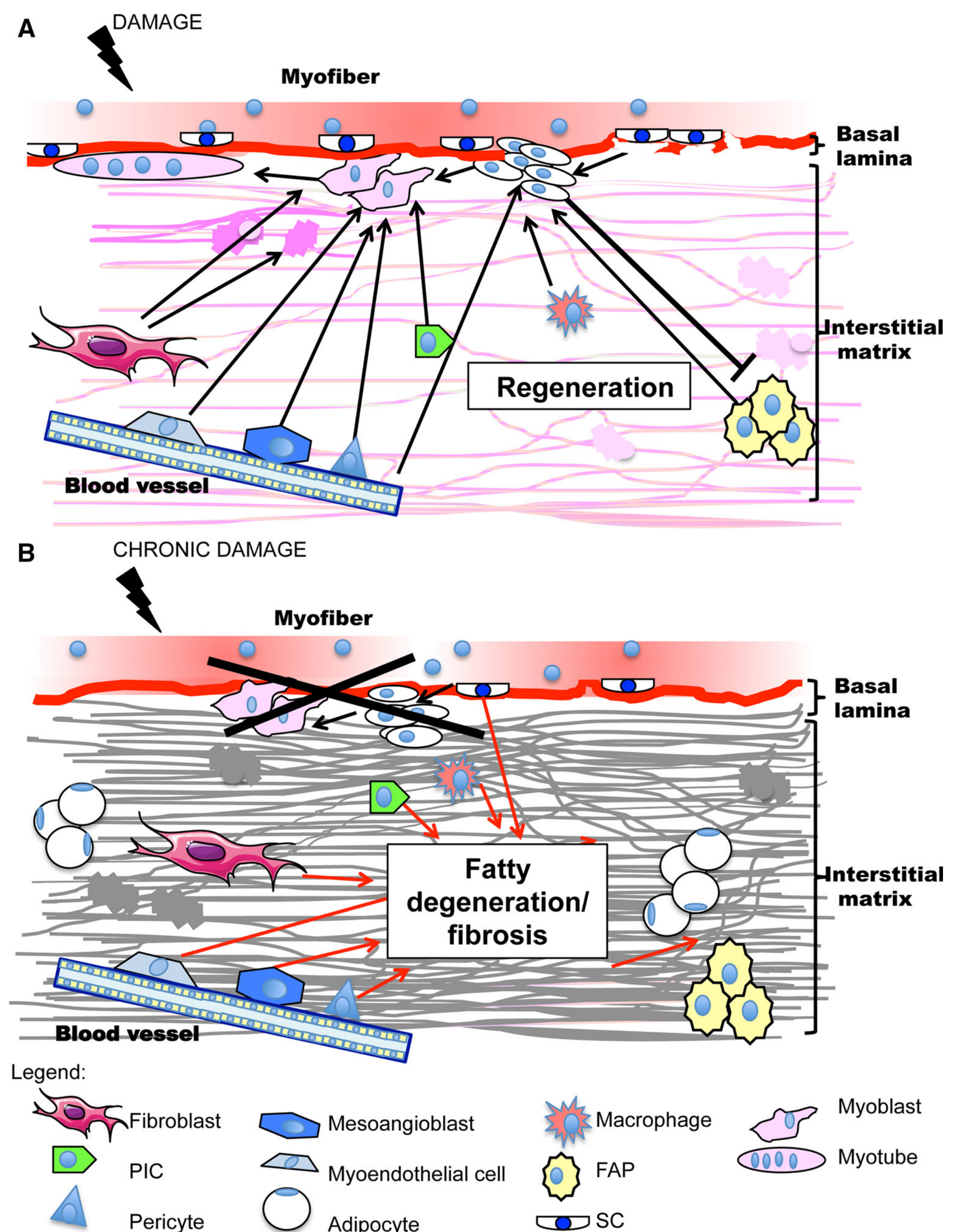

Fig. 1 Schematic representation of muscle resident cells involved in regeneration or in adipo-fibrous tissue formation and of their interactions. **a** In physiological conditions, fiber damage induces satellite cells (SC) activation after basal lamina breakage, proliferation and differentiation into myoblasts that fuse to form new myotubes. Fibro-adipogenic precursors (FAP), located in the interstitial matrix, sustain SC cell regeneration by soluble factors and conversely SC inhibit their differentiation into adipocytes. Interstitial cells (PW1-expressing cells or PIC, pericytes, mesoangioblasts and myoendothelial cells) differentiate into myoblasts and contribute to regeneration. Fibroblasts, endothelium and infiltrating macrophages sustain myogenesis. Fibroblasts are also responsible of ECM remodeling, which in addition favors myoblast migration and fusion. **b** In a situation of persistent damage regeneration fails because of SC number reduction and/or functional impairment. In this case, ectopic fibro-adipose tissue deposition comes into view: it is sustained by interstitial mesenchymal cells, including FAP, as well as by fibroblasts and chronic inflammation. SC transdifferentiation along the adipocyte program also occurs

specific proteins and revert to quiescence to maintain the stem cell compartment [82, 83]. Both quiescent and activated SC express two isoforms, different in length, of the stem cell marker CD34 [84]. Based on this stem cell potential, some authors suggest the possibility that SC transdifferentiate to non-myogenic cells including adipocytes. Several in vitro studies indicate that immortalized myoblasts (such as C2C12 and L6 myoblasts) and primary SC can differentiate into non-myogenic cells, including adipocytes. This SC transdifferentiation has been investigated using both isolate cells and single fibers maintained in culture [54, 85–88]. Differentiation appears more likely to occur in SC isolated from damaged muscle, such as in experimental models of obesity or aging, and in cells cultured in conditions of high oxygen pressure [89–91]. The SC isolation methods as well as the use of immortalized cell lines represent a potential criticism of these experiments. SC are usually isolated by enzymatic digestion of muscle followed by propagation in myogenic-selecting conditions or by clonal proliferation. This carries risk of contamination, depending on the method employed. Moreover, usually cell clones are cultured for long periods of time with a certain risk of spontaneous transformation with the generation of cells that are defective for differentiation [79].

