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Comprehensive Invited Review

REDOX CONTROL OF SKELETAL MUSCLE REGENERATION

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Abstract

Skeletal muscle shows high plasticity in response to external demand. Moreover, adult skeletal muscle is capable of complete regeneration after injury, due to the properties of Muscle Stem Cells (MuSCs), the satellite cells, which follow a tightly regulated myogenic program to generate both new myofibers and new MuSCs for further needs. Whereas reactive oxygen species (ROS) and reactive nitrogen species (RNS) have long been associated with skeletal muscle physiology, their implication in the cell and molecular processes at work during muscle regeneration is more recent. This review focuses on redox regulation during skeletal muscle regeneration. An overview of the basics of ROS/RNS and antioxidant chemistry and biology occurring in skeletal muscle is first provided. Then, the comprehensive knowledge on redox regulation of MuSCs and their surrounding cell partners (macrophages, endothelial cells) during skeletal muscle regeneration is presented in normal muscle and in specific physiological (exercise-induced muscle damage, aging) and pathological (muscular dystrophies) contexts. Recent advances in the comprehension of these processes has led to the development of therapeutic assays using antioxidant supplementation, which result in inconsistent efficiency, underlying the need for new tools aimed at precisely deciphering and targeting ROS networks. This review should provide an overall insight of the redox regulation of skeletal muscle regeneration while highlighting the limits of the use of non-specific antioxidants to improve muscle function.

1. Introduction

Skeletal muscle is a contractile tissue that accounts for 30-50% of body mass in humans (92). Besides its high plasticity and adaptation to various demands, adult skeletal muscle has the remarkable ability to regenerate *ad integrum* following injury. This regenerative property is mainly due to the Muscle Stem Cells (MuSCs), also called satellite cells, which are activated upon muscle injury and which follow a myogenic differentiation program to fuse and form new myofibers and restore the muscle tissue. Intrinsic and extrinsic cues drive MuSCs through these sequential steps of the regenerative process (15,63,290). Several cell types present in the injured muscle tissue play various roles in the regulation of muscle regeneration (359). Interestingly, functions of MuSCs and their neighboring cells are altered by oxidative stress modulation (44,255,348). For instance, macrophages are a major source of reactive oxygen species (ROS) and reactive nitrogen species (RNS) upon inflammation (16,38). Moreover, ROS and RNS have been recently proposed to be crucial actors of stem cell biology through the modulation of various cellular processes (34).

Concerning skeletal muscle biology, oxidative stress has been mainly investigated in skeletal muscle physiology, myofiber adaptation and contractile properties (22,156,206). It is likely that ROS/RNS-dependent modulation of the various cell types involved in skeletal muscle regeneration play important roles that may be altered in pathological contexts and may be the target of development of therapeutic strategies. The present review focuses on redox regulation of skeletal muscle regeneration. After a presentation of this complex biological process, basics of ROS/RNS and antioxidant chemistry and biology are described in the context of skeletal muscle. Then, the current knowledge related to redox regulation of skeletal

muscle regeneration is presented. Finally, specific alterations of redox regulation are described in various pathophysiological contexts, and therapeutic opportunities to improve skeletal muscle repair are discussed.

2. MuSCs and skeletal muscle regeneration

Skeletal muscle is a highly stable tissue that shows a high capacity to adapt to various physiological demands due to the properties of the myofiber, a multi-nucleated cell extending along the length of the muscle, which is capable of atrophy or hypertrophy. Adaptation is also possible because of the properties of different types of muscle fibers, adapted for different purposes, including slow-twitch type I and fast-twitch type II myofibers. In addition to its adaptive properties, skeletal muscle is capable of full functional recovery after an injury through the process of muscle regeneration, which goes beyond healing as the parenchyma recovers its functionality (**Figure 1**). Skeletal muscle regeneration relies on the properties of MuSCs, which are the adult muscle stem cells that normally reside in a quiescent state between the muscle fiber and its surrounding basal lamina (54). MuSCs are activated upon muscle injury and enter the cell cycle to proliferate. After an expansion phase, a large majority of MuSC progeny enters the myogenic differentiation program. The differentiated myogenic cells fuse to each other to form new myofibers and restore the tissue. A small subset of the expanding MuSCs does not commit into the terminal differentiation program but self-renews to replenish the pool of quiescent MuSCs for further needs. A general presentation of the cellular aspects of skeletal muscle regeneration is provided below since the reader can refer to excellent recent reviews on the topic (92,359). The molecular aspects of skeletal

muscle regeneration will be specifically addressed in the sections dedicated to the redox regulation of muscle regeneration (sections 7 and 8).

2.1. MuSCs and adult myogenesis

While the signaling pathways controlling the activation of MuSCs, *i.e.* their entry into the cell cycle, are still poorly understood and are the subject of intensive investigations, the gene expression program driving activated MuSCs along adult myogenesis is quite well known. MuSCs sequentially express a series of specific transcription factors from their exit from quiescence until their full differentiation and fusion (**Figure 2**) (92,359). At the quiescent state and during the expansion phase, they express Pax7, a transcription factor that is now considered as a canonical marker of MuSCs in various species. Once entered into the cell cycle, MuSCs sequentially express a series of specific muscle transcription factors, the Myogenic Regulatory Factors (MRFs). Structurally, MRFs bind DNA through their basic domain, while a helix–loop–helix motif allows their heterodimerization with E proteins, which mediate the recognition of genomic E-boxes, a motif present in the promoters of muscle-specific genes. While Myf5 is expressed by quiescent/early committed MuSCs, expanding MuSCs express MyoD, which is a strong determinant of their myogenic nature. After the expanding phase, MuSCs exit the cell cycle and downregulate the expression of Pax7 and MyoD while increasing that of Myogenin, which is considered a marker of irreversible terminal myogenic differentiation. Then, differentiated myocytes are capable of fusing together and with pre-existing fibers to reconstitute functional myofibers. At the time of fusion, differentiated MuSCs start to express the sarcomeric machinery of proteins required for contraction. Before exiting the cell cycle to differentiate, a subset of expanding MuSCs self-renews by

decreasing the expression of MyoD and increasing that of Pax7, therefore returning to a G0 quiescent state. The exact time (at the time of activation, during expansion or at the end of the expansion phase before differentiation) at which MuSC self-renew *in vivo* is still debated.

MuSCs are associated with all fiber types albeit with unequal distribution (264). Slow-twitch muscles express two- to three-fold higher MuSC density compared to fast- twitch muscles (284,296). These findings have been recently confirmed *in vivo* in mice using genetically labeled MuSCs (164). The phenotype of MuSCs from adult muscle fibers seems to be similar to their fiber of origin and to the fiber they tend to form in terms of myosin expression and contractile capacity (21,142). Aging is associated with skeletal muscle atrophy and a switch from fast-twitch to slow-twitch fiber type. Concomitantly, the number of MuSCs is reduced in some muscles (gastrocnemius, diaphragm, extraoccular muscles) at 20 months from 16 to 25% in mice (164). So far, no study has reported an impact of the type of myofiber – or the corresponding MuSCs – on the efficiency of muscle regeneration.

2.2. MuSC cellular neighborhood

MuSCs are absolutely indispensable for adult skeletal muscle homeostasis, since the specific depletion of Pax7-expressing cells completely prevents post-injury regeneration (188,292). However, numerous studies have recently shown that cells present in the neighborhood of MuSCs are an important support of adult myogenesis (**Figures 3,4**). One can distinguish between resident and non-resident muscle cells.

Among the resident non-myogenic muscle cells that have been shown to participate in muscle regeneration are vascular cells and fibro-adipogenic progenitors (FAPs). Vascular cells comprise endothelial cells (ECs) that specifically interact with

expanding MuSCs in a reciprocal way to promote concomitant myogenesis and angiogenesis (63,177) (**Figure 4**). Vascular cells also include peri-ECs such as pericytes and smooth muscle cells that promote the return of MuSCs to quiescence (2,170). Another cell type that has been recently described in skeletal muscle is the fibro-adipogenic progenitor, which also participates in the regulation of MuSC expansion and differentiation (157,329).

Non-resident muscle cells invade the injured muscle in a tightly organized sequence, as in any other tissue repair process (**Figure 3**). Infiltrating leukocytes exert various actions on FAPs and other immune cells, while macrophages have been shown to exert specific and direct effects on MuSCs (55,56). Invading circulating monocytes become inflammatory macrophages that stimulate the expansion of MuSCs while inhibiting their differentiation. Later on, at the time of resolution of inflammation, macrophages skew their phenotype to an anti-inflammatory status. These restorative macrophages exert specific pro-myogenic effects on MuSCs by stimulating their differentiation (expression of Myogenin) and their fusion (**Figure 4**).

2.3. Time and space orchestration of muscle regeneration – link with muscular diseases

Recent studies have shown that asynchronous injuries (76) or desynchronized delivery of anti-inflammatory cues (257) trigger similar defects in skeletal muscle regeneration, resembling what is observed in some muscle diseases. It is therefore admitted that the MuSC niche - the quiescence niche, as well as the so-called transient niche for a proper implementation of the myogenic program - is tightly regulated in space and time. It is therefore likely that this tight regulation operates

through subtle changes in the cues MuSCs receive all along the regeneration process (56).

Inherited muscular dystrophies are a group of heterogeneous diseases characterized by permanent cycles of necrosis/regeneration that lead to the replacement of muscle fibers by connective and fat tissue, and eventually to the loss of muscle function (57,214). Most of these diseases are caused by a genetic alteration targeting proteins of the dystrophin-glycoprotein complex (DGC), which is located at the sarcolemma and links the myofiber cytoskeleton to the extracellular matrix (6,214) **(Figure 5)**. It is believed that a weaker transmission force through the DGC triggers fragility of myofibers, which are therefore more susceptible to damage upon contraction/use (93). This primary event (mutation in a gene encoding for a DGC protein) leads to permanent regeneration processes throughout the tissue, triggering asynchronous delivery of regenerating cues to MuSCs and their close environment. This leads to failure in myogenesis and to secondary events including elevated oxidative stress, altered calcium homeostasis, chronic inflammation and endomysial (between myofibers) fibrosis which eventually replaces the atrophied myofibers (6) (**Figure 6**). Our knowledge of the cellular events in this context is still fragmented. It has been recently shown that in the Duchenne Muscular Dystrophy (DMD) mouse model mdx, FAP number is increased and macrophages exhibit altered inflammatory status while inhibition of the TGFβ pathway leads to the apoptosis of FAPs, improving the muscle state (187). The molecular pathways that are altered in such an unbalanced tissue environment are only beginning to be defined for each cell type. For instance, NF-κB signaling is deregulated in both myogenic cells and macrophages in DMD (3). Notch signaling, which has been shown to regulate MuSC fate, can be targeted to improve muscle homeostasis in this

disease (342). Given that the various cell types in the muscle no longer operate in a synchronized manner, one of the future challenges will be to identify the key pathways controlling the equilibrium of each cell type in degenerative myopathies.

3. Definition of free radicals, oxidative stress and redox signaling

ROS are a family of molecules that are continuously generated and consumed in all living organisms as a consequence of aerobic life (85). In 1985, Helmut Sies proposed oxidative stress as "a disturbance in the pro/antioxidant balance in favor of the former" (306). This definition has been widely associated with the view that ROS exert major biological effects as purely deleterious molecules causing damage to DNA, proteins and lipids, notably during aging (131), chronic diseases (335) or metabolic syndrome (151). During the early nineties, ROS-dependent signaling was discovered (review in (297)), shedding light on the beneficial role of ROS (316). Thus, oxidative stress has been redefined as "a disruption of redox signaling and control" (158). This notion implies that there is an optimal level of ROS/RNS to sustain both cellular homeostasis and adaptive responses, and that both too low and too high levels of ROS/RNS are detrimental to cell functions (108,235). This contemporary definition corresponds to a now well-accepted view that ROS production is crucial for cell functions (75,85,152,230,235). Recent developments in technological tools to detect and quantify ROS and ROS molecular targets, notably *in vivo*, have helped to better understand the role of ROS in numerous cellular processes (30,61,62,230). In the present review, the terms "ROS" and "RNS" include the oxygen and nitrogen radicals as well as some non-radical derivatives of O_2 and N, respectively (129) and are used when the specific entity is not described, as recently recommended by Forman and colleagues (104).

4. Major ROS and RNS in skeletal muscle

From its generation to the terminal product, ROS can undergo a wide range of transformations (**Figure 7**) that elicit specific effects on cell functions.

4.1. Major chemical entities

ROS and RNS can be classified depending on their chemical properties (reactivity, half-life) into two groups. Radical compounds (one electron oxidant) form the first group, whereas non-radical compounds (two electrons oxidant) form the second one (17). These chemical entities can also be classified based on the primary or secondary nature of the radicals.

4.1.1. Superoxide

The radical superoxide ($O_2^{\bullet-}$) is an anion produced by a monoelectronic reduction of an oxygen molecule ($O_2 + 1e^- \rightarrow O_2^{\bullet-}$). $O_2^{\bullet-}$ can diffuse within the cell but, despite a relatively longer half-life as compared with other radicals, $O_2^{\bullet-}$ is neither a strong oxidant, nor a powerful reductant. This is explained by a low standard reduction potential E_h of $O_2^{\bullet-}$/hydrogen peroxide (H_2O_2) above 0.94V (E_h value *i.e.* measurement of the tendency of chemical species to acquire electrons and to be reduced; the more positive the potential, the higher the tendency to be reduced). By a dismutation reaction, $O_2^{\bullet-}$ can produce H_2O_2 ($O_2^{\bullet-} + O_2^{\bullet-} + (2H^+) \rightarrow 2\ H_2O_2 + O_2$). In skeletal muscle as in other tissues, two distinct pools (inner and outer membrane) of mitochondrial $O_2^{\bullet-}$ have been observed (355).

4.1.2. Hydrogen peroxide

H_2O_2 is a two-electron oxidant, non-radical molecule generated from the dismutation of $O_2^{\bullet-}$. This molecule is stable and diffuses within and between cells and

therefore acts as a signaling molecule (338). However, it is a weak oxidizing and reducing agent, as evidenced by its inability to directly oxidize DNA and proteins as well as to induce antioxidant defenses in mammals (83). H_2O_2 is also described as a crucial player in tissue damage and repair (review in (337)). It acts as an alarmin to trigger inflammatory cell recruitment required for appropriate healing (238). However, H_2O_2 may also contribute to chronic tissue injury and subsequent inflammation by inducing irreversible oxidative damage (222). H_2O_2 is now recognized as a key molecule involved in the control of skeletal muscle function such as insulin control and glucose transport (135,338).

4.1.3. Hydroxyl radical

Hydroxyl radical ($HO^{\bullet}$) is a powerful oxidant (E_h value $O_2^{\bullet-}/H_2O_2$ = 2.34V) that is generated by reaction of metal ions with H_2O_2. The high reactivity related to its short half-life (10^{-9} sec) restricts its damaging effects to a local area close to the generation site (129). Additionally, $HO^{\bullet}$ can be formed from H_2O_2 in the presence of transition metals (iron) through the reactions of Fenton and of Haber-Weiss ($O_2^{\bullet-} + H_2O_2$ ($+Fe_3/Fe_2$) $\rightarrow HO^{\bullet} + O_2 + {}^{-}OH$) to induce oxidative damage (144,165,352).

4.1.4. Nitric oxide

Nitric oxide ($NO^{\bullet}$) is a free radical that easily crosses membranes and diffuses between and within cells. $NO^{\bullet}$ is synthesized from L-arginine by NO synthases (NOS), which are neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) NOS. It acts as a signaling molecule and exerts its effects *via* interactions with heme or non-heme iron and copper or by S-nitrosylation of cysteine redox centers to modulate protein functions (137). Moreover, $NO^{\bullet}$ reacts with $O_2^{\bullet-}$ to form peroxynitrite (128). nNOS and eNOS are the main NOS found in skeletal muscle (97,311). $NO^{\bullet}$ is a key

molecule in many physiological functions in skeletal muscle, including muscle regeneration (101,311).

4.1.5. Peroxynitrite

The reaction of $O_2^{\bullet-}$ with $NO^{\bullet}$ forms peroxynitrite, which is a strong oxidant capable of crossing cell membranes. Cell exposure to peroxynitrite produces many effects such as inhibition or depletion of antioxidant enzymes, modification of receptor functions, lipid peroxidation and DNA injury. Recently, peroxynitrite has been shown to induce the activation of mTOR signaling *via* a calcium-dependent signaling leading to skeletal muscle hypertrophy (147).

4.2. Major sources of ROS

In 1954, Commoner and colleagues identified free radicals in skeletal muscle (70). Since then, several sources of ROS production have been identified in various subcellular locations (148) (**Figure 8**).

4.2.1. Mitochondria

Mitochondria are one of the most important sources of ROS in muscle. At least ten sites capable of producing considerable amounts of ROS have been described (123). Early studies suggested that 2-5% of the total oxygen consumed by mitochondria undergo a one-electron reduction and generate $O_2^{\bullet-}$ (39). However, depending on different mitochondrial states of respiration, the percentage of oxygen that forms superoxide ranges from 0.1% in state 3 to 2% in state 4 (266,305). Other studies have shown that both complexes I and III of the electron transport chain are particularly involved in ROS production through a leakage of electrons (20,229). This was demonstrated by the use of specific inhibitors of complex I and III-derived ROS such as rotenone and antimycin, respectively (364).

4.2.2. NADPH oxidases

The NADPH oxidases (NOXs) transfer electrons across biological membranes and form $O_2^{\bullet-}$. NOX family includes 7 NOX isoforms (NOX1, NOX2/gp91^{phox}, NOX3, NOX4, NOX5, DUOX1, DUOX2), 2 organizer subunits (p47^{phox}, NOXO1), 2 activator subunits (p67^{phox}, NOXO1) and 2 DUOX-specific maturation factors (DUOXA1 and DUOXA2). NOX enzymes are primary known to produce $O_2^{\bullet-}$ in both phagocytic and non-phagocytic cells (26). A small GTPase named Rac1 has been described as an important activator of NOX1 and 2 in various cell types including skeletal muscle (317) and also leads to superoxide production in non-phagocytic cells (140). In skeletal muscle cells, NOX isoforms 2 and 4 are found at the plasma membrane, transverse tubules and sarcoplasmic reticulum where they modulate calcium release (261). They are described as the major source of ROS during skeletal muscle contraction (217). In this context, pharmacological inhibition of NOX-induced ROS production has been widely used, although these inhibitors are mainly unspecific and not isoform selective (review in (8,65)).

4.2.3. Xanthine oxidase

Xanthine oxidoreductase (XOR) is an intracellular enzyme involved in purine catabolism, which includes 2 isoenzymes: xanthine dehydrogenase (XDH) and xanthine oxidase (XO). XOR catalyzes the reduction of hypoxanthine and xanthine in uric acid. The oxidase form is used as an electron acceptor and both hypoxanthine and xanthine are reduced into uric acid and $O_2^{\bullet-}$ (210). The role of XO as a source of ROS generation has been demonstrated using allopurinol, a specific inhibitor. In both animals and humans, allopurinol prevents oxidation of glutathione (GSH) and lipid peroxidation (343). Interestingly, XO is a major source of ROS in skeletal muscle atrophy following unloading and in sarcopenia (82).

4.2.4. Other sources

The endoplasmic reticulum is another organelle involved in ROS production (50,199). In addition, various enzymatic systems such as phospholipase A2 induce ROS production by stimulating NOX. Studies reported both calcium-dependent and calcium-independent forms of phospholipase A2 as key actors in ROS generation in skeletal muscle (124,236).

5. Major antioxidants in skeletal muscle

Enzymatic and non-enzymatic antioxidant systems exist to detoxify excess ROS, prevent and repair oxidative damage, and maintain redox homeostasis in the cell (127). Halliwell and Gutteridge have defined an antioxidant as "any substance that delays, prevents or removes oxidative damage to a target molecule" (129). This definition suggests that this evolutionary conserved system exerts its antioxidant functions in 3 distinct ways: by converting ROS into less deleterious molecules, by reducing pro-oxidant molecules such as metallic ions, and by scavenging ROS. Thus, the complex antioxidant network works synergistically to protect cells from redox disturbances and oxidative damage. Interestingly, there is a growing body of evidence indicating that antioxidants are not only ROS-scavenger molecules, but also act as signaling molecules that are required both for the maintenance of cell homeostasis and for adaptive cell responses (277). Human Atlas Proteome (78,330) and the Redox Atlas of the Mouse (120) provide a global analysis of tissue-specific proteome. Based on these large-scale projects and the available literature, we present hereafter the skeletal muscle tissue-specific antioxidant system.

5.1. Endogenous

Among endogenous antioxidants, GSH, superoxide dismutases (SODs), catalase (CAT) and members of the thioredoxin (Trxs) family contribute to cell protection and regulation of redox signaling.

5.1.1. Superoxide dismutases

SODs are enzymes that catalyze the dismutation of $O_2^{\bullet-}$ into H_2O_2. SODs are divided into 3 isoenzymes (SOD1, SOD2, SOD3), which all require redox active transition metals in the active site (261). SOD1 (CuZnSOD) is a dimer of 32.5 kDa associated with one copper ion and one zinc ion and is mainly localized in cytosol and the mitochondrial intermembrane space (**Figure 8**). SOD2 (MnSOD) is a tetramer of 24.7 kDa, which is associated with one manganese ion. SOD2 is mainly located in mitochondria. SOD3 is an extracellular tetradimer of 30 kDa, which uses copper-zinc as a cofactor. Deletion of SOD1 in skeletal muscle induces a phenotype of accelerated age-related loss of skeletal muscle mass and function (291).

5.1.2. Catalase

CAT is a homotetramer of 240 kDa composed of 4 subunits, which requires iron as a cofactor. CAT is mainly localized in peroxisomes (**Figure 8**), which were originally named to highlight their content in enzymes either producing (oxidases) or breaking down (catalase) H_2O_2 (24,336). Therefore, it was originally thought that the primary function of these organelles was to control H_2O_2 metabolism (review in (194)). However, peroxisomes also have an established role in lipid metabolism, as well as emerging functions in cellular signaling relevant to metabolism (194). The function of CAT is to catalyze the dismutation of H_2O_2, which leads to the formation of one molecule of H_2O and of O_2. It has been shown that a decline of CAT protection, associated with elevated H_2O_2 levels, is observed at the onset of sarcopenia, which is the loss of muscle mass observed during aging (315).

5.1.3. Glutathione

GSH is the most abundant low molecular weight component in the cell. It functions as a buffer of the cellular redox status and as an antioxidant in skeletal muscle (126). This tripeptide synthetized in liver exerts its antioxidant functions through several features: as a substrate for glutathione peroxidases (Gpxs) and as a reducing agent for vitamin C and E radicals. GSH is predominantly found in the cytosol but is also distributed in various organelles such as the nucleus, mitochondria and endoplasmic reticulum (107). GSH is involved in $NO^{\bullet}$ metabolism since GSH and peroxinitrite lead to the formation of S-nitroglutathione (GSNO) that is able to generate $NO^{\bullet}$ (129). In skeletal muscle fibers, GSH is present at millimolar concentrations. Higher levels of GSH are found in type I muscle fibers, which have an oxidative/aerobic metabolism, as compared with type II fibers, which rely mainly on a glycolytic/anaerobic metabolism (185).

5.1.4. Family of thioredoxins

The thioredoxin family of proteins encompasses Trxs, Gpxs, glutaredoxins (Grx) and peroxiredoxins (Prx). These proteins share common structural sequences and are characterized by their active site motifs, containing either one or two cysteinyl residues. They are essential for the reduction of protein disulfides, protein de/glutathionylation and de/trans/nitrosylation (130,242). The Trx system includes 2 oxidoreductases Trx1 and Trx2 and a thioredoxin reductase (TrxR). Trx1 and TrxR1 isoforms are found in the cytosol, Trx1 is translocated into the nucleus to alter the redox state of transcription factors, while Trx2 and TrxR2 isoforms are located in the mitochondria (283) (**Figure 8**). Trxs serve as donors for Prxs to reduce peroxides. In the Trx system, electrons are transferred during a multiple step process from NADPH to Trxs to ultimately reduce disulfides and generate antioxidants (208). Trx1 and

related redox proteins are constitutively secreted by skeletal muscle cells and have been suggested to belong to myokines (203). Moreover, a decreased expression of Trx1 is observed during muscle atrophy that may be responsible for the enhanced oxidative damage during the late phase of disuse muscle atrophy (207). Grxs also modulate the thiol redox state of several proteins but no study has reported a role in skeletal muscle (130). Gpxs catalyze the reduction of H_2O_2 and organic hydroperoxides to water and alcohol, respectively (42,136). In skeletal muscle, Gpx activity is strongly decreased in cancer cachexia (314) and Gpxs have been involved in the regulation of MuSC function (see section 7). Prxs play multiple roles as peroxidases, sensors and regulators of peroxides. Interestingly, a role for Prx in H_2O_2-mediated cellular signaling has been described (354). The expression of Prx3 is decreased during muscle atrophy (207).

5.2. Exogenous

Exogenous molecules with antioxidant properties are generally classified as vitamins, trace elements or phytochemicals. Vitamin E is a term encompassing 4 tocopherols (α,β,δ,γ) and 4 tocotrienols (α,β,δ,γ) molecules. The α-tocopherol is the most abundant form in nature. Vitamin E, considered a master antioxidant, has the ability to prevent lipid peroxidation and other radical-driven events. It is mainly located in the plasma membrane (**Figure 8**) and acts as a chain-breaking antioxidant that prevents the propagation of free radical-reactions (43). Vitamin E reacts with oxidants to form tocopheryl radicals that are further reduced by vitamin C into non-radical α-tocopherols. This process requires GSH that gives an electron, leading to the formation of oxidized GSH. Vitamin E is required for skeletal muscle plasma membrane repair (141,176). However, the underlying cellular and molecular

mechanisms of this phenomenon remain to be determined (166). Ascorbate is the most common form of vitamin C found in the organism. This water-soluble molecule, localized in the extracellular compartment has special functions in redox reactions, as a powerful antioxidant and enzyme cofactor, notably of hydroxylases (204,325). Vitamin C has the ability to protect against lipid peroxidation by acting as a ROS scavenger and by one-electron reduction of lipid hydroperoxyl radicals *via* the vitamin E redox cycle (129). The dehydroascorbic acid (oxidized form of vitamin C) thereby formed can be reduced by TrxR and GSH (189), underlying the synergy within the antioxidant network. Finally, trace elements such as selenium, copper, manganese and zinc play important roles to support antioxidant functions and redox exchanges of many enzymes. Generally, these molecules play a role of substrate or cofactor and their deficiency leads to oxidative stress (116,246). They are likely important in skeletal muscle function, as exemplified by the reduced expression of selenoprotein N (*sepn1*) that triggers a congenital muscle disease (14), highlighting the role of trace elements in skeletal muscle homeostasis.

6. General mechanisms of redox signaling

In skeletal muscle, ROS/RNS have been identified as playing key roles in numerous physiological processes including glucose uptake (163), excitation/contraction coupling (261), mitochondrial biogenesis (145), training adaptations (122,281) and cell death (295). This underlines the need to understand how ROS/RNS act as determinant regulators of biological effectors involved in the control of homeostasis (90,302). It is suggested that oxidation and reduction of the thiol side chain of cysteine amino acids are the major mechanisms by which oxidants integrate signal transduction pathways (85).

6.1. Thiol oxidation as a major mechanism of oxidation-related post-translational modifications

Oxidants mainly exert their effects by targeted modifications of thiol residue (SH) of cysteine amino acids resulting in reversible or irreversible oxidations that depend on the state of oxidation (**Figure 9**). It results in the formation of sulfenic acid (SOH) that can be further oxidized in sulfinic (SO_2H) and in sulfonic (SO_3H) acids. Additional post-translational modifications involving $NO^{\bullet}$ or GSH are also observed and identified as S-nitrosylation and S-glutathionylation, respectively (17,35,85). Such chemical reactions are under the control of pKa, pH and local protein environment (127).

6.2. From signal transduction to post-translational modifications

The role of ROS in signaling has emerged with the observation that H_2O_2 production was necessary for normal signaling of growth factors, suggesting that ROS/RNS may act as secondary messengers (316). More recently, Jones highlighted the role of "radical-free" biology of oxidative stress, indicating that the free radical chain reactions are almost completely prevented in biological systems due to the very high concentrations of antioxidant proteins (159). He reported that 10% of unbalanced pro- and antioxidant reactions involve free radical intermediates and that less than 0.1% induce macromolecular damage. Hence, the non-radical H_2O_2 appears to be an ideal candidate for redox-dependent signal transduction (*e.g.* large diffusion, weak oxidant, reversible oxidation inductor) (105,152). Several mechanisms are described for H_2O_2-dependent signal transduction. One proposes H_2O_2-dependent signaling *via* a relay system involving a redox sensor (Prx2), which

oxidizes the target and forms a transient disulfide bond (310). Another mechanism is the floodgate model (354). It is based on the local inactivation of antioxidant defenses, such as Prx1, that allows low H_2O_2 levels to mediate signaling in a specific cell compartment. Although less well-described, two other mechanisms have been proposed. The first includes an intermediate step involving the oxidation of Trx or GSH to the redox relay mechanism, and the second one likely results from the dissociation and subsequent activation of a target protein following H_2O_2 oxidation of a scavenger enzyme (272). In addition, $NO^{\bullet}$ and its derivatives (S-nitrosothiols (SNO) and GSNO) are involved in signal transduction (103,152,276,338). These molecules induce modifications of protein structure, thus affecting protein functions as well as the signaling pathways in which these proteins are involved.

Technological advances such as proteomic and redox proteomics (60) have recently revealed that 500 proteins contain reactive and potentially modulatory cysteine residues (162,345). Among the targeted proteins detected in the "redoxome", are transcription factors (NFκB, FOXO), kinases (AMP Kinase, mTOR), protein tyrosine phosphatases, proteases (caspases, metalloproteases), and chaperones proteins. This illustrates the broad impact that oxidation of conserved cysteine residues may have in various physiological processes from signal transduction to gene expression (review in (152)).

In response to oxidative stress, cells modulate their gene expression (7). Several transcription factors have been described as redox-sensitive and are involved in various cellular signaling pathways with distinct outcomes. Nuclear factor kappa B (NF-κB) is the first eukaryotic transcription factor which has been shown to respond directly to oxidative stress in the L6 skeletal muscle myoblasts upon exposure to the non-radical ROS hydrogen peroxide and for which activation was controlled by the

cell glutathione disulfide/glutathione (GSSG/GSH) ratio (301). Additionally, NF-κB is involved in the regulation of a wide array of cellular processes including immune response, cellular survival, proliferation, and differentiation (review in (18)). Interestingly, NF-κB activity is impaired as a consequence of S-glutathionylation of IKK-β cysteine 179 and is associated with a decrease of pro-inflammatory signals (275). Redox factor-1 (Ref-1) contains in its N-terminal region a redox regulatory domain, which is characterized by 2 cysteines that are involved in the redox-dependent modification (reduction) of NF-κB and of other transcription factors such as activator protein-1 (AP-1) (Fos and Jun), p53, and hypoxia-inducible factor (HIF)-1α. The oxidized form of AP-1 exhibits poor DNA-binding activity, due to the cysteine residues within the DNA-binding domain of both Fos and Jun. Inversely, AP-1 is mainly activated by antioxidants (192,300). Of interest, these redox-sensitive transcription factors NF-κB and AP1 trigger the synthesis of cytoprotective regulatory proteins such as SODs, catalase and heat shock proteins after the delivery of ROS generated by the skeletal muscle itself upon contractile activity (149).

The transcription factor NF-E2 p45-related factor 2 (Nrf2) is another well-known redox-sensitive master regulator that controls the expression of networks of genes encoding proteins with diverse cytoprotective activities. Nrf2 itself is primarily controlled at the level of protein stability. Under basal conditions, Nrf2 is a short-lived protein that is subjected to continuous ubiquitination and proteasomal degradation. The most common ubiquitin ligases that contribute to the degradation of Nrf2 are Kelch-like ECH-associated protein 1 (Keap1) and a substrate adaptor protein for Cullin 3 (Cul3)/Rbx1 ubiquitin ligase. Keap1 is considered to be a sensor for a wide array of small-molecule activators of Nrf2 including ROS. These latter block the cycle of Keap1-mediated degradation of Nrf2 by modifying specific cysteine residues within

Keap1 (87) or by directly disrupting the Keap1:Nrf2 interaction. As a consequence, Nrf2 is not degraded, and the transcription factor accumulates and translocates to the nucleus, where it forms a heterodimer with a small Maf protein and binds to antioxidant-response elements in the upstream regulatory regions of its target genes, and initiates transcription (133).

In skeletal muscle, which is an active metabolic tissue, insulin signal transduction is crucial. Insulin signaling has been shown to be altered by ROS (such as 4-hydroxy-2-nonenal and peroxynitrate) (13) and to be regulated by the selenoproteins Gpx1 and Sepp1 (through the inhibition of energy metabolic mediators AMPK and Akt) (312).

7. Redox regulation of MuSCs during skeletal muscle regeneration

During skeletal muscle regeneration, MuSCs undergo a series of processes called adult myogenesis, which includes activation and entry in the cycle, proliferation, exit from the cell cycle and commitment into terminal differentiation, followed by fusion into multinucleated cells (myotubes *in vitro* and myofibers *in vivo*) (**Figure 2**). Various aspects of adult myogenesis have been shown to be, or are likely redox-regulated (264,361) (**Figure 10**).

7.1. Viability - apoptosis

ROS and redox reactions have been described to regulate cell survival through the modulation of transcription factors (NF-κB, AP-1, Nrf2…) and pro-apoptotic proteins (caspases, Bcl-2) (247,270). The role of oxidative stress in cell death pathways is supported by the fact that antioxidants can protect cells against apoptosis (247). In skeletal muscle, long-term exercise has been shown to reduce

ROS generation, to improve antioxidant status and to decrease oxidative stress that should serve to attenuate any ROS-induced activation of apoptosis (265). Elevated apoptotic signaling has been reported in skeletal muscle during aging, stress-induced states and diseases; a phenomenon that plays a role in muscle dysfunction, degradation, and atrophy (265). During fetal myogenesis, analysis of double conditional Pitx2/3 mouse mutants in primary muscle cell cultures reveals excessive upregulation of ROS levels leading to DNA damage and apoptosis of differentiating cells (175). In adult male rats, GSH depletion in skeletal muscle increases caspase-independent signaling and increases mitochondrial-associated apoptotic events to subsequent cell death stimuli (77). Whereas apoptosis in skeletal muscle is low at rest, skeletal muscle injury causes apoptosis and necrosis (307) and is associated with ROS production (341). Therefore, protection of MuSCs from cytotoxic damage is crucial to ensure efficient regeneration. MuSC antioxidant levels have been described as a major determinant of MuSC cell therapy efficiency (91). Several antioxidant pathways have been involved in oxidative stress detoxification of MuSCs. Retinoic acid-Gpx3 pathway (96) and aldehyde dehydrogenase activity (153) improve MuSC survival. Recently, cytoglobin, a heme containing protein has been involved in the resistance to oxidative stress-induced apoptosis that results in incomplete skeletal muscle regeneration (307). Apoptosis being a highly conserved process playing a critical role in tissue homeostasis (**Figure 10**), its putative role in skeletal muscle regeneration remains to be elucidated.