Cell sorting by fluorescence-activated flow cytometry provides a powerful tool for cell isolation. The selection of specific SC markers (e.g., Pax7) limits contamination by non-myogenic cells [92, 93]. Similarly, quiescent SC can be also isolated using SM/C-2.6 [94, 95], anti-integrin α7 [96, 97] or anti-syndecan 3/4 [98] antibodies. Some authors have employed CD34 for myoblast isolation [84]. However, the use of this antigen for SC's retrieval is debatable; in human muscle, $CD34^{negative}$ precursors show an in vivo and in vitro myogenic potential higher than $CD34^{positive}$ cells [99]. These progenitors indeed comprise cells with adipogenic or myogenic commitment and myoadipogenic bipotent precursors and can be separated by the expression of CD56 and CD15 [100]. CD34 is also expressed, together with the stem cells antigen 1 (Sca-1), by a recently identified population resident in skeletal muscle, localized in the interstitial space, and able to differentiate into adipocyte, endothelial and myogenic cells. These cells are defined as myoendothelial cells [101]. It should be taken into account that all the isolation methods based on quiescence markers may need validation for SC purification from damaged muscles: activated cells have a reduced expression of Pax7 as well as of the other quiescence markers [6]. Some recent papers report investigations about SC transdifferentiation employing the Cre-loxP recombination system for lineage tracing. Using the Cre–loxP recombination system with the *cre* gene driven by the MyoD promoter, Goldhamer and colleagues demonstrated in vitro that the YFP clonal cells derived from MyoD $(cre^{+})R26R(YFP^{positive})$ muscles (that also represent 98 % of isolated $Pax7^{positive}$ cells) only undergo myogenic differentiation [102]. These cells accumulate lipids within the cytoplasm, but do not activate an adipogenic program and do not express terminal differentiation markers of mature adipocytes, such as adipsin or FAB4 [103]. The same group investigated the origin of isolated adipocytes in Tie2–Cre mice muscle fibers. Since Tie-2 identifies a population of interstitial cells with adipogenic potential, they demonstrated that the majority of adipocytes were Cre-recombinant, underlining that the origin of muscle adipocytes is different from that of SC. Consistently with the previous studies, using a Cre/LoxP tracing system for Pax3 (a paired-box gene expressed during muscle development), it has been demonstrated that intramuscular fat in leg muscles (e.g., *soleus* and *extensor digitorum longus* muscles) is derived from a lineage that is Pax3 negative, non-SC lineage [80].

While these recent studies seem to call the SC transdifferentiation theory into question, the possibility that SCs could adopt an adipogenic fate in vivo during repeated rounds of damage or under different death stimuli remains to be clarified.

Brown adipocytes are present in muscles [104]. SC and brown adipose tissue precursors derive from progenitors that have common gene determinants: both cells originate from a mesenchymal precursor that expresses Myf-5. In 2001, the group of Rudnicki demonstrated that lacZ+ cells isolated from Myf-5–nlacZ mice undergo myogenic, osteogenic and adipogenic differentiations [86]. More recently using a Cre/LoxP-based system for SC lineage tracing (Pax7–CreER; R26R-tdTomato), they showed that SC could become brown adipocytes. The choice between myogenic or brown adipose differentiation is tightly controlled in physiological conditions by regulation of the expression of nuclear Prdm16, a transcription factor required to establish brown adipocyte lineage [105]. Myf-5 expression in adipose tissue is heterogeneous and some Myf-5-positive cells differentiate in vitro and in vivo into adipocytes or myofibers according to the expression levels of stem cell antigen (Sca-1) [106]. Accordingly, mesenchymal stem cells derived from adipose tissue differentiate into functional skeletal muscle cells when intramuscularly or intravenously injected in murine dystrophic muscle [107].

Fibro-adipogenic precursors (FAP)

Back to back papers published in 2010 identified very similar cells located in the interstitial space between fibers (but outside of vessels) that have adipogenic potential and are important in skeletal muscle regeneration [78, 108].

These cells, called fibro/adipogenic precursor cells (FAP), can be identified and isolated from both wild-type (WT) and dystrophic mice muscles as CD45^{negative}, CD31^{negative} and Sca-1^{positive} cells. They also express the platelet-derived growth factor receptor α (PDGFRα) in the absence of SC markers such as α-7 integrin, SMC 2.6 and Pax7 [78, 92, 108]. FAP and SC do not share common progenitors, reside in close proximity within damaged muscle and are both important for regeneration. Bromodeoxyuridine incorporation experiments reveal that FAP proliferate more quickly than SC during the first 72 h after injury. Their number returns to those measured in pre-damaged tissue 4–5 days later, concomitantly with SC-initiated muscle regeneration [78]. This suggests that, when regeneration physiologically occurs, FAP proliferate to sustain myogenesis. They produce soluble factors that stimulate SC. Among these, IL-6 has been identified in cells isolated from WT animals [78], while FAP from leg dystrophic muscles of young but not old *mdx* mice (a mouse model of DMD) produce follistatin [109]. FAP also seem to play a role in the phagocytic clearance of necrotic cells and debris. This event is essential for effective muscle regeneration [110].

FAP yield adipocytes in vitro when challenged with adipogenic factors and in vivo under conditions that favor adipogenesis, such as glycerol injection [79] or conditions characterized by SC failure (e.g., DMD) [92]. Conversely, efficiently proliferating myoblasts inhibit FAP differentiation into adipocytes [79].

FAP isolated from healthy animals can also differentiate into other cells of mesenchymal origin, including osteoblasts, chondroblasts and smooth muscle cells when cultured under appropriate conditions. They apparently never yield myogenic cells [78, 79]. Transforming growth factor (TGFβ1) acts on FAP to yield collagen expressing fibroblasts: they are abundant in the fibrotic area of the diaphragm muscle of *mdx* mice, indicating that FAP are the possible source of fibrosis in these muscles [111]. Sca-1^{positive}, α-7 integrin^{negative} cells isolated from the leg muscle of *mdx* mice that received Tricostatin (TSA), a histone deacetylases inhibitor, acquired myogenic potential with the expression of MyoD and of the SWI/SNF chromatin-remodeling factor BAF60C (important for MyoD-mediated transcription). These cells form MHC-positive structures and less adipocytes when cultured for 6 days in adipogenic conditions [112] However the expression of PDGFRα, expressed by Sca-1^{positive}-FAP, is not reported. These data indicate that in chronically damaged muscles, the environment profoundly modifies the FAP behavior. Interestingly, a human counterpart of PDGFRα^{+} progenitors described in mice has been recently identified and seems to aberrantly accumulate in muscle diseases [113]. This might open the possibility of developing new approaches for DMD patient therapy based on the regulation of FAP differentiation: the efficacy of treatment has been already demonstrated in pre-clinical studies [92, 109, 114].

Myoendothelial cells, pericytes and mesoangioblasts, and PW1-expressing cells (PIC)

Besides FAP, other mesenchymal cells that differentiate into adipocytes have been identified. This is a heterogeneous group of cells located in the *interstitium* between fibers (outside the basal lamina). Most of these cells are tightly associated with muscle vessels and express the platelet endothelial cell adhesion molecule. This stem cell population is distinct from SC, but may have a myogenic fate in vivo. It was identified in 1999 and purified based on fluorescent dye Hoechst 33342 efflux; these cells have been named side population (SP) [115]. SP cells express Sca-1 and c-Kit and may be either CD45^{negative} or CD45^{positive}: despite the absence of Pax7 and desmin expression, they can differentiate into myoblasts within muscle or in co-culture with SC. They become hematopoietic cells after intravenous injection [51, 115–119]. All these results indicate that SP cells, though possessing a constitutive haematopoietic potential, yield myoblasts upon appropriate conditions. Tamaki and co-workers showed that CD34^{positive} cells are present outside the basal lamina. Among these, some CD34^{positive}/Sca-1^{positive}/CD45^{negative} cells differentiate into myoblasts and endothelial cells when injected into a recipient muscle and become adipocytes in vitro. These cells have been named myoendothelial progenitors [101]. They have been recently characterized for the high expression of PDGFRβ and absence of PDGFRα, and have showed a multi-lineage potential (i.e., myogenic, endothelial and adipogenic) by clonal analysis. Hence, these cells represent a subpopulation of SP cells endowed with more pronounced myogenic potential. Myoendothelial cells are also able to inhibit in vivo, under physiological conditions, muscle adipogenesis via bone morphogenetic protein (BMP) [120]. A human putative counterpart able to differentiate in chondrocytes and osteoblasts has also recently been identified [121].

Pericytes, also known as aka mural cells, surround endothelial cells in capillaries and microvessels. Like myoendothelial cells, pericytes are characterized by the expression of the neural/glial antigen 2 (NG2), α-smooth muscle actin, CD146 and PDGFRβ. They exhibit multi-lineage developmental potential and differentiate into skeletal myofibers, bone and cartilage [122, 123]. They express PPARγ2 and form lipid droplets when cultured in adipogenic medium [124]. Proliferating pericytes do not express Pax7, Myf5 and MyoD, but up-regulate rapidly these pro-myogenic markers, together with myogenin

before forming myotubes, in myogenic-inducing condition [123]. Recently, two pericyte subtypes have been identified using a double transgenic nestin–GFP/NG2-DsRed mouse. Type 1 cells express nestin and type 2 do not [125]. Both types re-enter into cell cycle and proliferate in vivo after damage; however only type 2 pericytes, which are characterized by the lack of CD34 and Sca-1 expression, generate muscle cells. In contrast, type 1 nestin^{negative} pericytes express Sca-1 and PDGFRα and differentiate into adipocytes, but not into myogenic cells. This indicates that a subset of pericytes may correspond to the previously described FAP [126].

Mesoangioblasts, possibly a sub-fraction of pericytes, have been first initially isolated from murine aorta and have been found in skeletal muscle vessels of different species [127]. Freshly isolated mesoangioblasts in culture express several early endothelial markers such as fetal liver kinase-1, Sca-1, CD34 and VE (vascular endothelial)-cadherin but not the von Willebrand factor. A fraction of mesoangioblasts consistently express smooth muscle actin [128]. Both murine and human mesoangioblasts differentiate into skeletal myoblasts under condition permissive for myogenesis, in osteoblasts after exposure to BMP-2, into adipocytes and into chondrocytes and in smooth muscle too [128, 129]. These cells express Pax3, which is required for both myogenic and adipogenic differentiation [130]. After long-term culture they lose myogenic differentiation capacity, but remain myogenesis inducible upon co-culture with myoblasts. Therefore, they have a therapeutic potential in the treatment of skeletal muscle disease [128, 131]. Interestingly, mesoangioblasts apparently depend on the interaction with polarized macrophages to yield effectively functional contractile tissue [132].

Finally, Mitchell and co-workers identified another mesenchymal population with adipogenic potential in the early mouse postnatal muscle *interstitium*. These cells, called PIC, express PW1, a protein coded by a zinc finger gene which as a role in the myogenic and neuronal lineage development [133]. PIC share with SC the PW1 expression, but do not express Pax3 or Pax7 and display myogenic potential in vitro. They form new fiber after in vivo engraftment while retaining the ability to differentiate into α-smooth muscle actin-positive myofibroblasts in vitro [133]. A recent paper by the same group of researches revealed that postnatal PIC were a heterogeneous population. They express different levels of PW1 and sca-1 and are endowed with different differentiation potentials [134]. A fraction of cells express a high level of sca-1 (Sca-1^{high}), and 60 % of these positive for PW1 show also PDGFRα. Another group of PW1^{+} cells expressing low (or middle, Sca-1^{med}) level of sca-1 is abundant in the early mouse postnatal stages, but rapidly declines later on. They are no longer detectable after 5–7 weeks of age. When analyzed in early postnatal stages, Sca-1^{med}PW1^{+} cells have both myogenic and adipogenic potential, higher than that of PW1^{+}/Sca-1^{high} cells. In adult muscles, following a decline of Sca-1^{med}PW1^{+}, PW1^{+}/Sca-1^{high} cells increase their myogenic potential. It is important to note that the fraction of PDGFRα^{+}/PW1^{+} cells, which is not myogenic, could overlap at least in part with already described FAP. All PIC seem to express mRNA for NG2, indicating a possible overlap of these cells also with muscle pericytes.

Fibroblasts

Stromal fibroblasts synthesize ECM proteins including elastin, laminin, fibronectin, proteoglycans (PG) and the various isoforms of collagen [135]. Fibroblasts are also sensitive to mechanical loading and synthesize different amounts and types of collagen. Moreover, they express different levels of MMP according to the need of each specific muscle [136]. They play an active role in reparative myogenesis through ECM remodeling; fibroblasts proliferate and migrate after damage and provide new ECM components. These elements stabilize the damaged tissue, provide a scaffold to new fibers and drive the formation of neuromuscular junction. Upon injury resolution, they undergo apoptosis. Concomitantly, they regulate matrix remodeling and degradation by the expression of proteases and regulation of their specific inhibitors [137].

Chronic diseases, including DMD, are characterized by recurring cycle of damage and regeneration, alongside persistent inflammation. Those events lead to the development of fibrosis, an accumulation of aberrant ECM within the tissue [138]. In non-muscle organs, activated/fibrogenic fibroblasts can be easily identified by the expression of vimentin and, in particular, of α-smooth muscle actin (αSMA), a contractile protein of stress fibers. It exerts mechanical tension on the ECM, providing a mechanically resistant support, and hence the name of ‘myofibroblasts’. In tissues such as of liver or lung, myofibroblasts may derive from various cells including resident mesenchymal cells and epithelial and endothelial cells or from circulating progenitors derived from the bone marrow. Myofibroblasts are activated by a variety of mechanisms, such as cytokines produced by lymphocytes and macrophages, autocrine factors and pathogen-associated molecular patterns [137]. Notably, one greatest limitation of the study of fibrosis in the muscle is the lack of markers for activated fibroblasts; vimentin or αSMA are also expressed by myoblasts, albeit at lower levels. Recently, transcription factor 4 (Tcf4) has been identified as a newly identified fibroblast marker. The key role of fibroblasts in regeneration has been recently underlined, inducing their ablation using a Tcf4CreERT2 system: this leads to depletion of the SC pool with premature

differentiation and formation of smaller regenerated myofibers [139].