7.2. Quiescence

At rest, MuSCs are maintained in quiescence (G_0 phase) (58). Transcriptomic analysis revealed an antioxidant molecular signature (250) involving the up-

regulation of Gpx3 and TrxR1, suggesting a protective effect of quiescent cells from oxidative insults. During muscle regeneration, while most of MuSCs commit to a myogenic fate and differentiate, a small population self-renews to maintain a stem cell pool for further needs (**Figure 2, 10**). $NO^{\bullet}$ was shown to increase MuSC self-renewal in a cGMP-independent pathway. This requires enhanced expression of Vangl2, a member of the Wnt planar cell polarity pathway that controls the homeostatic number of MuSCs (179).

7.3. Activation

Upon muscle injury, MuSCs rapidly enter the cell cycle. Although not fully understood yet, several pathways involved in the control of MuSC activation are redox-regulated (**Figure 10**).

7.3.1. Calmodulin-nitric oxide pathway

Several lines of evidence indicate that $NO^{\bullet}$ is involved in MuSC activation. *In vivo* inhibition of NOS activity by the pharmacological inhibitor N(G)-nitro-(L)-arginine methyl ester (L-NAME) prevents the immediate injury-induced MuSC activation and their subsequent proliferation (10,100,319). In the single myofiber model, $NO^{\bullet}$ donation (SIN-1) or inhibition (L-NAME) increases and decreases the number of $Pax7^{+}$ quiescent MuSCs, respectively (46). Similar changes of the number of activated MuSCs are observed in NOS deficient mice ($nNOS^{-/-}$ or $iNOS^{-/-}$) after muscle injury (280). *In vitro*, stretching myofibers induces Hepatocyte Growth Factor (a potent MuSC mitogenic factor) release in a metalloprotease and $NO^{\bullet}$-dependent manner (356). Upstream, calcium-calmodulin, known to associate with NOS in other cell types (347), activates NOS activity in MuSCs; its specific inhibition abrogates the

calcium ionophore-induced MuSC activation (320). Collectively, these data argue for a positive role of $NO^{\bullet}$ in MuSC activation following muscle injury.

7.3.2. Sphingolipids

Sphingolipids are a group of lipids playing a crucial role in cell membrane structure and lipid signaling. They may act as second messengers to stimulate oxidant production (11,241). Notably, ROS production from mitochondria has been identified as regulator of sphingosine 1-phosphate (254). Interestingly, sphingolipid signaling is required for the entry of MuSCs in the cell cycle and sphingosine 1-phosphate regulates MuSC proliferation *in vitro* and *in vivo* (233). Consequently, inhibition of sphingolipid signaling impairs skeletal muscle regeneration (119).

7.3.3. p38 MAPK

Mitogen-activated protein kinases (MAPKs) are members of many signaling pathways in eukaryotic cells and are known to be activated in response to oxidative stress *in vitro* and *in vivo* (161). p38δ/β MAPK are required for MuSC activation, their specific inhibition leading to a reduced expression of MyoD and the acquisition of a quiescent state (327). This may support a role of ROS in p38δ/β MAPK-dependent MuSC activation.

7.3.4. Notch

The Notch pathway is critical for multiple stages of post-natal myogenesis. Inhibition of Notch signaling abolishes MuSC activation and impairs muscle regeneration (72). In addition to MuSC activation, Notch signaling is also involved in MuSC proliferation, while it must be shut down for their differentiation through antagonism with the Wnt pathway (see below) (40,72). While there is not yet any evidence in muscle, it was recently shown that Notch is modulated by the ROS-producer NOX-1 to control the fate of colonic precursor cells (67), indicating that this

important signaling pathway in myogenesis may also be regulated by ROS during muscle regeneration.

7.3.5. Wnt

The Wnt/βCatenin pathway plays important various roles in MuSC biology and during regenerative myogenesis (review in (344)). Collectively, several Wnts, through the classical pathway and the planar cell polarity pathway, have been shown to control MuSC fate (179), their motility (31), their proliferation (248) as well as their differentiation (40). In non-muscle systems, several redox molecules regulate Wnt signaling. Trx nucleoredoxin is a putative interactor of Dishevelled along the Wnt/βCatenin pathway that would suppress the signaling in a redox-dependent manner (115). Another redox molecule, selenium, is associated with the Wnt pathway through Gpx1. Selenium restriction is associated with Wnt activation, whereas high selenium levels inhibits Wnt signaling (41,169). These studies show that ROS regulate the Wnt/βCatenin pathway. Given its importance in MuSC biology, it is likely that this pathway is also the target of redox regulation in skeletal muscle.

7.4. Proliferation

The cell cycle has emerged as a redox cycle (review in (48,59)). Twenty years ago, Burdon and colleagues stated that low concentrations of oxidants ($O_2^{\bullet-}$, H_2O_2) stimulate mammalian cell proliferation (47). Similarly, $O_2^{\bullet-}$ production by MuSCs depends on NADPH oxidases (Nox2, Nox4, p22(phox), p47(phox), and p67(phox)), and MuSC proliferation is inhibited by antioxidants (N-acetylcysteine (NAC), apocynin) or NOX silencing by controlling cyclin D1 level through Erk1/2, PI-3 kinase/AKT and NF-κB pathways (223). Furthermore, it has recently been shown that zinc promotes the MuSC proliferation in a dose-dependent manner through the

positive control of PI3K/Akt and ERK signaling pathways, although it is not known whether this occurs through a redox-dependent mechanism (244). Direct evidence for the involvement of ROS comes from genetically deficient mouse strains. Gpx1-deficient MuSCs exhibit lower proliferation rates and decreased differentiation capacity compared with wild-type MuSCs (183). MuSCs from SOD1-G93A mice (a mutation that leads to amyotrophic lateral sclerosis) demonstrate lower proliferation potential, triggering lower differentiation while the expression of cyclin-dependent kinase inhibitor 1A, which is known to promote myoblast differentiation, is reduced (205).

7.5. Myogenic differentiation

Myogenic differentiation is an important step in myogenesis that arises from reprograming of MuSCs to generate differentiated cells that eventually fuse into plurinucleated myotubes/myofibers. Detoxification and DNA repair appear to be crucial steps before the induction of skeletal muscle differentiation to avoid propagation of genomic abnormalities (263). This suggests that antioxidant and cytoprotective genes could be crucial players involved in the control of efficient myogenic differentiation, and several studies support this notion (**Figure 10**). *In vitro*, MuSC differentiation into myotubes elicits a rapid decline in NOX4 and $p67^{phox}$ expression, suggesting a change in ROS production at the time of myogenic commitment (223). Overexpression and inhibition of DUOXA1 in MuSCs reduces and enhances *in vitro* myogenic differentiation, respectively (293). *In vivo*, genetic deletion of the $p66^{ShcA}$ protein adaptor that promotes ROS formation, results in a lower mitochondrial ROS (mtROS) production and a better muscle regeneration (360). In the same line, differentiation defect is observed in a mouse model deleted

for *sepn1* gene (52), confirming previous reports on selenium deprivation of MuSCs (328). A molecular mechanism may be through NF-κB, which has been shown to regulate myogenic differentiation (18). In addition to NF-κB, the Nrf2-glutamate-cysteine ligase (GCL)/glutathione reductase (GR)-GSH signaling pathway is involved at the time of myogenic differentiation to regulate the GSH redox status after endogenous H_2O_2 is produced (259). Moreover, it has been shown that the Nrf-2 target gene carbonyl reductase 1 (CRB1), a NADPH-dependent cytosolic enzyme that reduces highly reactive aldehydes and prevents both protein carbonylation and DNA damage, is upregulated during MuSC differentiation. CRB1 inhibition is associated with an increase of ROS production, and with impaired differentiation and skeletal muscle regeneration *in vivo* (190). Consistently, skeletal muscle regeneration is strongly delayed in Nrf2 deficient animals, where alteration in the time course of myogenesis is observed and has been attributed to an alteration of MuSC proliferation/differentiation balance (4,304). Similarly, *in vitro* experiments showed that MuSCs cultured with dithiothreitol (a reductant agent) display a higher expression of the contractile protein myosin heavy chain as compared with those cultured under oxidized conditions (H_2O_2 in medium) (341). Similar results were observed on myogenic differentiation *in vitro* using the antioxidant resveratrol (160,224).

However, other studies support pro-differentiating effects of ROS in skeletal muscle. MuSC differentiation is accompanied by an increase of mtROS production (200), especially H_2O_2 (184,353). As a response, an upregulation of antioxidant enzymes, mainly with peroxidase function such as Prx2, is also observed (109,184,353). Additionally, treatment of MuSCs with mitochondria-targeted antioxidants such as MitoQ, MitoTEMPOL or MCAT alters their differentiation (184),

as does treatment with nordihydroguaiaretic acid (NDGA) (146). Particular attention was given to $NO^{\bullet}$ effects on myogenic differentiation. First, experiments using chick MuSCs showed that NOS activity transiently increases at the time of differentiation and requires Ca^{2+}, calmodulin, and NADPH (182). This transient activity is associated with the activation of NF-κB sites in the promoter region of the gene encoding iNOS. Interestingly, only differentiating, and not proliferating, cells exhibit these properties (182). Consequently, a $NO^{\bullet}$ donor (sodium nitroprusside) induces precocious MuSC fusion, while a $NO^{\bullet}$ competitor (NG-monomethyl-L-arginine) delays fusion (181). These results were confirmed in a mouse model in which L-Arginine, the substrate for endogenous synthesis of $NO^{\bullet}$, transiently increases $NO^{\bullet}$ production and triggers MuSC fusion. L-Arginine effects are prevented by the NOS inhibitor L-NAME and by L-Lysine, a competitive inhibitor of L-Arginine uptake (195). More recently, it has been shown that these $NO^{\bullet}$-mediated effects rely on mitochondria fusion at the time of MuSC fusion since $NO^{\bullet}$ inhibition enhances the activity of a pro-fissing GTPase dynamin-related protein-1 that triggers mitochondrial dysfunction (81).

Collectively, the major concern of all the above studies is the lack or inappropriate characterization and/or quantification of ROS entities. Moreover, opposite conclusions may result from the non-linear dose-response relationship of oxidative stress on biological effects. Indeed, pioneer studies showed that $NO^{\bullet}$ exerts positive or negative effects on MuSC proliferation in a dose-dependent way *in vitro* (331). Moreover, several studies showed transient ROS upregulation at the time of differentiation (181,195) suggesting a tight regulation of their release in the MuSC neighborhood. A recent study, using muscle conditional mutants of the homeodomain transcription factors Pitx2/3, highlights the importance of ROS levels in myogenesis (175). Double pitx2/3 mutants show excessive upregulation of ROS levels leading to

DNA damage and apoptosis of differentiating MuSCs, both during fetal and adult myogenesis. Pitx2/3 deficiency triggers the downregulation of antioxidant genes (such as Nrf1) and impairment of mitochondrial function. Interestingly, premature muscle differentiation occurs in single Pitx3 mutants. These various effects are directly linked to dose-dependent changes in ROS levels and can be reversed by lowering ROS with NAC. Thus a controlled increase in ROS is required for MuSC differentiation (175).

Although the impact of oxidative stress on MuSC fate is recognized, only a few studies have explored the regulation of the master genes MyoD and Myogenin by the redox balance. Cellular redox status is reflected in the balance between a number of reduced or oxidized molecular pairs including NADH/NAD. Some transcription factors and DNA modifying enzymes are sensitive to NADH/NAD ratio, implying that cellular redox status regulates gene transcription (192). At the time of MuSC activation, reprograming of cellular metabolism decreases intracellular NAD^+ levels, decreasing in turn the activity of Sirtuin 1, a NAD^+-dependent histone deacetylase. This leads to elevated H4K16 acetylation and activation of MyoD transcription. SIRT1 forms a complex with the acetyltransferase PCAF and MyoD and, when overexpressed, delays muscle differentiation (113,286). These results indicate that the redox status can control the myogenic gene expression program. Later on during myogenesis, exposing differentiating MuSCs to oxidative stress increases the nuclear localization of Focal adhesion kinase (FAK), which interacts with MBD2 (methyl CpG-binding protein 2). This leads to a decreased MBD2 association with HDAC1 (histone deacetylase complex 1) and methyl CpG sites in the myogenin promoter, thus inducing myogenin expression (198). Finally, Heme oxygenase-1 (HMOX1), which is a cytoprotective enzyme and whose expression is induced in response to oxidative

stress, inhibits miRNA processing leading to a decrease of several myomiRs (miR-1, miR-133a/b, miR-206), which triggers a reduced expression of MyoD, myogenin, and myosins. However, it is important to note that this activity is independent of the antioxidative properties of HMOX1 (172).

ROS are clearly involved in the numerous signaling pathways that control the sequential steps of MuSC fate following skeletal muscle injury **(Figure 10)**. The recent development of new tools (redox sensors, redox proteomics) will allow for a more accurate identification and quantification of these ROS and for the identification of which entity, at which level, and during which time-period, controls a specific stage of MuSC fate. Finally, MuSCs evolve in a continuously changing environment during muscle regeneration, and receive cues from their immediate microenvironment, which has been shown to be necessary for efficient regeneration, and which is also subjected to redox regulation.

8. **Redox regulation of the cells present in the MuSC microenvironment**

Skeletal muscle regeneration requires the involvement of many cell types, including vessel cells, interstitial cells and inflammatory cells, such as monocytes/macrophages (55) (**Figures 3,4**).

8.1. Myeloid cells

After skeletal muscle injury, leukocytes rapidly invade the damaged area. While neutrophils rapidly disappear in few days, monocytes convert into macrophages that exert various roles on MuSCs and on the regeneration process (289) (**Figures 4,11**). Numerous data on redox signaling in macrophages are available in the context of infection where they are shown to actively produce ROS (44,106). Inversely, only a

few studies have investigated the redox status and redox-regulation of macrophage biology during tissue injury/repair. In wound healing models in zebrafish and drosophila embryo, leukocyte recruitment depends upon a H_2O_2 signal gradient generated by DUOX activation (237,271). In skeletal muscle, as in other tissues, entering monocytes/macrophages exhibit a damage-associated inflammatory profile and release pro-inflammatory cytokines (TNF-α, IL-1β, IL-6). In skeletal muscle the most studied redox regulated factor is high-mobility group box 1 (HMGB1), an ubiquitous nuclear protein acting as a Damage Associated Molecular Pattern to signal tissue damage when released extracellularly. When cysteine residues are reduced, HMGB1 promotes the recruitment of inflammatory cells *via* its interaction with the CXCL12 chemokine to bind the CXCR4 receptor. When cysteine residues are oxidized, HGMB1 promotes the release of proinflammatory cytokines (339). After injury, an oxidized form of HMGB1 is detected in the muscle within 6 h (339). Then, an antioxidant response is observed in the regenerating muscle, notably in leukocytes, which express high levels of SOD1 and Trx, suggesting that a biphasic expression of oxidized/reduced forms of HMGB1 is required for skeletal muscle regeneration. In accordance, HMGB1 oxidation abrogates migration of MuSCs and their ability to differentiate into myotubes *in vitro* (341). To date, no study has characterized ROS production in macrophages in the context of skeletal muscle regeneration or tissue repair. iNOS is expressed by inflammatory macrophages that infiltrate a tissue upon infection. It is also expressed by inflammatory macrophages during the first steps of muscle regeneration, its expression declining with time, and notably in restorative anti-inflammatory macrophages (227). It has been shown that mice deficient for iNOS (which is mainly expressed by macrophages infiltrating the injured muscle) fail to regenerate properly because of a defect of MuSC proliferation

and differentiation, associated with an increase of inflammatory markers (280), indicating a role of macrophage-derived iNOS in skeletal muscle regeneration.

The pro-inflammatory phase ends with phagocytosis of muscle debris, at which point macrophages switch their phenotype to give rise to anti-inflammatory/restorative macrophages (15,227). These restorative macrophages produce soluble factors (IL-10, TGF-β , VEGF, IGF-1) to dampen inflammation and stimulate tissue repair, notably triggering myogenic differentiation (**Figure 11**) (15,227,290). The switch (skewing) of macrophage phenotype occurring during skeletal muscle regeneration is a key feature of efficient repair (227). Interestingly, all effectors identified so far as regulators of this process are redox-sensitive. The MKP-1/p38MAPK axis, which belongs to the redox-sensitive MAPK family (7), regulates macrophage phenotype skewing in a timely way to promote the resolution of inflammation (257). Another redox sensitive pathway, CREB-C/EBPβ (27) is required for the expression of the anti-inflammatory program in macrophages (285). A third effector is AMPKα1, which is required in macrophages for the acquisition of the anti-inflammatory phenotype, ensuring efficient muscle regeneration (227). AMPK is linked to redox-based signals, notably it is targeted by pro-oxidant species, suggesting a role for AMPK in the tuning of redox-dependent processes (review in (51)). A fourth molecule has been involved in the skewing of macrophages during skeletal muscle regeneration. Insulin Growth Factor-1 (IGF-1), which is a master regulator of myogenesis and skeletal muscle growth, is expressed mainly by macrophages during muscle regeneration and is required for their skewing in an autocrine way (324). One study reported that ROS suppression (UK-134 and α-lipoic acid) restores IGF-1 sensitivity of diabetic fibroblasts and improves their healing functions *in vitro* and *in vivo* (37).