In muscle chronic disorders, fibroblasts continue to proliferate, leading to a progressive and self-perpetuating ECM deposition known as fibrosis, with a mechanism resembling and often accompanying muscle fat deposition [140, 141].

Fibroblasts could also directly convert to adipocytes. Green and Kehinde demonstrated this in 1974; when 3T3 fibroblasts are cultured in adipogenic medium they increase their content in fatty acid, precursors of triglyceride synthesis, and activity of lipogenic enzymes. This cellular differentiation occurs when cells stop growing [142, 143]. In 2006, these cells were shown to be pluripotent and could be reprogrammed to pluripotency by transduction of four stem cell-specific transcription factors. The discovery led to the award of the 2012 Nobel Prize to Prof. Takahashi [144]. The fate of skeletal muscle stromal fibroblasts in vivo remains unclear and the lack of specific markers for these cells increases the problem complexity. In human tissues including skeletal muscle, TE-7 has been recently validated as a fibroblast-specific protein [145]. TE-7^{+}/CD56^{-} cells have been isolated from muscle biopsies. They express collagen IV, fibronectin, vimentin and PDGFRα and respond to fatty acid treatment with full adipocyte differentiation [146]. Interestingly, FAP can be identified in mdx diaphragm fibrotic areas and differentiate in vitro into collagen type I-producing cells upon TGFβ stimulation [111]. Accordingly, fibroblast-activated protein-α^{+} stromal cells express PDGFRα and sca-1 like FAP [147], and PDGFRα knockin mice, characterized by chronic activation of the receptor, have diffuse fibrosis in the skeletal muscle, as well as other organs [148].

The interplay between muscle fibrosis and adipogenesis in pathological conditions as well as the possible connection of stromal skeletal muscle fibroblasts and FAP during physiological regeneration remain to be clarified.

Regulators of skeletal muscle adipocytes-generating cells

The differentiation of pre-adipocytes into fully differentiated adipocytes (endowed with lipid droplet and expressing adipocyte proteins) is finely regulated and already described in non-muscle tissue [149]. Some molecular regulators of muscle resident mesenchymal precursor differentiation in adipocytes have been identified. Table 1 summarizes the best-characterized pathways of intramuscular pre-adipocyte differentiation describing their suggested sources and signaling pathways.

Table 1 Regulators of muscle pre-adipocyte differentiation

Regulators	Source/origin	Molecular pathway	Effect	Experiments	References
High glucose	Diet	Oxidative stress, mTOR activation	Adipogenesis induction	In vitro	[151, 152]
Protein synthesis reduction	Dietary restriction	n.d.	Adipogenesis induction	In vitro	[153]
PPARγ	Expression/up-regulation	Gene transcription	Adipogenesis induction	In vitro	[54, 85]
WNT	Skeletal muscle	cCreB, PPARγ inhibition	Adipogenesis inhibition	In vitro/in vivo	[159, 160]
IL-6	Skeletal muscle/adipose tissue	Akt phosphorylation/c-Jun terminal kinase	Lipolysis induction/adipogenesis inhibition	In vitro	[78]
Myostatin	Skeletal muscle/adipose tissue	Smad 3	Adipogenesis induction/inhibition	In vitro	[170–173, 177]
Follistatin	Skeletal muscle/adipose tissue	n.d.	Brown adipogenesis induction	In vitro	[176]
BMP	Skeletal muscle/(other organs?)	Smad 1, 5, 8	Adipogenesis induction/inhibition	In vitro/in vivo	[104, 180]
IGF-1	Skeletal muscle	Rho GTPase	Adipogenesis inhibition	In vitro	[183]
MMP	Fibroblasts/other cells	n.d.	Adipogenesis inhibition	In vivo	[185]
Collagen (V and VI)	Fibroblasts/adipocytes	Adipose gene transcriptional inhibition	Adipogenesis induction	In vitro	[193, 196]
PG	Fibroblasts/adipocytes	n.d.	Adipogenesis induction/inhibition	In vitro	[197, 198]
Macrophages-secreted factors	Macrophages	n.d.	Adipogenesis inhibition	In vitro	[201]
NO	Skeletal muscle pharmacological	microRNA-27b	Inhibition of FAP differentiation	In vitro/in vivo	[92]
HDAC inhibitor (TSA)	Pharmacological	miR1. miR2, miR133, miR206	Adipogenesis inhibition	In vivo/in vitro	[229, 236]

Nutrient availability

Nutrient and energy availability is crucial for muscle homeostasis. Myofibers burn substrates to produce energy and have a relatively high content of mitochondria, depending on the type of fiber. Adipocytes are responsible for energy storage and are endowed with few oxidative organelles [54]. SC activation and differentiation is also hinged on mitochondrial biogenesis [150]. Muscle-derived stem cells and SC undergo adipogenic differentiation when exposed to persistent hyperglycemia via oxidative stress or mTOR activation [151, 152]. Moreover, restriction of protein synthesis by essential amino acid reduction seems to favor SC's transdifferentiation into adipocytes, without affecting their viability [153]. The efficacy of dietary interventions in attenuating muscle loss and restoring muscle mass in sports and geriatric medicine and in the treatment of neuromuscular disease [154] may also be grounded on the regulation of muscle adipose tissue deposition.

PPARγ

PPARγ is a key regulator of adipogenesis. A number of diverse lipids, lipid-like compounds and drugs activate PPARγ and induce pre-adipocytes to generate fully differentiated adipose cells. Up-regulation and activation of PPARγ in mesenchymal cells induce adipogenesis and modulate insulin sensitivity [54, 85]. PPARγ expression and activation induce repression of MyoD [85]. Recent data indicate the existence of a mutual regulation between PPARγ and myogenic factors such as MyoD. The simultaneous expression of the proteins in mesenchymal cell generates myotubes or adipocytes, but not hybrid cells. In adipocytes, the ubiquitin–proteasome system induces MyoD degradation. In myotubes, PPARγ histone acetylation is inhibited in several loci including that of C/EBPα, the essential pro-adipogenesis PPARγ partner [155].

WNT

The wingless-type mouse mammary tumor virus integration site family (WNT) was first identified for a role in carcinogenesis. The proteins also have important function in myogenesis, where the integration of multiple WNT signals allows the self-renewal and the differentiation of muscle precursors. WNT signals deregulation leading to the disruption of muscle homeostasis and to fibrosis [156, 157]. The WNT-activated canonical-β catenin pathway negatively controls adipogenesis and favors myogenic differentiation [158]. Interestingly, WNT signaling is down-regulated in aging [159]. WNT 10 has an important role in the control of intramuscular adipogenesis. Its deficiency/inhibition is involved in adipose tissue deposition after injury caused by diverse triggers, including cardiotoxin, low temperatures and rotator cuff tear [159, 160].

Myokine and adipo-myokine

The skeletal muscle has been identified as an endocrine organ. It releases soluble factors called myokines, at least in part responsible for the beneficial effect of exercise in neuromuscular disorders [161]. They are synthesized by skeletal muscle tissue especially during contraction and exercise and can act within the muscles in an autocrine/paracrine manner and in distant tissues in an endocrine fashion. Most myokines are also secreted by adipose tissue and are therefore referred to as adipo-myokines [162]. The adipo-myokine family includes angiopoietin-like 4, fibroblast growth factor 21, follistatin-like 1, interleukin 6 (IL-6), interleukin 8, monocyte chemoattractant protein-1, myostatin, and vascular endothelial growth factor. IL-6, myostatin and follistatin may control differentiation of mesenchymal precursor in adipocytes within the muscle as described in this review.

Interleukin-6 (IL-6)

Mechanical load increases IL-6 production depending on exercise duration, intensity and the muscle mass. It is the best-characterized adipo-myokine: IL-6 may act locally within the muscle in an autocrine/paracrine manner or may be secreted. IL-6 is produced during inflammation. It has well-described roles in the adipose tissue and in the liver including the inhibition of insulin-signaling pathways [163, 164]. In myotubes, recombinant IL-6 enhances insulin-stimulated Akt phosphorylation and seems to have a beneficial effect on insulin-stimulated glucose disposal and fatty acid oxidation [165]. Muscle-derived IL-6 may locally inhibit the effects of other inflammatory cytokines such as tumor necrosis factor-alpha. Recently, it has received significant attention for its regulatory role in muscle wasting during cachexia [166]. Its role in muscle resident pre-adipocytes differentiation remains to be elucidated. Interestingly, FAP up-regulate expression of IL-6 after muscle damage. The cytokine is the possible regulator of FAP-induced stimulation of myogenic differentiation [78]. Production of not yet identified soluble factors (probably including IL-6) during pre-adipocytes/myoblasts interaction results in adipogenic inhibition via suppression of lipogenic genes such as lipoprotein lipase, adipsin and glycerol-3-phosphate dehydrogenase [167]. These findings support the hypothesis for a role of IL-6 in the control of metabolism during contraction with a pro-myogenic effect. Conversely, the chronic elevation of IL-6 released from adipocytes may induce muscle insulin resistance.

Myostatin/follistatin

Myostatin is a developmental protein which acts as a negative regulator in myogenesis. It is a member of the TGFβ protein family produced by both skeletal muscle cells and adipose tissue. It inhibits muscle differentiation and growth. Accordingly, myostatin knockdown accordingly promotes myogenesis [168]. Based on these observations during the last decade, experimental therapies with myostatin blockers to treat DMD have been developed [169]. Myostatin stimulation or inhibition of adipogenesis was alternatively described depending on cell types and culture conditions. Its expression in mesenchymal cells promotes its adipogenic differentiation via Smad3 with a negative cross talk with β catenin [170, 171]. Myostatin inhibits lipid accumulation in pre-adipocytes cell lines and fibroblasts [172, 173]. The inhibitory role of myostatin in muscle is counterbalanced by the endogenously produced follistatin [174]. White and brown adipocytes also produce follistatin [175, 176]. In particular in brown adipocytes, follistatin induces differentiation of pre-adipocyte cells. In sharp contrast, myostatin inhibits this process [176, 177]. Interestingly, FAP of young mice exposed to the histone deacetylase inhibitor TSA do not differentiate into adipocytes and efficiently stimulate SC differentiation producing a high level of follistatin [110].

Bone morphogenetic proteins (BMP)

Sequence similarities link TGFβ to the BMP family of proteins. In spite of this, the BMP pathway is a positive regulator of muscle mass [178]: BMP-2/4 up-regulates inhibitors of TGF-β-induced myogenesis repressors to block the TGF-β1-negative effect on myogenesis [179].

Like myostatin, BMP could both induce and repress adipogenesis. Ablation of BMP receptor 1 in mouse muscle mesenchymal precursors (myoendothelial progenitors) increases their adipogenic differentiation upon BMP4 stimulation, while the myogenic differentiation is reduced [180]. In apparent contrast, sca-1-positive cells isolated from skeletal muscle adopt an adipogenic fate after BMP7 treatment even if they seem to differentiate into brown instead of white adipocytes [104]. To gain a full appreciation of the role of TGFβ and BMP, further studies are needed. However, a crucial point has already emerged: both proteins regulate target genes via Smad. The manner by which the two subfamilies of ligands recruit different Smad proteins in various cell types is important for the skeletal muscle physiology [181]. Unraveling the mechanism may be advantageous for developing suitable inhibitors or mimetic agents to treat adipose tissue-related dysfunctions.

Insulin-like growth factor 1 (IGF1)

IGF1 is ubiquitously expressed in all tissues. Blood concentrations are high, because of its production by liver, bone and adipose tissue. The expression level of IGF-binding proteins is tissue specific. In the case of skeletal muscle, IGF-I signaling is a requisite for development [182]. Studies on precursor clonal cells have revealed that IGF1 is a crucial player in both adipocyte and myoblast differentiation. Although growth arrest is necessary for differentiation, IGF1 paradoxically stimulates both proliferation and differentiation of the cells. IGF-1 acts through Rho GTPase to switch in the adipogenesis–myogenesis fate. To alter the differentiation process it is sufficient to manipulate the activation of the Rho GTPases;.a reduction in the levels of the Rho inhibitory protein, p190-B RhoGAP, results in the reduction of adipogenesis and in increase of myogenesis. IGF-1 receptor directs the downmodulation of Rho GTPase activity by regulating the subcellular distribution of p190-B RhoGAP. This leads to increased IGF-1 signaling to downstream proteins previously implicated in adipogenesis [183].

Extracellular matrix

Muscle cells are surrounded by the basal lamina composed of collagen IV, laminin and heparin sulfate-containing PG. These are directly linked to sarcolemma and sustain muscle structural integrity. These enable the tissue mechanotransduction and act together with other components of muscle ECM named endomisium (around muscle cell) perimysium (around groups of muscle cells) and epimysium (around whole muscle) [184]. These elements have quite different compositions, but contain fibrils of collagen I and III in close association to collagen V, PG and glycoproteins (such as perlecan and fibronectin). ECM is mainly produced primary by fibroblasts: however other muscle cells within the muscle such as myoblasts are able to synthesize various ECM proteins upon activation [185].

Recent evidence pinpoints a role for ECM in the regulation of SC growth and differentiation. Data suggest that a tightly regulated dynamic interplay between intrinsic SC factors and extrinsic molecules of the microenvironment exists. Therefore, ECM, the adjacent vascular system, the intramuscular fibroblasts and preadipocytes are defined as SC niche [185, 186]. Isolated SC quickly change their fate and lose self-renewal capacity. Hence, the recent quest for appropriate scaffold to culture of SC [187]. Genetic and pharmacological studies in both animals and humans demonstrated that loss of almost any ECM components can lead to a myopathy often accompanied by fibrosis and to further fatty degeneration [138].