In vitro studies have shown that ROS/RNS production is needed for macrophage skewing. Inhibition of $O_2^{\bullet-}$ production specifically blocks the differentiation of alternatively activated (G-CSF+IL4) (so-called M2) macrophages. *In vivo*, continuous administration of a ROS inhibitor (butylated hydroxyanisole) efficiently blocks the occurrence of Tumor Associated Macrophages, which exhibit a M2 phenotype in mouse cancer models (362). Similarly, macrophages issued from $SOD1^{-/-}$ mice display a pro-inflammatory profile as compared with WT macrophages (134). Inversely, in SOD1-Tg mice, where SOD1 is overexpressed and H_2O_2 generation is enhanced, macrophages exhibit an anti-inflammatory status, associated with an increased activation of STAT6 (134), which is known to trigger the expression of anti-inflammatory genes in macrophages (178).

8.2. Endothelial cells

Skeletal muscle regeneration is associated with the angiogenic process, in order to rebuild the vascular bed after injury. Privileged interactions have been described between MuSCs and ECs. MuSCs are located close to capillaries and their number increases with the capillarization of myofibers (63). MuSCs express several angiogenic factors, including VEGF, and exhibit angiogenic properties (278). Similarly, ECs release factors supporting MuSC growth. Thus MuSCs and ECs interact to support skeletal muscle regeneration (63,177,226). While the role of ROS in these processes has not yet been investigated in skeletal muscle, there is a considerable amount of evidence coming from other models that ROS modulate angiogenesis through the regulation of redox-sensitive signaling pathways. This results in the control of EC growth, proliferation and migration (review in (95,111)). ECs generate ROS such as $O_2^{\bullet-}$ and H_2O_2. NOX is the main source of ROS in ECs

and is activated by growth factors, cytokines, hormones and hypoxia (125). Exogenous ROS, as well as overexpression of NOX4, increase the expression of VEGF, VEGFR2 and Angiopoietin 1 and stimulate EC proliferation, migration and angiogenesis *in vitro* (1,64,79,334). Furthermore, Angiopoietin 1-induced angiogenesis is enhanced in CAT deficient mice, suggesting an involvement of H_2O_2 in angiogenesis (168). Conversely, in Tie2-driven CAT transgenic mice (where CAT is constitutively expressed in ECs), impairment of neovascularization and reduced vessel sprouting following injury are observed (333). In the same line, silencing the 66^{ShcA} protein adaptor, which promotes ROS formation and by which phosphorylation is enhanced by VEGF, inhibits both VEGF-induced ROS generation and EC proliferation and migration (245).

Few studies have directly investigated the role of ROS in the environment of MuSCs during skeletal muscle regeneration. However, evidence from other models of tissue repair argues for the redox regulation of both immune cells and ECs, which likely takes place in the skeletal muscle milieu.

9. Skeletal muscle regeneration in pathophysiological contexts

9.1. Exercise-induced muscle damage

In a pioneering paper, Davies and colleagues demonstrated an increase in free radical concentrations in rat skeletal muscle following exercise to exhaustion (80). Since then, aerobic and anaerobic exercises have clearly been shown to increase ROS/RNS production in humans (102)). The relationship between ROS and contractile activity has also been widely investigated. $NO^{\bullet}$ derivatives and ROS

modulate contractile function of skeletal muscle (273). At rest and during exercise, skeletal muscle produces both species (261) and produces antioxidant defense systems as an adaptive response (154). It is now acknowledged that low and physiological levels of ROS are required for force production, while high levels of ROS promote contractile dysfunction, as a result of oxidative damage of proteins and lipids, triggering muscle weakness and fatigue (261).

Exercise-induced muscle damage (EIMD) is particularly induced by eccentric contractions, which require lengthening of the muscle (112,143,262). Following physical exercise with repeated eccentric contractions, such as long-distance or downhill running, soreness appears within a few hours following the effort, a phenomenon called delayed-onset muscular soreness (DOMS). Despite an unclear etiology, DOMS is characterized by various markers such as histological changes (Z-line disruption), biochemical changes (increased creatine kinase and lactate dehydrogenase in plasma/serum) and altered clinical scores (increased soreness, reduced specific force, decreased range of motion). Several studies showed that damaging exercises are temporally associated with an increase in ROS production (66) and oxidative stress markers (239). Lipid peroxidation markers such as malondialdehyde (MDA), thiobarbituric-acid reactive substances, or protein carbonyls are increased in the blood (99,240) and in the skeletal muscle (218). In parallel, no meaningful alteration of antioxidant enzyme activity is reported in the blood (review in (180)). In whole muscle lysates, the GSH/GSSG ratio is slightly decreased at 2 and 4 days post-exercise, while the levels of antioxidant enzyme activitity only slightly increase, most notably when compared with markers of oxidative stress/damage (218,240,313). Moreover as Insulin Receptor Substrate-1 oxidation is induced after a strenuous exercise, it was proposed as a possible explanation for the impairment of

insulin-induced glucose uptake observed in damaged muscle, through the alteration of insulin signaling (12).

A difficulty of such skeletal muscle physiology studies is to evaluate the level of myofiber damage, which is dependent not only on the type of exercise, but also on the status of the subject (healthy/pathologic, trained/sedentary, young/old, etc.) (253). Thus, the link between EIMD and MuSC activation is still unclear. Several levels of EIMD, classified according to the loss of muscle force, have been proposed and linked to the relative extent of inflammation (253). Similar comparative investigations should help to determine the role of ROS in EIMD.

9.2. Aging

Aging is a process characterized by an impairment of functional capacities. Many studies on ROS during aging were inspired by Harman's "free radical theory of aging" (131), which reports that "aging and the degenerative diseases associated are attributed basically to the deleterious side attacks of free radicals on cell constituents and on the connected tissues". Later on, the identification of mitochondria as the main source of ROS and oxidative damage during aging strengthened this free radical theory of aging (221). Interestingly, aging is not only associated with an increase of oxidative damage, but also with an up-regulation of antioxidant enzyme levels in skeletal muscle. Indeed, aging increases skeletal muscle content of Trx1, Trx2, TrxR2, cytosolic and mitochondrial SOD, catalase and Gpxs (86,155).

Aggregation of damaged proteins, mitochondrial dysfunction, epigenetic alterations and accumulation of toxic metabolites (ROS) and of DNA damage are hallmarks of aged stem cells (243). In skeletal muscle, proteomic investigations revealed that aged skeletal muscle is targeted by oxidative protein post-translational

modifications such as carbonylation that alters protein functions (19). Moreover, aging causes a reduction in redox-sensitive proteins involved in energy metabolism, indicating a loss in the flexibility of the redox energy response (211). While numerous studies have investigated the impact of ROS and its regulation on aged skeletal muscle (review in (12,148)), few experimental data establish a link between MuSC aging and ROS **(Figure 12)**. In aged muscle, MuSCs exhibit a reduced capacity to proliferate and self-renew while they are prone to differentiation. This is explained by a deregulation of several signaling pathways, including decreased Notch signaling (71), increased Wnt signaling (36), increased JAK-STAT3 (322) and p38 MAPK (32) signaling pathways. All these pathways are redox-sensitive as discussed above (see section 7.3). Only a few studies directly investigate ROS in aged MuSCs. *In vitro*, old MuSCs are refractory to stretch and $NO^{\bullet}$ induced activation, a property that is rescued by $NO^{\bullet}$ donors (33). Increase in oxidation of DNA and lipids is observed in the elderly MuSCs, together with a reduction in CAT and glutathione transferase activities (114). Similarly, an increase in lipid peroxidation is observed in myotubes derived from aged MuSCs, and is associated with a defective status of the excitation-contraction coupling apparatus (25). Cultured aged MuSCs exhibit increased $O_2^{\bullet-}$ /mitochondrial mass and H_2O_2/mitochondrial mass ratios. The increased ROS production correlates with the corresponding decreased mitochondrial ATP production (220). The antioxidant Nrf2 signaling is impaired in aging muscle (219). Of note, in Nrf2 deficient mice subjected to acute endurance exercise stress, alteration in the expression of the MuSC markers Pax7 and MyoD is observed along with the activation of ubiquitin and apoptotic pathways (234). More recently, studies demonstrated that autophagy has an important role in sustaining the higher bioenergetic demands required for MuSC activation (318) and maintaining MuSC

stemness by preventing the senescence caused by mitochondrial dysfunction and oxidative stress during aging (117). Hence, it is clear that ROS and autophagy participate in skeletal muscle homeostasis (193,268) and are involved in muscle dystrophies (249); however, it is still unclear whether ROS are crucial for the induction of autophagy and whether up or down regulation of these processes induces a detrimental or a beneficial response (282). Another redox sensitive mechanism has recently been involved in the maintenance of aged MuSCs. Reduction of cellular NAD^+ pool (which is required for mitochondria function) blunts the adaptive mitochondrial unfolded protein response (mtUPR) pathway that leads to the loss of MuSCs and decrease of their regenerative capacities (361). Although the precise mechanisms are still to be deciphered, these studies show that in aged skeletal muscle, higher ROS provide an unfavorable environment, which can adversely affect MuSC survival and functions and limit skeletal muscle repair (9).

9.3. Muscular dystrophies

The increase of oxidative stress in muscular dystrophy, which was acknowledged a long time ago (132,228), has been shown to correlate with the severity of the pathology (274) and is considered to contribute to the pathology of several muscular dystrophies (173). Interestingly, mutations in antioxidant *sepn1* gene cause SEPN1-related myopathy, a different class of muscle disease (14). While oxidative damage is assumed to participate in the pathogenesis of the disease, the exact mechanisms are still poorly understood, particularly at the level of the sarcolemma, where there is no direct experimental evidence of myofiber damage by oxidative stress (review in (321)). Calcium homeostasis is perturbed in muscular dystrophies (6). In this context, it has been shown that S-nitrosylation of the Ryanodine Receptor 1 (RyR1), an

intracellular calcium channel essential for the excitation-contraction coupling of the myofiber, induces a leak of sarcoplasmic reticulum Ca^{2+}, contributing to muscle weakness (28,29,94). Nevertheless, cellular and molecular mechanisms involving ROS signaling in the MuSC defects observed in muscular dystrophies are still poorly understood **(Figure 13)**. The first evidence came from cultured MuSCs derived from either normal or dystrophic muscles. In mice, dystrophin deficient MuSC-derived myotubes are more susceptible to free radical-induced injury (269). The relative susceptibility of MuSC-derived myotubes to oxidative stress is associated with the severity of the dystrophy (assessed by the expression of different lengths of the dystrophin protein), and the extent of protein oxidation, suggesting that the DCG complex has important functions in antioxidant defense (88). It was shown that nNOS, which binds to DGC, has no impact on MuSC susceptibility to oxidative stress (363). Inversely, increased susceptibility of dystrophin-deficient-derived myotubes to oxidative stress is associated with altered phosphorylation of MAPKs, including JNK, p38 and ERK1/2 (309), which are both targeted by ROS and involved in myogenesis (see above, section 7). Susceptibility to oxidative stress seems to be a common feature in several muscular dystrophies. Indeed, MuSCs derived from affected muscles in facioscapulohumeral muscular dystrophy (FSHD) patients exhibit higher susceptibility to an induced oxidative stress than MuSCs derived from non-affected muscles of the same patients (350). This is associated with a defect in the size and branching of the differentiated myotubes (23). At the regulatory levels, it was shown that the transcriptional repressor Stra13, which is elevated in dystrophin deficient mdx mice, protects MuSCs from oxidative damage while Stra13 deficiency exacerbates muscular dystrophy (340). Epigenetic regulation has also been reported to control redox signaling and MuSC fate. The Polycomb group protein Bmi1, which

is an essential epigenetic regulator of stem cell function, is upregulated during normal skeletal muscle regeneration, whereas the number of MuSCs expressing high Bmi1 level is reduced in mdx muscle. Upregulation of Bmi-1 in MuSCs in mdx triggers the increase of metallothionein that confers MuSCs the ability to resist oxidative stress and to limit DNA oxidative damage, thus improving the pathology (84). These data confirm that ROS are elevated in muscular dystrophies and that MuSCs are more sensitive to oxidative stress, altering their functions and participating in the skeletal muscle deterioration in these progressive diseases.

Only a few studies have investigated the role of oxidative stress in the non-myogenic cell types in muscular dystrophies. It is assumed that NO deficiency in DMD contributes to the pathogenesis of the disease (69). In transgenic mdx animals expressing normal NO levels (through the constitutive expression of nNOS), an improvement of the muscle state is observed with a decrease of inflammation and of sarcolemma injury (346). In this context, long term treatment of dystrophic mice with HCT 1026, a nitric oxide-releasing derivative of flurbiprofen, ameliorates the morphological, biochemical, and functional phenotype of the diseased muscle (45) through a decrease of the number of inflammatory cells and FAPs, thus reducing fibrosis (74).

10. Therapeutics opportunities

Various strategies have been used to improve recovery of skeletal muscle function. Here, we present data related to exercise and antioxidant supplementation as approaches to improve skeletal muscle homeostasis.

10.1. Exercise as therapy

Skeletal muscle is a secretory organ. Upon contractile activity, it releases myokines that are known to control hypertrophy (LIF, IL-4, IL-6, IL-7, IL-15) (review in (256)). Some myokines have also been identified as crucial molecules involved in skeletal muscle regeneration (290). Few studies have investigated the role of exercise/contractile activity following skeletal muscle injury. It appears that exercise (running) increases skeletal muscle mass and cross sectional area of regenerating muscle as well as recovery of metabolic properties (171,279). These effects can be explained by a higher activation of the mTOR pathway, the master regulator of cell growth and protein synthesis previously described as a redox-regulated molecule (294) and of PGC-1α (171), the master regulator of oxidative metabolism (191,196).

Exercise, through ROS production, initiates the transcription of antioxidant genes and specific signaling pathways (122,154). Thus, an increased production of ROS may act as an activator of the adaptive response in skeletal muscle, resulting in the increased production of antioxidant enzymes that could facilitate rapid muscle remodeling and provide protection against subsequent periods of damaging exercise (209). However, no study specifically investigated the effect of exercise on ROS in MuSCs. In contrast, endurance exercise of long duration and/or high intensity appears to generate much higher levels of ROS that can overwhelm cellular antioxidant defenses, and may cause tissue damage. This paradoxical effect of physical exercise refers to the hormesis theory (267). Such ROS-induced damage requires effective repair systems, and sufficient recuperation, before exercise adaptation can recommence (288). However, the over generation of ROS observed during acute exercise is blunted in endurance trained subjects, and most studies demonstrate that post-exercise oxidative stress is reduced after endurance exercise training (267). The main mechanism for this reduction in oxidative stress is likely the

upregulation of enzymatic antioxidants in response to an exercise-training program (261). SOD, one of the initial antioxidant enzymes expressed in response to oxidative stress, has been regularly reported to increase in response to exercise training (185) whereas activity of Gpx demonstrates a muscle fiber-specific pattern with type IIa being the most responsive to training (260). On the contrary, measurement of CAT activity in skeletal muscle in response to exercise training gives inconsistent results.

Aged skeletal muscle is more susceptible to damage, particularly following exercise and is unable to rapidly adapt following sequential periods of exercise. However, the elderly who are physically active benefit from exercise-induced adaptation in cellular antioxidant defense systems (326). Improved skeletal muscle mechanics, strength, and endurance make them less vulnerable to acute muscle injury and chronic inflammation (138). Therefore, many critical questions remain regarding the relationship of aging, ROS and exercise. At this time, there is no recommendation for exercising in dystrophic patients. It could be hypothesized that patients receiving pharmacological/gene/cell therapy could practice adapted exercise with exclusive concentric contraction to release beneficial myokines and adaptive responses to free radicals.

10.2. Antioxidant supplementation

10.2.1. In EIMD

The use of antioxidant in the field of physical exercise is still a controversial issue (review in (122,212,258)). Some groups have reported that antioxidant supplementation has a negative effect on training-induced adaptations and health (73,121,201,202,252,281). On the contrary, other groups reported no alterations of

training-induced performance improvements (225,357,358). These conflicting results have to be considered in the light of training status, type of exercise, qualitative and quantitative supplementation. Further studies are needed to elucidate this complex relationship. A wide range of studies has used nutritional antioxidant supplementation as a countermeasure to improve recovery following EIMD (212,231). Indeed, studies have investigated the role of vitamin C, vitamin E, combined antioxidant supplementation or NAC as potent protectors against skeletal muscle damage after eccentric contraction. Antioxidant supplementation before and 7 days after intensive knee maximal eccentric contraction reduces the loss of force following exercise as compared with the control group (303). However, some data indicate that antioxidants may delay muscle recovery (66,218). Indeed, prolonged vitamin C supplementation two days before and fourteen days after downhill running exercise reduces muscle soreness and MDA levels, but is associated with a delayed recovery of muscle function (66). In 2013, a double-blind study suggested that the modulation of redox biology by NAC supplementation for 8 days following quadriceps eccentric contractions at low velocity may interfere with skeletal muscle repair and myogenic program through the modulation of transcription factors involved in inflammation and myogenic program (218). In view of these results, there is little evidence to support a beneficial role of antioxidant supplementation in response to skeletal muscle damage (231).