Differentiation of multipotent stem cells into various lineages is influenced by their interactions with ECM components. Muscle mesenchymal stem cells sense mechanical properties of their matrix (i.e., strain, shear stress, substrate rigidity and topography) and respond to environmental changes differentiating into mature myoblasts [188]. Activation of the muscle adipogenesis process occurs rapidly when ECM is disrupted. In healthy mice, ECM damage can be induced by nylon mesh material implanted to create space between fibers. The mice show abundant adipocytes at week 2 that invade and replace fibrous material. By week 4 granulation tissue typical of wound healing is detected [189].

MMP are a heterogeneous group of zinc-containing, calcium-dependent endopeptidases. They are part of the ECM and they have been extensively studied for their role in muscle regeneration. MMP differ in substrate specificity, cellular localization and regulation. After damage, their expression is rapidly up-regulated. Their activation favors a number of processes, namely myogenesis and angiogenesis. MMP control the migration of inflammatory and endothelial cells and fibroblast and the proliferation and differentiation of myogenic precursors. Specific inhibitors finely regulate them. MMP activation needs the cleavage by membrane-type 1 MMP or plasmin. Once activated, MMP are regulated through covalent binding by specific inhibitors. Collagenases and MMP further remodel ECM when fiber integrity is restored. [185].

Upon administration of GM6001, a broad-spectrum MMP inhibitor, in vitro muscle-derived stem cells display a reduced migration capacity as well as a reduced myogenic and adipogenic differentiation. Accordingly, in vivo treatment of injured mice with the MPP inhibitor jeopardizes skeletal muscle healing [190].

Another important ECM component is collagen. The muscles of DMD patients that show increased ECM deposition express lower levels of the PG decorin and significantly higher levels of collagens I and VI. Expression of collagen VI α5 and α6 chains has been recently identified [191]. Collagen's role in regulating pre-adipocyte differentiation has been well investigated in vitro [192]. Pre-adipocytes synthesize various ECM proteins in vitro; they produce type II, V and VI collagens and some glycoproteins. When stimulated with adipogenic-inducing medium, these cells arrange type III and IV collagen and laminin in a non-fibrous structure, increase expression of type V and VI and reduce the expression of type II and type I collagen and of fibronectin [193–195]. Bovine pre-adipocytes derived from the muscle and 3T3-L1 cells have been treated with modulators of type V and VI collagen. As a consequence, they reduce triglyceride synthesis and their adipose differentiation is inhibited [193, 196]. In vivo, the role of collagen in the control of adipogenic differentiation in conditions of jeopardized repair is still unclear.

The role of PG in pre-adipocyte proliferation and differentiation has been studied in vitro using 3T3-L1 with dishes coated with biglycan and decorin and fibronectin. Biglycan and decorin reduce proliferation of pre-adipocytes, partly by induction of apoptosis. Co-treatment with fibronectin restores normal proliferation [197]. In vivo, co-injection of an extract of basement membrane proteins and basic fibroblast growth factor into mouse leg or masticatory muscle induced angiogenesis followed by fat pad formation [198]. Nevertheless, the exact role of muscle PG as a regulatory factor for muscular pre-adipocytes proliferation and differentiation remains to be investigated.

Inflammatory cells

Inflammatory cells including macrophages are recruited into the extra-fiber environment after injury to remove debris and sustain SC activity [43, 199]. Alterations of macrophage responses (e.g., an imbalance between the different macrophage sub-types) have profound effects on muscle regeneration and induce fibrosis as in DMD [138].

The absence or the alteration of macrophage metabolism has important consequences also for adipose tissue deposition within the muscle. The administration of an inhibitor of macrophage colony-stimulating factor and reduction of the differentiation and proliferation of macrophage/monocyte lineage induce intramuscular adipogenesis and abundant collagen deposition after cardiotoxin damage [200]. The role of macrophages is at least in part due to secreted factors; mesenchymal stem cells isolated from mouse skeletal muscle and incubated with macrophages conditioned medium show reduced tendency to differentiate into adipocytes [201]. Macrophages are also important for the storage and recycling of iron, a function that is tightly dependent on their polarization state [202] and that might play a role in the effective regeneration of the tissue (G. Corna and PRQ, unpublished results). Effective recycling of myoglobin-associated iron might be a relevant step to prevent fat deposition. It might require the effective recruitment and in situ activation of leukocytes able to properly support the reconstitution of effective vasculature in the regenerating tissue [203, 204].

Adipose tissue of obese subjects is infiltrated by inflammatory macrophages positive for F4/80, CD11b and CD11c which predominantly display a classically activation, (M1-like macrophages) [205, 206]. Skeletal muscle macrophages that classically infiltrate dystrophic muscle could contribute locally to the adipose intrafiber deposition: therapeutic interventions that control the shift between M1 and M2 macrophages exert a beneficial effect

in animal models of dystrophy and might also regulate muscle adipogenesis [207, 208].

Heredia and co-workers have recently demonstrated that eosinophils are also important for muscle adipogenesis and in particular for FAP regulation. IL-4α receptor null mice are defective in muscle regeneration. Using a GPF reporter construct for IL-4 gene, they identified eosinophils as the dominant cell type producing the cytokine [110]. Accordingly, eosinophil-deficient mice showed defective regeneration after injury. A specific up-regulation of IL-4α in FAP is detected and inhibits their differentiation into adipocytes, thereby preventing muscle fatty degeneration. Finally, FAP stimulated via eosinophil-derived IL-4 show an increase ability to clear cell and tissue debris by phagocytosis [110].

Nitric oxide (NO)

NO is a key signaling molecule synthesized from L-arginine by a family of NO synthases (NOS). Neuronal NOS (called NOSμ) is the most important NOS in skeletal muscle and is located at the sarcolemma of fibers in the dystrophin complex. As a small, hydrophobic gas molecule NO readily diffuses into cells. It acts on different targets by cGMP-dependent or -independent pathways and in cross talks with other molecules [209–212]. In muscle, it controls the structure, bioenergetics, mitochondrial function and number, energy and oxygen supply, and excitation–contraction coupling [213–216]. Alteration of NO synthesis has an important role in the pathophysiology of muscle diseases and in particular of DMD [217, 218]. Restoration of NO generation, by genetic or pharmacological interventions with NO donating drugs, ameliorates dystrophic phenotype increasing regeneration and preserving muscle morphology in animals [219–223]. This intervention has high profiles of safety and tolerability with promising signs of efficacy in humans [224].