As for the investigation of the ROS mechanisms in MuSCs, few studies explored the effects of antioxidant treatment on MuSC behavior following EIMD. In fact, to our knowledge, only one group has investigated the effects of antioxidant administration during post-contusion skeletal muscle regeneration at the cellular level. *In vivo* administration of polyphenol in rats 2 weeks before and after the injury induces an

accelerated activation of MuSCs, and consequently, a rapid neofiber formation that is completed earlier than in the control group (232). Furthermore, polyphenol treatment blunts the entry of neutrophils into the injured muscle, while the kinetics of macrophages and of their skewing to restorative macrophages is accelerated, and the production of the anti-inflammatory cytokine IL-10 is increased (174,232).

10.2.2. In aging

So far, most studies performed to counteract oxidative stress in aged muscle have targeted sarcopenia and muscle atrophy. Results consistently report significant improvements of antioxidant biomarkers after antioxidant supplementation while the effects on muscle function are more mitigated (review in (53)) (**Figure 14**). However, increased mitochondrial antioxidant activity, using transgenic mice with targeted overexpression of the human *cat* gene to mitochondria (mtCAT mice), restores age-dependent skeletal muscle dysfunction (332). This suggests that the preservation of muscle function in aging may be specific to the type of antioxidant and their site of action. Studies investigating the functions of MuSCs in aging muscle are rare and relatively new, due to the new development of tools allowing the specific analysis of these cells. *In vitro* treatment of human senescent MuSCs with tocotrienol rich fraction (Vitamin E) reduces their senescent phenotype and increases their proliferation capacity (190). In aged mice, antioxidant treatment (using Trolox, a vitamin E analog) prevents the appearance of senescence markers and restores the expansion capacity of MuSCs, and therefore their regenerative properties, through the recovery of an effective autophagy (117). Similarly, treatment with the NAD precursor nicotinamide riboside restores MuSC concentration of NAD^+, improves mitochondrial homeostasis and maintains MuSC functions in aging mice (110,361). Moreover, in mice deficient for SIRT1 (an NAD^+-dependent deacetylase that

increases mitochondrial biogenesis) in MuSCs, nicotinamide riboside beneficial properties are dampened in regenerating muscle, making a link between the NAD^+-SIRT1 pathway, mitochondrial activity and MuSC function (361) (**Figure 14**).

10.2.3. In muscular dystrophies

More than 30 years ago, thus before the discovery of the dystrophin gene, supplementation with antioxidants (SOD precursors, vitamin E, selenium) was carried out in DMD patients since vitamin E deficiency triggers similar skeletal muscle damage as that observed in DMD patients (6). Since, antioxidants have been shown to reduce necrosis in skeletal muscle of dystrophin deficient mdx mouse (215). NAC administration results in decreased numbers of regenerating myofibers (349). FSHD patients supplemented with vitamin C, vitamin E, zinc gluconate and selenomethionine increase their antioxidant level and improve some physical performance (251). However, it should be acknowledged that most of the results obtained with antioxidant treatments are inconsistent (167), highlighting the complexity of redox biology (**Figure 14**).

Few studies have explored the effects of antioxidant treatment on MuSC behavior (**Figure 14**). *In vitro*, NDGA (inhibitor of Lysyl oxidase) treatment of primary MuSCs issued from a DMD patient inhibits their proliferation while stimulating their differentiation and myotube formation in a dose-dependent manner (118). Green tea polyphenol blend and its major polyphenol (EGCg) efficiently protect mdx myotubes from oxidative damage since mdx MuSCs express 7 fold more 67LR, a receptor for EGCg, than normal MuSCs (89). In desminopathies, mutations trigger the formation of desmin aggregates in MuSCs upon stress conditions, including redox-associated (H_2O_2 and cadmium chloride) stress. Antioxidants (fisetin, NAC, α-tocopherols,

Trolox) prevent desmin-aggregation during stress in MuSCs expressing specific desmin mutants (49,299). Still *in vitro*, it was shown that supplementation of MuSCs with α-tocopherol promotes plasma membrane repair upon oxidant challenge (141). *In vivo*, various antioxidant treatments trigger an improvement of the muscle phenotype of mdx or *sgca* KO (α-sarcoglycan null mice, a severe mouse model of dystrophy) mice, together with an increase in the muscle function. Some studies evaluated the impact of such treatments on MuSCs. Mdx treatment with flavocoxid for 5 weeks increases the number of myogenin-positive cells (216). Similarly, oral administration of the flavonol quercetin during 6 months to adult mdx mice increases the number of new fibers as well as the number of MuSCs, beside a general improvement of the muscle (139). In *sgca* KO mice, NCX 320 (cyclooxygenase inhibiting NO donator) treatment for 8 months leads to an increase in the number of newly formed fibers, as well as an increase of the number of MuSCs. Moreover, MuSCs exhibit an increased ability to differentiate *in vitro* and to express the differentiation markers myogenin and myosin heavy chain, indicative of their good regenerative potential (298). Finally, mdx mice supplemented for 10 weeks with nicotinamide riboside exhibit an increased number of MuSCs while muscle regeneration is improved, indicating that nicotinamide riboside also prevents MuSC senescence in muscular dystrophy (287,361), as it does in aging muscle (see above) (**Figure 14**).

On the whole, these studies indicate that antioxidant supplementation can benefit muscle health in muscular dystrophies. It is worth mentioning that while the importance of inflammation and inflammatory cells in the establishment and maintenance of the pathology during muscular dystrophies is recognized, the phenotype and functions of these cells have been overlooked. In this context, it is

interesting to mention that antioxidant treatment has been shown to reduce the amount of inflammatory cells in these diseases, and this decrease is associated with the improvement observed in the muscle (298).

11. Perspectives

While redox signaling has long been known to play a role in skeletal muscle homeostasis, it has recently been established that the cellular and molecular processes at work during skeletal muscle regeneration are redox-regulated. Numerous signaling pathways that control MuSC biology as well as their close environment, including immune cells, are targeted by ROS/RNS and are altered in physiological (EIMD, aging) or pathological (muscular dystrophies) situations. Attempts to counteract oxidative stress in such contexts have led to inconsistent results that highlight the complexity of redox biology (6,167). This complexity has begun to be deciphered at the cellular and molecular levels. Measuring ROS concentrations, as well as redox modifications is difficult, notably *in vivo*, given the short half-lives, high specificities and high reactivity of the involved compounds. Very recent years have seen the development of tools to decipher redox complexity. Given the current knowledge, the field should move toward accurate identification of sources of ROS and their molecular targets. Indeed, sensor reporters will help to precisely identify and quantify the ROS/RNS that are produced in a tissue and in a given cell (**Figure 15**). Additionally, techniques of redox proteomics will help to decipher molecular targets of ROS/RNS and the nature of their post-translational modifications (**Figure 15**). Such technological improvements already form the basis of new therapeutic advancements. For example, the development of selective antioxidant probes has led to therapeutic efficiency in preclinical animal studies, and

in some cases, such as with the mitochondria-targeted antioxidant MitoQ, in phase II human trials (308). The design of a specific mitochondria-selective S-nitrosating agent, MitoSNO, has been found to be cardioprotective *in vivo* in mice after ischemia/reperfusion, through the S-nitrosation of mitochondrial complex I, which is the entry point for electrons from NADH into the respiratory chain (62). A genome-wide Cas9-mediated screen enabled the identification of the hypoxia response as protective during mitochondrial respiratory chain inhibition. Genetic or small-molecule activation of the hypoxia response is therefore protective against mitochondrial toxicity (150). NOX are also potent targets for therapeutic developments. New NOX inhibitors, identified through high-throughput screen after structure–activity relationship analysis, showed improved specificity for NOXs and were validated in *in vivo* animal studies although they require further developments to improve isoform selectivity. For instance, the NOX4 inhibitor GKT137831 has been shown to reduce fibrosis in several animal models of chronic diseases and is under clinical development (8). Inversely, after an acute injury, inducing a compartment-specific increase of the NAD^+/NADH ratio (using LbNOX water-forming NOX from Lactobacillus brevis) allowed to decipher the NAD^+/NADH ratio driven signaling and to improve proliferative and metabolic defects in mitochondrial deficient cells (323). Several studies recently confirmed that the redox couple NAD^+/NADH is a privileged target to improve cell and tissue homeostasis, including in aged and diseased skeletal muscle (see above, section 10.2) (110,287,361). Therefore, such an integrated approach would be an interesting option to better understand the underlying mechanisms linking redox regulation and skeletal muscle regeneration. Thus, the identification of redox-regulated networks will be useful to design

specifically targeted antioxidant probes in order to improve skeletal muscle phenotype and function.

Author Disclosure Statement

No competing financial interests exist.

Abbreviations

CAT: catalase
DGC: dystrophin-glycoprotein complex
DMD: Duchenne Muscular Dystrophy
DOMS: delayed-onset muscular soreness
ECs: endothelial cells
EIMD: exercise induced muscle damages
eNOS: endothelial NOS
FAPs: FibroAdipogenic Progenitors
FSHD: facioscapulohumeral muscular dystrophy
Gpx: glutathione peroxidase
Grx: glutaredoxin
GSH (GSSG): glutathione
GSNO: S-nitroglutathione
H_2O_2: hydrogen peroxide
HMGB-1: High mobility group box-1
HO•: hydroxyl radical
IGF-1: Insulin Growth Factor-1
iNOS: inducible NOS
L-NAME: N(G)-nitro-(L)-arginine methyl ester
MAPK: mitogen-activated protein kinase
MDA: malondialdehyde
mtROS: mitochondrial ROS
mtUPR: mitochondrial unfolded protein response
MuSC: muscle stem cell
NAC: N-acetylcysteine
NDGA: nordihydroguaiaretic acid
nNOS: neuronal NOS
NO•: Nitric oxide
NOS: NO• synthase
NOX: NADPH oxidase
$O_2^{\bullet -}$: superoxide
Prx: peroxiredoxin
RNS: reactive nitrogen species
ROS: reactive oxygen species
SEPN1: selenoprotein N
SNO: S-nitrosothiols
SOD: superoxide dismutase
Trx: thioredoxin
TrxR: thioredoxin reductase
XO: xanthine oxidase
XOR: xanthine oxidoreductase

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Figure legends

Figure 1. Overview of skeletal muscle regeneration. Left panel presents hematoxylin-eosin (HE) staining of uninjured and regenerating muscle (mouse *Tibialis Anterior* muscle) after an injury triggered by the injection of the venom toxin cardiotoxin. Day 1 after injury, myofibers undergo necrosis (pale pink staining) while the first inflammatory cells (neutrophils and monocytes/macrophages) enter the injured area (purple cells). Day 2 after injury, the number of infiltrating immune cells has increased and is mainly composed of monocytes/macrophages (large purple cells), which phagocyte the necrotic debris and necrotic myofibers. Meanwhile (non visible on HE staining), both MuSCs and FAPs activate and expand. Day 4 after injury, MuSCs differentiate and fuse to form new myofibers (dark pink structures containing one or two large nuclei with a "fried egg" appearance) while angiogenesis takes place (non visible on HE staining). Macrophages are still very numerous and they surround the regenerating myofibers. Day 7 after injury, the number of interstitial cells (FAPs and macrophages) has dropped while the regenerating myofibers grow in size (through both fusion of differentiated MuSCs and hypertrophy). One month after injury, the muscle has come back to its normal appearance, except that the regenerating myofibers are characterized by nuclei in a central - and not peripheral - location. In this model, functionality of the muscle is fully recovered 2 months after injury.

Figure 2. Adult myogenesis. At the quiescent state, most MuSCs express high levels of the transcription factors Pax7 and Myf5. Upon activation, MuSCs enter the cell cycle, still express Pax7 and Myf5 and express MyoD while they are proliferating. Self-renewing MuSCs (grey arrows towards the left) dowregulate MyoD expression

while upregulating Pax7 expression to return to quiescence. The majority of expanding MuSCs enter into myogenic differentiation that is sustained by the extinction of Pax7 and Myf5 expression together with the increase of Myogenin and MRF4 expression, which are synthetized once the cells have exited the cell cycle. Later on, contractile proteins are produced in the fully differentiated myogenic cells that fuse to form new myofibers. Maturation (not shown) of the myofibers is achieved by re-innervation through neuromuscular junctions.

Figure 3. Various cell types are present around MuSCs during skeletal muscle regeneration. During the time course of post-injury muscle regeneration, several cell types participate in the MuSC adult myogenesis program. In the model of cardiotoxin injury, MuSCs expand for a couple of days then start to differentiate. At day 4 new regenerating myofibers are visible. FAPs are also activated upon injury and rapidly expand during the first days of regeneration, after which their number rapidly decreases. After injury, the vascular bed is altered and a few days later a reorganization of ECs is observed to form new functional vessels concomitantly to the formation of the new myofibers. Monocytes from the circulation enter into the injured area a few hours after the injury and initially give rise to inflammatory macrophages during the first 1-2 days. Then, upon phagocytosis of the muscle tissue debris, they transform into anti-inflammatory/restorative macrophages that proliferate and stay numerous until the end of the regeneration process, then their number drops back to normal values.

Figure 4. Adult myogenesis during skeletal muscle regeneration is regulated by neighboring cells. The various cell types neighboring MuSCs (see Figure 3)

exert specific functions on MuSCs by delivering specific cues to MuSCs in a timely manner. Proliferation/expansion of activated MuSCs is sustained by FAPs and inflammatory (damage-associated) macrophages. The next step of myogenesis, *i.e.* differentiation, is stimulated by anti-inflammatory (restorative) macrophages as well as by ECs. Finally, the fusion of MuSCs into multinucleated structures is boosted by restorative macrophages. Return to quiescence of a small subset of MuSCs is supported by the action of peri-endothelial cells such as smooth muscle cells and pericytes, which also maintain the stability of vessels/capillaries.

Figure 5. Muscular dystrophies – the DGC complex. Inherited muscular dystrophies originate from mutations in one of the components of the DystroGlycan complex (DGC), which links the actin cytoskeleton of the myofiber to the extracellular matrix. The main proteins are the extracellular α-dystroglycan (α-DG) and laminin, the sarcolemmal β-dystroglycan (β-DG), sarcoglycans (SGCs) and sarcospan (Sar), and the cytosolic proteins dystrophin, syntrophins (SYN), dystrobrevin (DYB), actin and nNOS. The DGC complex provides stability to the membrane structure of skeletal muscle fibers during contraction/relaxation cycles. When mutations target DGC components, the stability is not ensured and fragility of the membrane leads to repeated injuries of the myofibers during contraction.

Figure 6. Permanent regeneration during muscular dystrophies. After an acute injury in normal muscle (top left part of the picture), the process of skeletal muscle regeneration occurs due to synchronous regenerating cues delivered by cells neighboring MuSCs. In muscular dystrophies, repeated injuries, occurring throughout the muscle, lead to the asynchronous delivery of regenerating cues, preventing the

various cell types from being able to carry out the timely orchestration of the process of skeletal muscle regeneration, which is thus profoundly affected. The permanent attempts of regeneration together with the cycles of necrosis lead to failed myogenesis, chronic inflammation, fibrosis and alterations in the vascular bed.

Figure 7. ROS metabolism and biochemistry. Chemical characteristics of ROS reveal their biological properties. Oxygen molecule (O_2) univalent reduction leads to the formation of reactive superoxide anion ($O_2^{\bullet -}$). This primary ROS is a weak oxidant and a weak reductant agent. $O_2^{\bullet -}$ interacts with $NO^{\bullet}$, transition metals and can naturally dismute in H_2O_2. Superoxide dismutases accelerate the rate of this reaction by 10,000. H_2O_2 is a non-radical molecule exhibiting a low reactivity and the ability to cross membrane, suggesting a crucial role in signaling. By contrast, hydroxyl radical ($HO^{\bullet}$) derived from metals and H_2O_2 interactions is highly reactive and is an unstable molecule that damages unspecified compounds that are located close to its site of production.

Figure 8. Intracellular sources of ROS and RNS and antioxidant network in skeletal muscle cell. Nucleus, mitochondria, endoplasmic reticulum (ER) and peroxisomes are the main sources of ROS in skeletal muscle cell. Additionally, enzymatic complexes generate ROS such as XO and NOX. Antioxidant molecules are mainly localized in the cytosol and in mitochondria. In addition, lipophilic antioxidants (vitamin E and tocopherols) are contained within the lipidic membrane. Dietary antioxidants such as vitamin C and carotenoids enter into cytosol following food ingestion.

Figure 9. Major oxidative thiol-modifications in redox-sensitive cysteine residues. Thiol moiety of redox-sensitive cysteine residues (SH) is vulnerable to oxidation and can lead to various post-translational modifications that are reversible or irreversible depending on the degree of oxidation. The initial reaction product with ROS is sulfenic acid (SOH). Further oxidation of sulfenic acid leads to the formation of irreversible modifications such as sulfinic acid (SO_2H) and subsequently sulfonic acid (SO_3H). Reaction of thiol with ROS can lead to the formation of both intra and intermolecular disulfide bonds. Thiol reaction with GSH and RNS leads to the formation of glutathionylation (-SGSH) and nitrosothiols (-SNO), respectively. Full and dotted arrows represent oxidation and reduction, respectively.