We and other groups have explored the mechanisms beyond the therapeutic potential of NO. It has been found that it has multiple actions on survival, self-renewal, activation and differentiation of SCs. Some of these effects depend on NO-induced increase of cGMP generation, while others are independent of it [222, 225–228]. We have recently demonstrated that NO influences FAP differentiation. Long-term treatment of *mdx* dystrophic mice with NO donor drugs inhibits adipose tissue deposition in tibialis anterior muscles in vivo and reduces the differentiation to adipocytes of both WT and *mdx* FAP [92] (Fig. 2a). NO inhibits the increase of PPARγ induced by adipogenic medium by controlling both its promoter activity and the expression of microRNA-27b (miR-27b), an important PPARγ post-transcriptional regulator [92] (Fig. 2b). In dystrophic muscles, the treatment with the drug, and the subsequent enhanced expression of miR-27b, reduced the expression of adipocyte markers [92]. These NO actions are cGMP independent and apparently not critically involved in the initial stages of FAP adipogenic differentiation; NO does not affect the expression of the early adipogenesis transcription factors KLF4, c-EBPβ and CHOP10. However, it regulates factors active at a later stage in adipogenesis, such as PPARγ, an adipogenic transcription factor active at later phases of the process. NO does not apparently affect the TGFβ-induced differentiation of FAP in fibroblasts (Fig. 2c). However, it regulates muscle fibrosis by controlling miR-133a, a known regulator of collagen type I expression [92]. The mechanism by which NO regulates microRNAs (miRNAs) 133a and 27b has not been clarified yet. NO might induce S-nitrosylation as demonstrated for other miRNAs (miR-1 and miR-29) and can function as a histone deacetylase (HDAC) inhibitor (see below) [229]. The role of NO in the control of adipose differentiation of other mesenchymal stem cells responsible for intrafiber adipose tissue deposition remains to be investigated.

Another interesting issue to explore is NO capacity to induce mitochondrial biogenesis and to regulate mitochondrial function in these cells. These effects are critically dependent on its concentrations, NOS localization and exposure of the target; in isolated mitochondria, high concentrations of NO inhibit complexes of the mitochondrial respiratory chain irreversibly, whereas physiological lower levels of NO reversibly inhibit cytochrome c oxidase [230, 231]. In intact cells, physiological levels of NO stimulate the uptake and oxidation of glucose and fatty acids by skeletal muscle and adipose tissue, while they inhibit the synthesis of glucose, glycogen and fat, and enhance white adipocyte lipolysis [232]. These effects could also control brown adipocyte generation and exert beneficial effects on energy production in defective muscles. NO stimulates cell expression of both PPARα and PGC-1α [216, 233]. PGC-1α, the master regulator of UCP1 expression, is involved in the development of brown adipocytes as well as in mitochondrial biogenesis [234].

Histone deacetylases and micro-RNA

Histone deacetylases (HDAC) are enzymes that remove acetyl groups from lysine residues of histones, thus regulating gene expression. Blockade of HCAC by drugs such as valproic acid or Tricostatin A (TSA) results in chromatin expansion, facilitating transcription. Among the HDAC family, HDCA2 seems to control the expression of many skeletal muscle genes such as follistatin, the endogenous antagonist of myostatin [235]. HCDAC inhibitor treatment of *mdx* mice ameliorates dystrophy by enhancing regeneration and preventing fibrotic scars and fat