Figure 10. Redox-sensitive targets during adult myogenesis. Each step of adult myogenesis during skeletal muscle regeneration (see Figure 2) is regulated in MuSCs by redox activity. Some redox-related factors have been shown to directly regulate MuSC survival, activation, proliferation, self-renewal and differentiation (upper panel). Moreover, various signaling pathways have been shown to control MuSC fate on the one hand, and are known to be targets of redox regulation on the other hand (lower panel).

Figure 11. Macrophages during skeletal muscle regeneration. Upon skeletal muscle injury, monocytes/macrophages are recruited from blood and invade the tissue as damage-associated macrophages ($Ly6C^{pos}$ cells). They release pro-inflammatory molecules (TNFα, IL6, IL1β, VEGF) to stimulate MuSC proliferation. Then, upon phagocytosis these macrophages skew their phenotype to acquire a restorative phenotype and secrete compounds that stimulate myogenic differentiation

(IGF-1, TGFβ). Macrophage skewing is regulated by a series of effectors, which are regulated by redox mechanisms. Moreover, restorative macrophages express high levels of SOD1 and Trx, which are associated with the reduction of the alarmin HGMB1 that participates to MuSC migration and differentiation. See text for details.

Figure 12. Redox alteration of myogenesis in aged muscle. Fate of MuSCs during post-injury myogenesis is altered in aged muscle, including reduced activation, reduced proliferation, reduced self-renewal and increased differentiation. A series of redox effectors are deregulated in aged MuSCs and are listed in the box. In italic are shown the signaling pathways that are altered in aged MuSCs and known to be redox regulated. ↗↘ stand for increase and decrease, respectively.

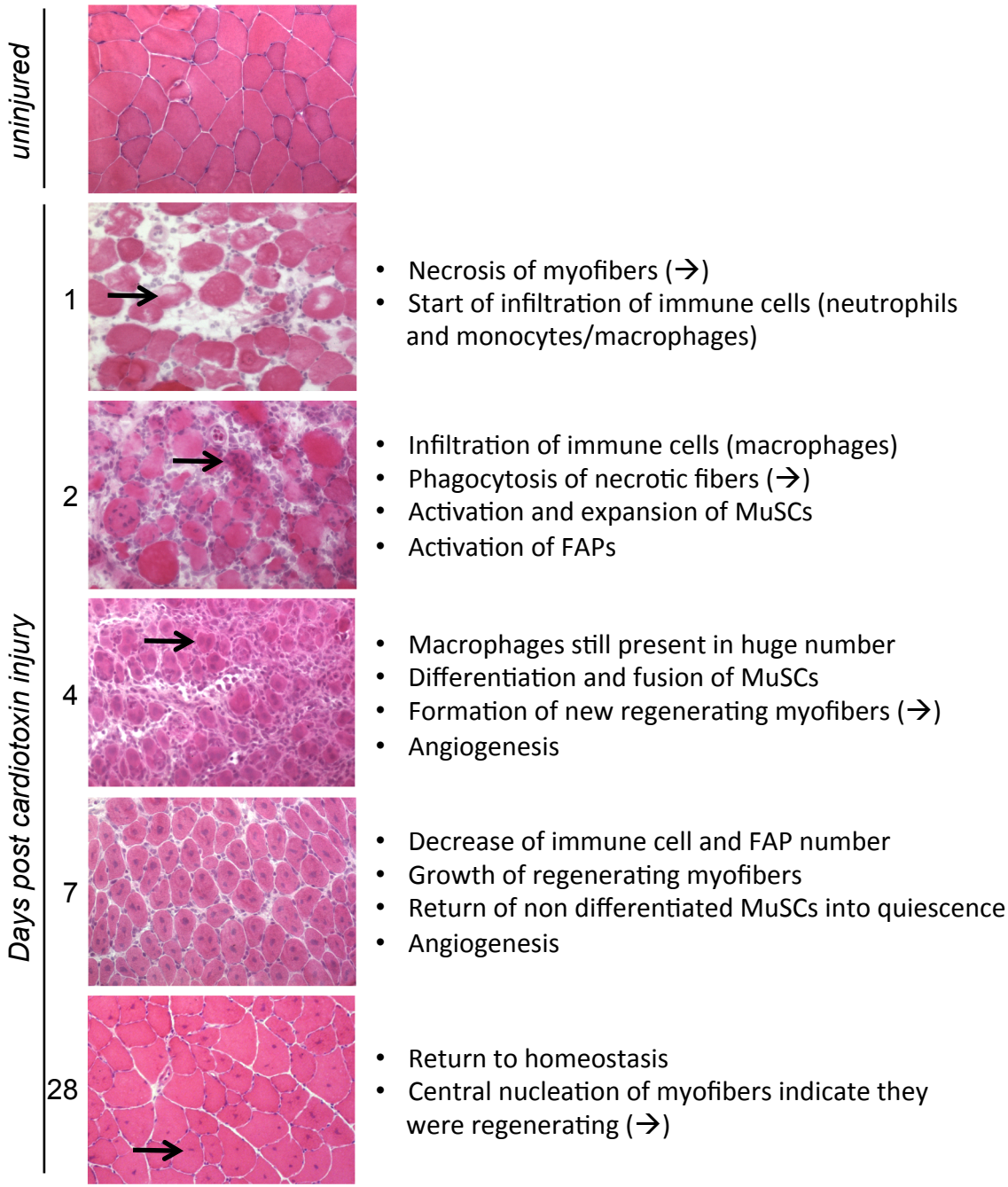
Figure 13. ROS in MuSCs in muscular dystrophies. The left circle represents a synchronous regeneration process triggered by a single injury. The right circle shows that in muscular dystrophies, repeated injuries trigger the delivery of asynchronous regenerative cues, leading to a failure of the regeneration process, associated with a whole increase of ROS. MuSCs from dystrophic muscle have been shown to be more susceptible to oxidative stress than normal MuSCs. However, only few molecular determinants underlying this susceptibility have been evidenced so far, those are listed in the box. See text for details. ↗↘ stand for increase and decrease, respectively.

Figure 14. Therapeutic strategies in aged and dystrophic muscles – effects on MuSCs. Antioxidant supplementation on whole body (upper panel) increases the detection of antioxidant biomarkers, triggers some beneficial effects in muscular

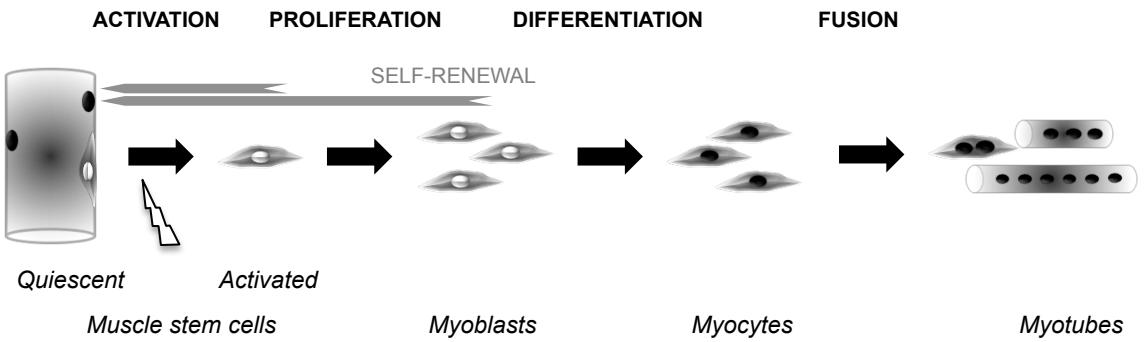
dystrophies, but no improvement of muscle function in both aged and diseased muscle is observed. A variety of antioxidant molecules exert beneficial effects on MuSCs *in vitro* (middle panel), notably they decrease some abnormalities observed in muscular dystrophies. The analysis of MuSCs *in vivo* after treatment in mouse models (lower panel) indicates an overall benefit for MuSC homeostasis via increased energetic metabolism and myogenesis. ↗↘≈ stand for increase, decrease and no effect, respectively.

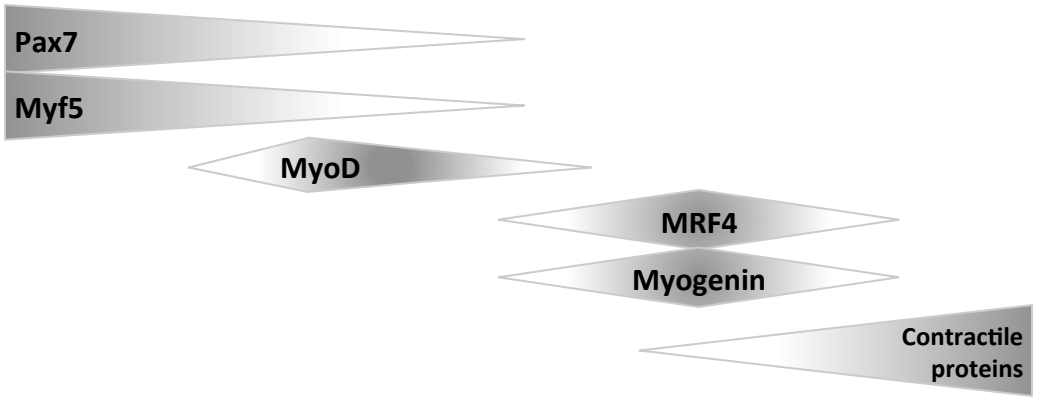
Figure 15. Future strategies for the study of ROS. The scheme presents interrelated strategies and developing tools that will help to identify precisely which ROS and ROS-dependent signaling are involved in a given biological context. Such knowledge will further allow to define specific targets potentially used in pharmacological trials. Detection and identification of ROS is performed through the development of specific probes (upper left). Several chemical probes have been developed to investigate redox mechanisms, mainly composed of a fluorophore that emits or is liberated upon oxidation (351). More recently, genetically encoded fluorescent redox sensors or injected exomarkers allow the detection and visualization of live variations of ROS or redox regulated molecules (*e.g.* GSH, NAD) *in vitro* and *in vivo* (upper right) (5,68,98,197). ROS-induced post-translational modifications (PTMs) have recently been described due to the development of redox proteomics, thanks to the setup of cysteine residue labeling (bottom right). Together with crystallography, which describes protein modifications upon redox reaction, these biochemical strategies allowed the identification of new molecular targets of redox reactions (bottom right) (60,61,186,213). The development of this large variety of tools for *in vitro* and *in vivo* investigation of redox biology, together with the

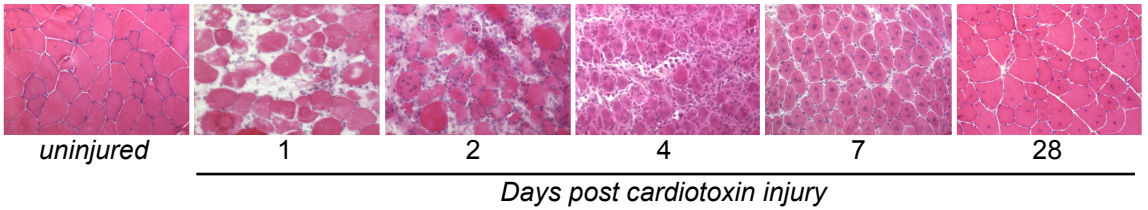
improvement of the probes and of their specificity will enable the development of antioxidant probes to be tested in preclinical and clinical trials (bottom left).

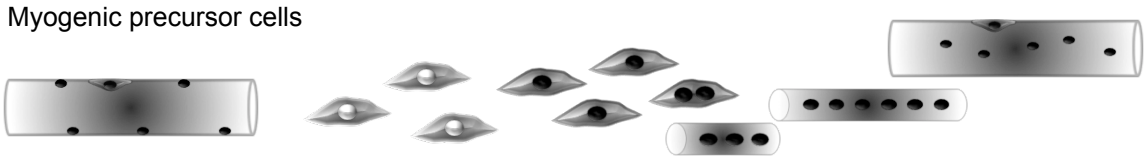

uninjured
Days post cardiotoxin injury
1
2
4
7
28
• Necrosis of myofibers (→)
• Start of infiltration of immune cells (neutrophils and monocytes/macrophages)
• Infiltration of immune cells (macrophages)
• Phagocytosis of necrotic fibers (→)
• Activation and expansion of MuSCs
• Activation of FAPs
• Macrophages still present in huge number
• Differentiation and fusion of MuSCs
• Formation of new regenerating myofibers (→)
• Angiogenesis
• Decrease of immune cell and FAP number
• Growth of regenerating myofibers
• Return of non differentiated MuSCs into quiescence
• Angiogenesis
• Return to homeostasis
• Central nucleation of myofibers indicate they were regenerating (→)

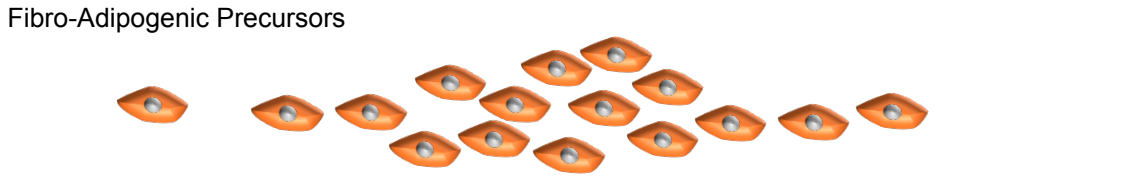
ADULT MYOGENESIS DURING SKELETAL MUSCLE REGENERATION

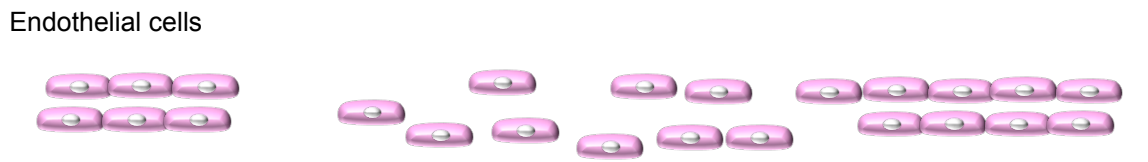


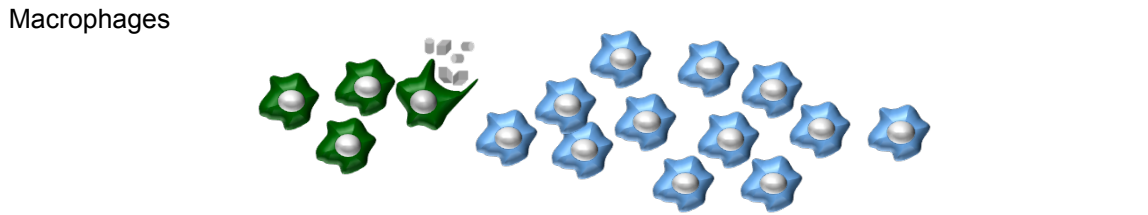


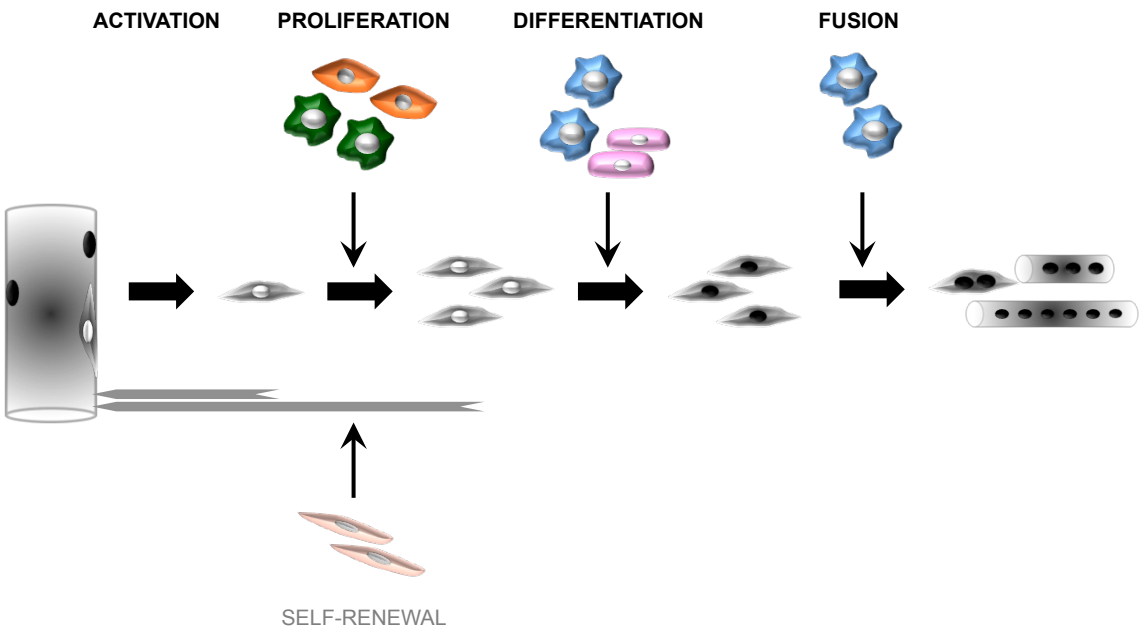

uninjured
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Days post cardiotoxin injury

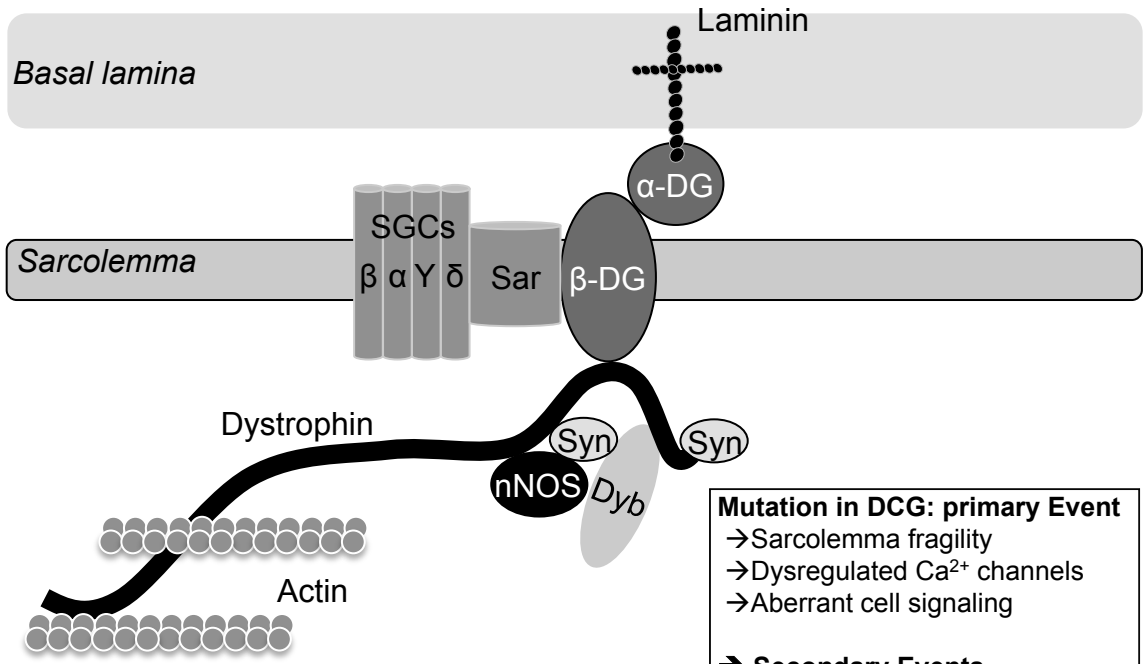

Myogenic precursor cells


Fibro-Adipogenic Precursors


Endothelial cells

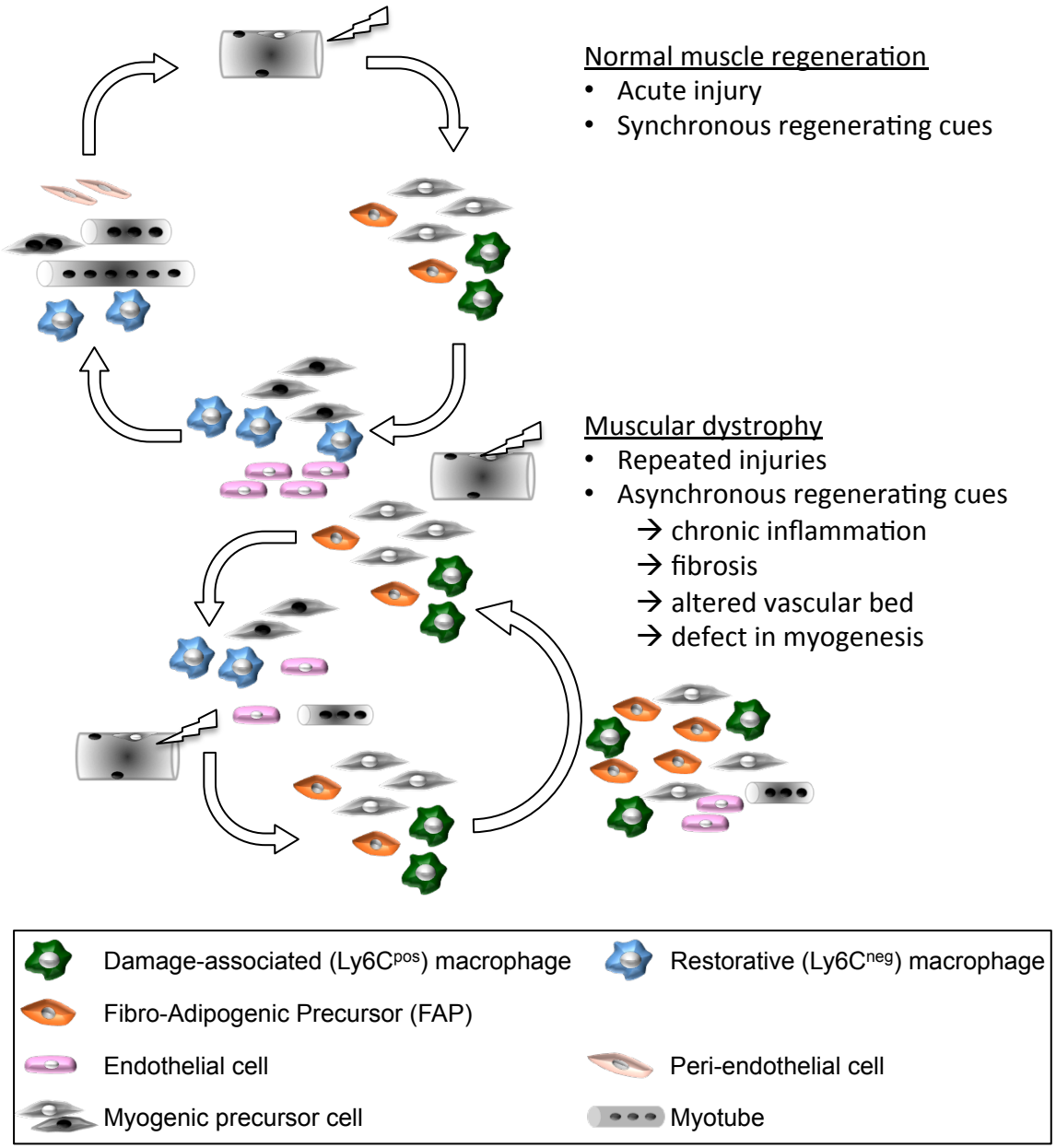

Macrophages

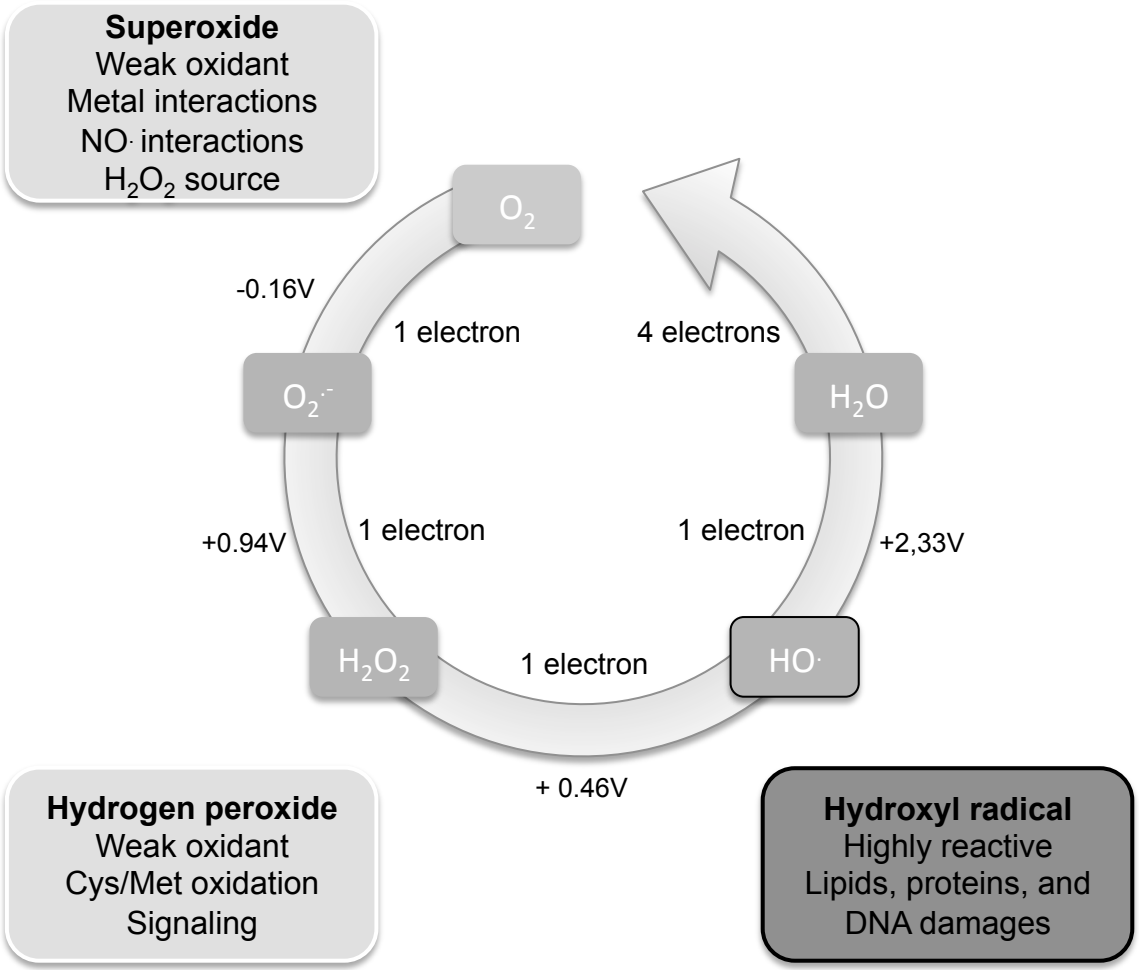

ACTIVATION
PROLIFERATION
DIFFERENTIATION
FUSION
SELF-RENEWAL
Damage-associated (Ly6C^{pos}) macrophage
Restorative (Ly6C^{neg}) macrophage
Fibro-Adipogenic Precursor (FAP)
Endothelial cell
Peri-endothelial cell
Myogenic precursor cell
Myotube

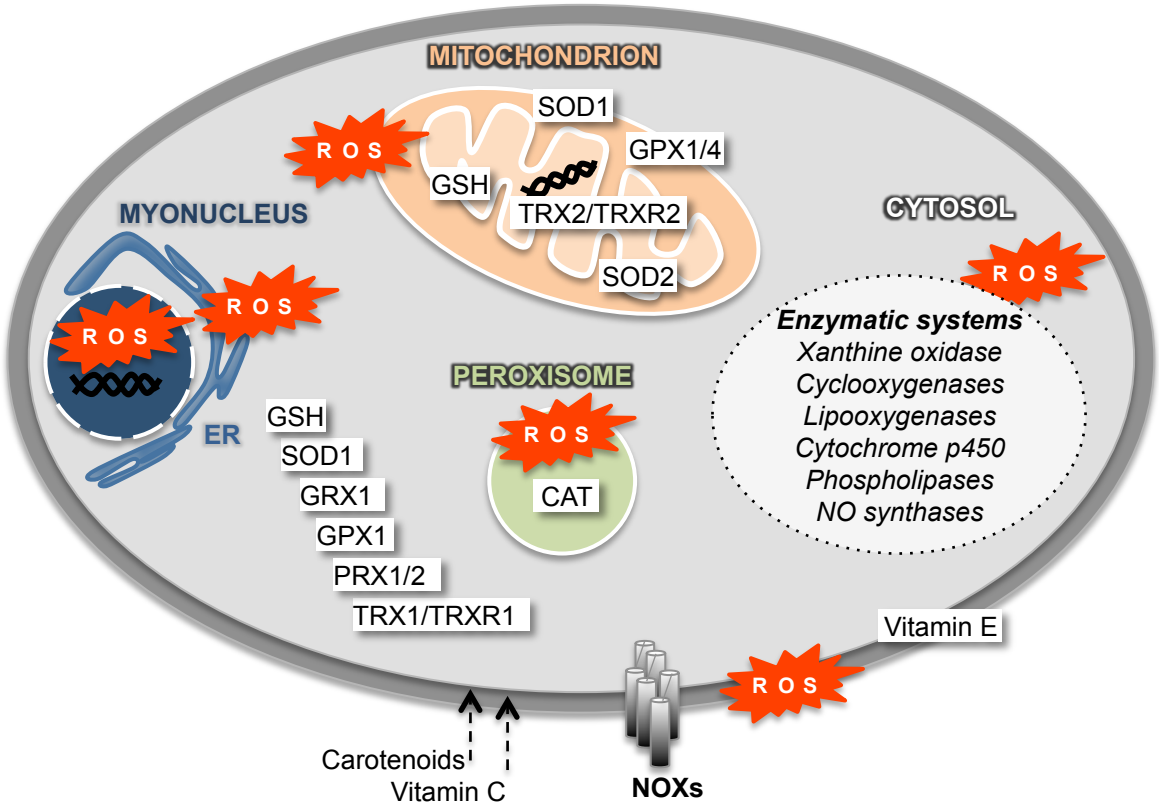


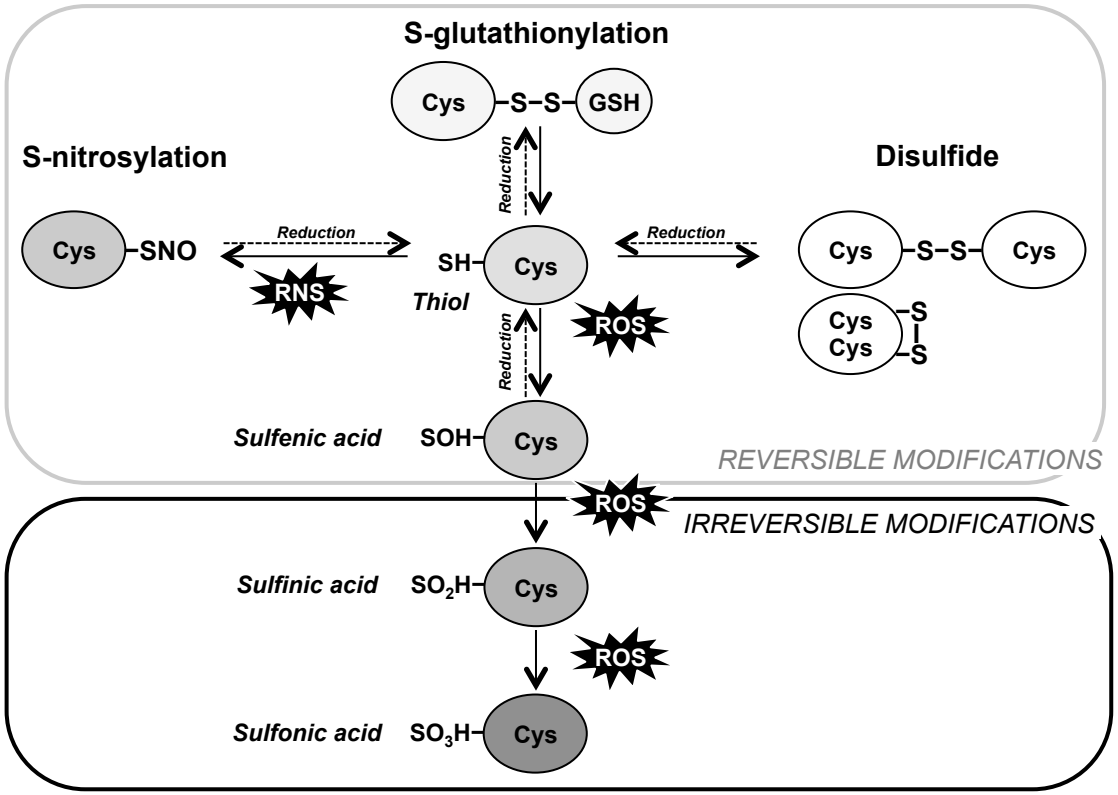
Mutation in DCG: primary Event
→Sarcolemma fragility
→Dysregulated Ca^{2+} channels
→Aberrant cell signaling

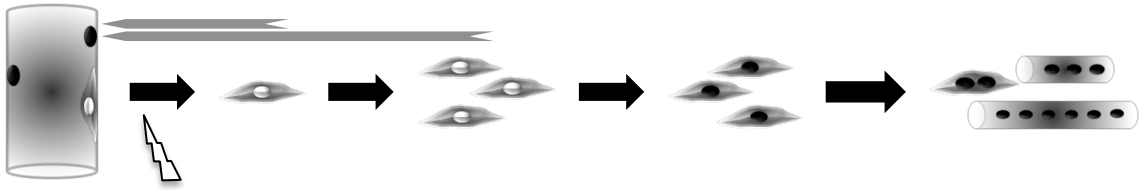
➔ **Secondary Events**
• Elevated intracellular Ca^{2+}
• Increased oxidative stress
• Chronic inflammation
• Fibrosis


Normal muscle regeneration
• Acute injury
• Synchronous regenerating cues
Muscular dystrophy
• Repeated injuries
• Asynchronous regenerating cues
→ chronic inflammation
→ fibrosis
→ altered vascular bed
→ defect in myogenesis
Damage-associated (Ly6C^pos) macrophage
Restorative (Ly6C^neg) macrophage
Fibro-Adipogenic Precursor (FAP)
Endothelial cell
Peri-endothelial cell
Myogenic precursor cell
Myotube