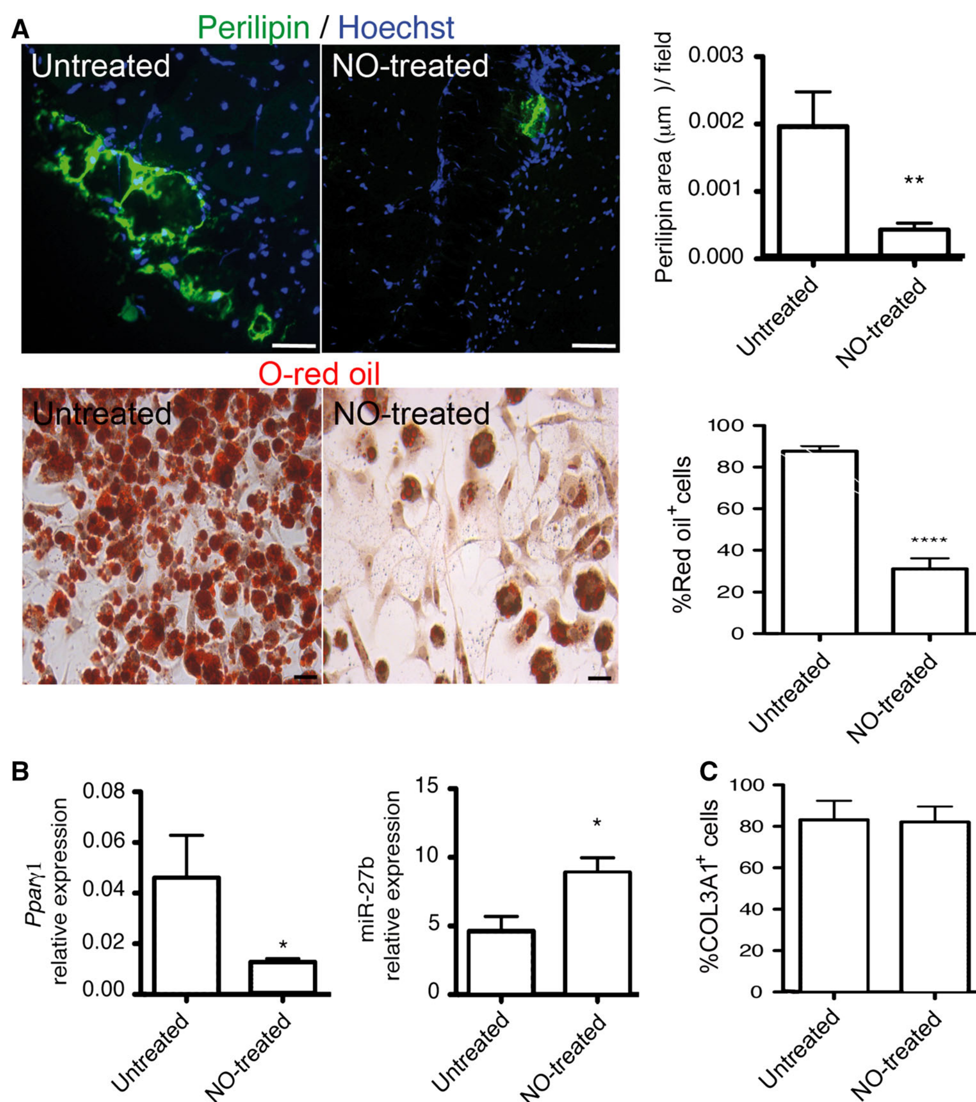

Fig. 2 Nitric oxide inhibits adipose tissue deposition and FAP adipocyte differentiation in dystrophic muscle. **a** Nitric oxide effect on adipose tissue deposition (*upper panels*) and on FAP differentiation in adipocytes (*lower panels*) for *tibialis anterior* muscles, cells isolated from untreated *mdx* mice (untreated) and from mice that received an NO donor drug (NO-treated). Adipose tissue in section was revealed using an anti-perilipin antibody (*upper panels*) while O-red oil was used to count adipocytes in culture (*lower panels*). Hoechst was employed for nuclei staining. Quantification of perilipin-positive area (*upper*) and adipocytes number (*lower*) are reported in the right graphs (mean ± S.D). $^{**}p < 0.001$ and $^{****}p < 0.0001$ vs. untreated, *scale bars* are 20 μm. **b** Expression of PPARγ mRNA and mir27b (*left* and *right*, respectively) in *tibialis anterior* muscles obtained from untreated *mdx* mice (untreated) and from mice that received an NO donor drug (NO treated); mean ± S.D. $^{*}p < 0.05$ vs. untreated. **c** Percentage of collagen-positive cells in FAPs cultured in the absence (untreated) or in the presence of an NO donor (NO treated) and stimulated with TGFβ; mean ± S.D

deposition [229, 236]. HDAC are effective only when administered to young mice. This indicates that a permissive environment is essential. In isolated cells, TSA increases SC differentiation capacity similarly in young and old mice, whereas FAP response to the drug changes dramatically with the age of the donor. FAP isolated from young animals and exposed to TSA fail to differentiate into adipocytes, produce high level of follistatin and efficiently stimulate SC differentiation. On the contrary, the adipogenic potential of FAP obtained from 12 months old animals was unaffected by treatment with HDAC inhibitors and unable to stimulate SC [109]. Furthermore, HDAC inhibitor treatment of FAP isolated from dystrophic mice at the early stage of the disease de-repressed their latent myogenic program inducing myogenic transcriptional machinery. HDAC-induced up-regulation of myogenic miRNAs (mir1.2, miR133 and miR206) seems to mediate the effect [237].

Micro-RNA

Micro-RNAs (miRNAs) are small non-protein coding RNAs, some of which act as post-transcriptional gene regulators in muscle development and function [238]. Among miRNAs specifically expressed in muscles, miR-1/206 and miR-133 are the most studied. miR-1 and miR-133 have distinct roles in modulating proliferation and differentiation of cultured myoblasts; miR-1 promotes myogenesis by targeting HDAC4 (histone deacetylase 4). Unlike miR-1, miR-206 expression is restricted to skeletal muscles where it plays a crucial role in the differentiation of activated SCs, by targeting Pax7 mRNA, and in myoblast differentiation by targeting multiple genes [239]. Their expression is altered in muscle disorders including DMD [240, 241]. MiRNAs have recently emerged as crucial determinants for cellular lineage decision. By a peculiar repressing activity on the 3′ UTR (untranslated region) of target mRNAs, miRNAs have been reported to confer proper timing and robustness or differentiation program. Mir129a is highly expressed in SP interstitial muscle cells and contributes to maintain their quiescent state, blocking proliferation and differentiation in adipogenic, osteogenic and myogenic cells targeting PPARγ, Runx1 and Pax3 [242].

Fat deposition versus fibrosis

Fibrosis reflects excessive accumulation of ECM components, particularly of collagen. Stromal fibroblasts, which play a fundamental role in normal repair, are also crucial in fibrosis that is a hallmark of chronic neuromuscular disorders. When regeneration fails, fatty degeneration occurs. Fibrosis and fatty degeneration of muscle are certainly strictly linked and share common precursor cells [111]. Nevertheless, it is not entirely clear whether fibrosis and adipose degeneration in muscle damage are the two sides of the same coin or if independent/alternative pathways sustain them. Some issues should be highlighted.

The two processes occur often, but not always together during muscle degeneration. In experimental models of acute injury, the type of insult is relevant. For example after glycerol injection, adipogenesis is predominant, while after acute ischemia fibrosis occurs [108, 243].

The link between fibrosis and aberrant inflammatory reaction is well established in muscles: virtually no fibrotic tissue can be identified in the absence of inflammation. A link with the infiltration by inflammatory, M1-like, macrophages has been suggested in adipose tissue [206]. However, detailed MRI analyses of DMD muscles indicate that inflamed and edematous muscles are not always affected by adipose tissue infiltration, while other muscles display fatty deposition in the absence of a prominent inflammatory reaction [72].

Finally, fibrosis appears to correlate with the loss of muscle locomotor activity and the reduction of contractile fiber force, at least in DMD [244]. On the contrary, MRI investigations of DMD patients fail to demonstrate a correlation between intramuscular adipose tissue and muscle strength.

Signals for fibroblast or adipocyte differentiation seem to act at least in vitro. TGFβ and connective tissue factors as well as myostatin easily induce fibroblast activation and fibrotic features [138]. Although the effect of TGFβ/myostatin has not been completely clarified, mesenchymal stem cells fail to differentiate in adipocytes in the presence of those proteins [172, 245]. Similarly, PDGF receptor inhibition reduces fibrosis in mdx mice diaphragm, but promotes in vitro adipogenesis in mesenchymal stromal cells [114, 246].

Concluding remarks

Progressive loss of muscle mass and contractile function is a common feature of skeletal muscle diseases characterized by impairment of the tissue repair process. This process is consistently accompanied by adipose intrafiber deposition. SC play a central role in regeneration; alteration of their regulatory signals within the tissue explain muscle adipogenesis. The close anatomic contact between fat and muscle cells suggests a reciprocal influence. In fact, several molecules coming from the muscle or from the regenerative milieu in turn affect the deposition of adipose tissue. Mesenchymal stem/progenitor cells are reported to exist in almost all mammalian organs. The ability to differentiate toward adipogenic, osteogenic and chondrogenic lineages is a hallmark of these cells. Strong evidence indicates that differentiating into a certain lineage is not an intrinsic property of mesenchymal cells in physiological condition. Their differentiation fate changes during disease progression and might be detrimental. Dissecting the mechanism of mesenchymal cells interactions and differentiation ought to be an important object of future studies. A better understanding of the molecular pathways that regulate gain or loss of muscle mass and muscle-to-fat conversion is crucial for treating muscle wasting-associated disorders along with their physical and metabolic complications. Fibrosis and fatty degeneration are a consequence of regeneration failure. Fibrosis is induced in disease characterized by chronic inflammation. Fibrosis and adipogenesis are often simultaneous and share the some precursor cells. However, fibrosis and inflammation can occur in the absence of adipogenesis and in vitro the differentiation of precursors in adipocytes or fibrogenic

cells seems to be regulated by different molecular pathways. These data suggest that fibrosis and fatty degeneration could be the outcome of independent programs recruited during skeletal muscle degeneration.

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