Superoxide
Weak oxidant
Metal interactions
NO· interactions
H2O2 source
O2
-0.16V
1 electron
4 electrons
O2·-
H2O
+0.94V
1 electron
1 electron
+2,33V
H2O2
1 electron
HO·
+ 0.46V
Hydrogen peroxide
Weak oxidant
Cys/Met oxidation
Signaling
Hydroxyl radical
Highly reactive
Lipids, proteins, and
DNA damages


MITOCHONDRION
SOD1
ROS
GPX1/4
GSH
TRX2/TRXR2
SOD2
MYONUCLEUS
ROS
ROS
ER
CYTOSOL
ROS
Enzymatic systems
Xanthine oxidase
Cyclooxygenases
Lipooxygenases
Cytochrome p450
Phospholipases
NO synthases
PEROXISOME
ROS
CAT
GSH
SOD1
GRX1
GPX1
PRX1/2
TRX1/TRXR1
Vitamin E
ROS
Carotenoids
Vitamin C
NOXs

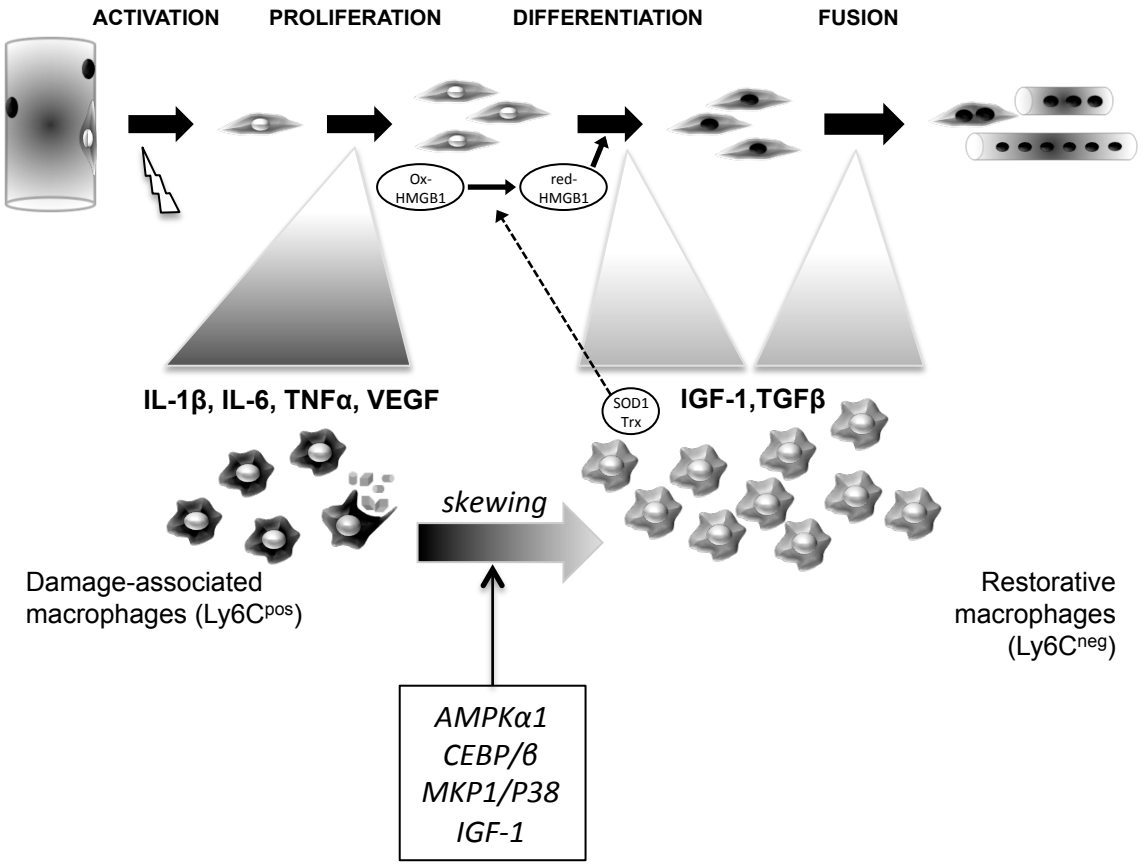

S-glutathionylation
Cys
S−S
GSH
Reduction
S-nitrosylation
Disulfide
Cys
SNO
Reduction
Reduction
SH
Cys
Thiol
RNS
ROS
Cys
S−S
Cys
Cys
S
Cys
S
Reduction
Sulfenic acid
SOH
Cys
REVERSIBLE MODIFICATIONS
ROS
IRREVERSIBLE MODIFICATIONS
Sulfinic acid
SO2H
Cys
ROS
Sulfonic acid
SO3H
Cys

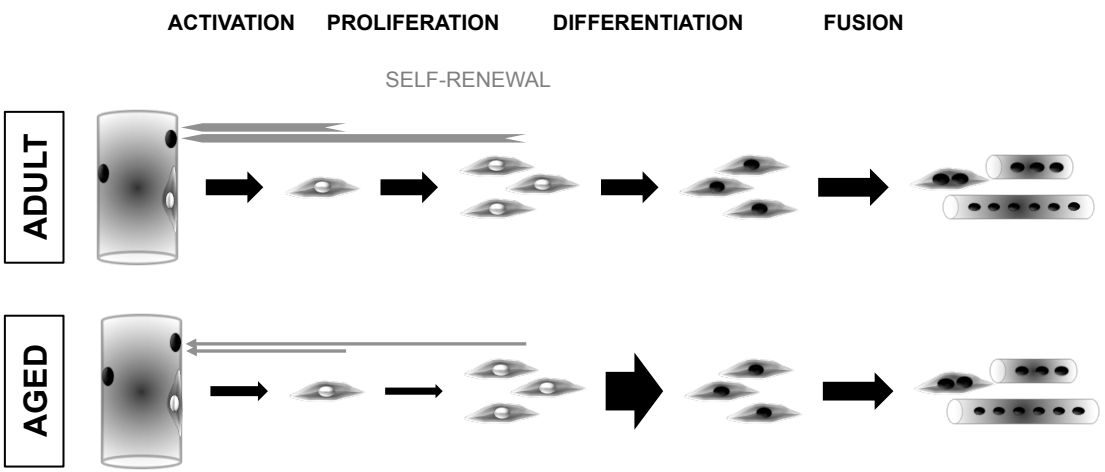


Quiescent *Activated*

Muscle stem cells *Myoblasts* *Myocytes* *Myotubes*

survival	activation	proliferation	self-renewal	differentiation
Redox related factors				
GPX3 ALDH Cytoglobin	CaM-NOS NO• (HGF, MMP)	NADH-$O_2^{\bullet-}$ GPX1 SOD1	GPX3 TrxR1 NO• (Vangl2)	DUOXA1 $p66^{ShcA}$ SEPN Nrf2-GCL/GR-GSH Nrf2-CRB1 NO• DRP1 Prx2
Redox regulated signaling pathways				
NF-κB AP-1 Nrf2 Caspases Bcl-2	S1P MAPKs (p38) Notch Wnt-Se-GPX1	ERK1/2 PI3K Akt/mTOR NF-κB CDKN1 S1P MAPKs (p38) Notch		NF-κB


ACTIVATION
PROLIFERATION
DIFFERENTIATION
FUSION
Ox-HMGB1
red-HMGB1
IL-1β, IL-6, TNFα, VEGF
SOD1
Trx
IGF-1,TGFβ
skewing
Damage-associated macrophages (Ly6C^pos)
Restorative macrophages (Ly6C^neg)
AMPKα1
CEBP/β
MKP1/P38
IGF-1



↘ activation upon stretch and NO
↘ autophagy

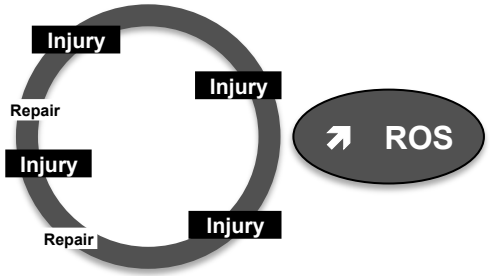
↘ catalase
↘ glutathione transferase
↘ Nrf2 signaling → Pax7 MyoD alteration
↘ Nrf2 signaling → ↗ubiquitin/ apoptotic pathways
↘ NAD^+ → ↘ mtUPR
↗ oxidation of DNA and lipid peroxidation
↗ superoxide/mitochondrial ratios → ↘mitochondria ATP production

↘ *Notch*
↗ *Wnt*
↗*JAK-STAT3*
↗ *p38 MAPK*

Normal muscle



Diseased muscle

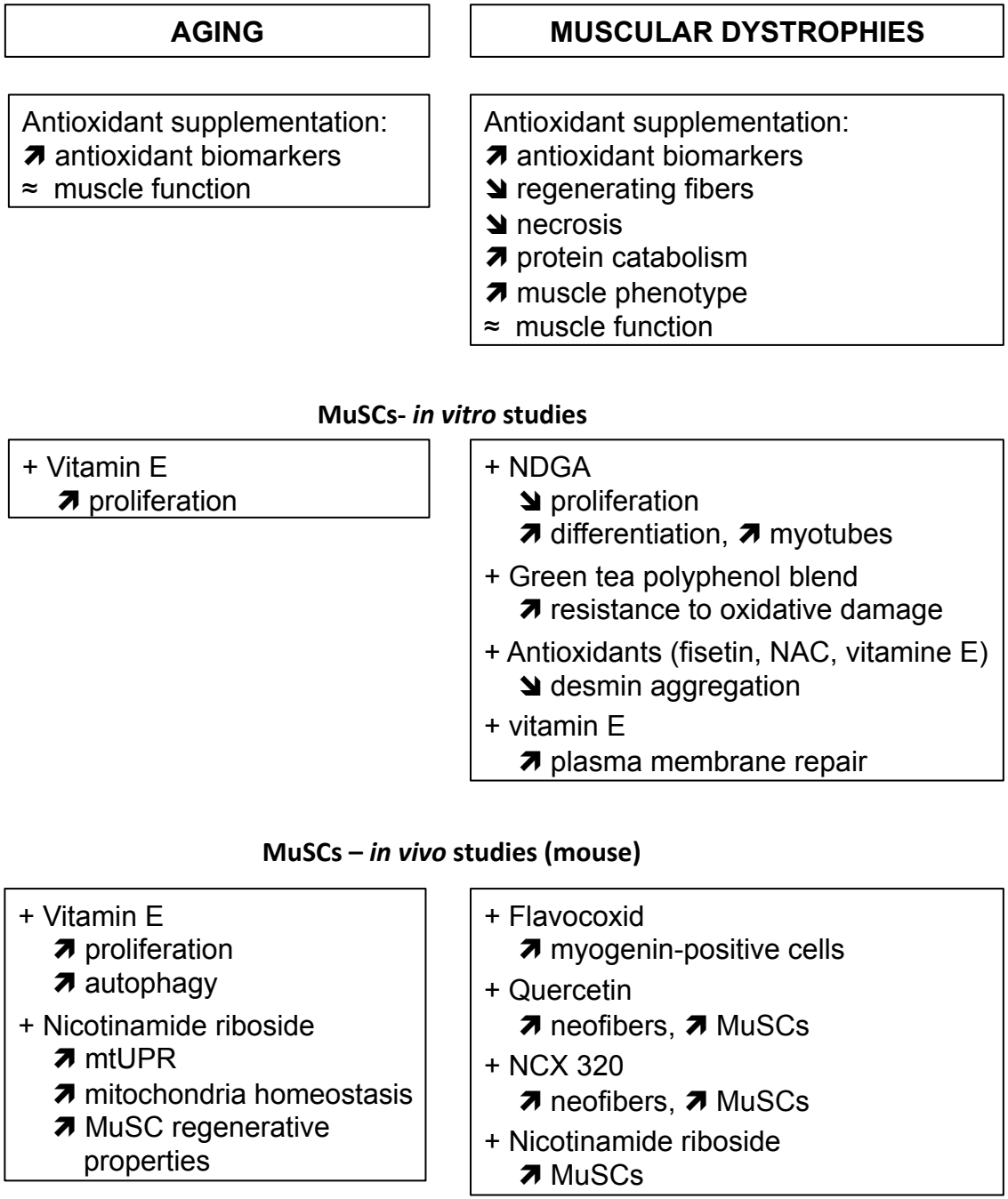


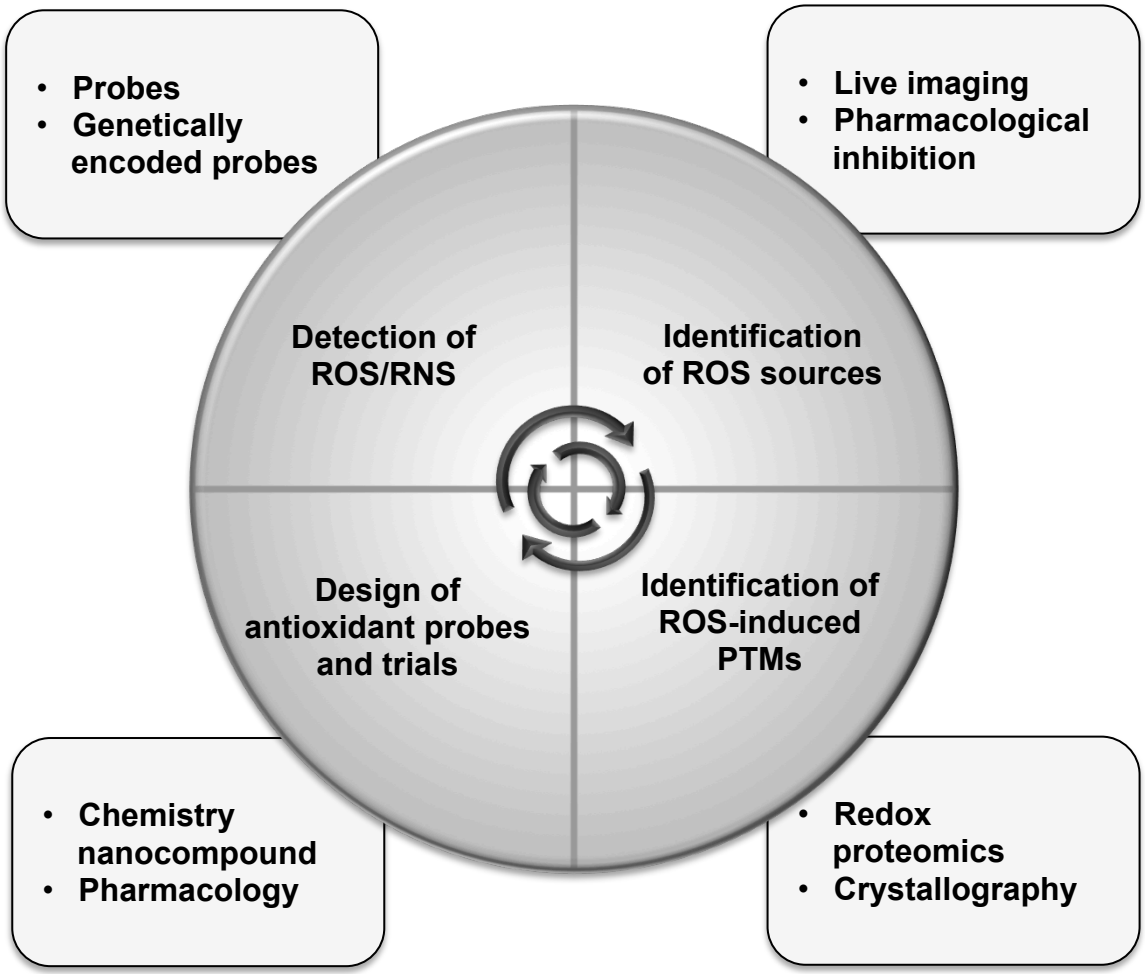
MuSCs:

↗ susceptibility to oxidative stress
- → altered MAPKs (JNK, p38 and ERK1/2) signaling
- → defect in myotube size
- → defect in branching myotubes/myofibers

↗Stra13

↘Bmi-1 → ↘MT1


AGING
MUSCULAR DYSTROPHIES
Antioxidant supplementation:
↗ antioxidant biomarkers
≈ muscle function
Antioxidant supplementation:
↗ antioxidant biomarkers
↘ regenerating fibers
↘ necrosis
↗ protein catabolism
↗ muscle phenotype
≈ muscle function
MuSCs- in vitro studies
+ Vitamin E
↗ proliferation
+ NDGA
↘ proliferation
↗ differentiation, ↗ myotubes
+ Green tea polyphenol blend
↗ resistance to oxidative damage
+ Antioxidants (fisetin, NAC, vitamine E)
↘ desmin aggregation
+ vitamin E
↗ plasma membrane repair
MuSCs – in vivo studies (mouse)
+ Vitamin E
↗ proliferation
↗ autophagy
+ Nicotinamide riboside
↗ mtUPR
↗ mitochondria homeostasis
↗ MuSC regenerative properties
+ Flavocoxid
↗ myogenin-positive cells
+ Quercetin
↗ neofibers, ↗ MuSCs
+ NCX 320
↗ neofibers, ↗ MuSCs
+ Nicotinamide riboside
↗ MuSCs


• Probes
• Genetically encoded probes
• Live imaging
• Pharmacological inhibition
Detection of ROS/RNS
Identification of ROS sources
Design of antioxidant probes and trials
Identification of ROS-induced PTMs
• Chemistry nanocompound
• Pharmacology
• Redox proteomics
• Crystallography