Chapter 1

Molecular and Cellular Mechanism of Muscle Regeneration

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http://dx.doi.org/10.5772/48229

1. Introduction

Skeletal muscle contractions power human body movements and are essential for maintaining stability. Skeletal muscle tissue accounts for almost half of the human body mass and, in addition to its power-generating role, is a crucial factor in maintaining homeostasis. Given its central role in human mobility and metabolic function, any deterioration in the contractile, material, and metabolic properties of skeletal muscle has an extremely important effect on human health.

Several possible mechanisms for age-related muscle atrophy have been described; however the precise contribution of each is unknown. Age-related muscle loss is a result of reductions in the size and number of muscle fibers [1] possibly due to a multi-factoral process that involves physical activity, nutritional intake, oxidative stress, and hormonal changes [2-4]. The specific contribution of each of these factors is unknown but there is emerging evidence that the disruption of several positive regulators [Akt and serum response factor (SRF)] of muscle hypertrophy with age is an important feature in the progression of sarcopenia [5-7]. In addition, sarcopenia seems to include the defect of muscle regeneration probably due to the repetitive muscular damage. Indeed, the group of Conboy [8-10] indicates that Notch-dependent signaling is impaired in sarcopenic muscle.

Upon tissue injury, the cues released by the inflammatory component of the regenerative environment instruct somatic stem cells to repair the damaged area [11]. The elucidation of the molecular events underpinning the interplay between the inflammatory infiltrate and tissue progenitors is crucial to devise new strategies toward implementing regeneration of diseased or injured tissues. Regeneration of diseased muscles relies on muscle stem cells (satllite cells) located under the basal lamina of muscle fibers [12], which are activated in response to cytokines and growth factors [13]. The current lack of knowledge of how

external cues coordinate gene expression in these cells precludes their selective manipulation through pharmacological interventions.

The inflammatory infiltrate is a transient, yet essential, component of the satellite cell niche and provides the source of locally released cytokines, such as interleukin (IL)-1, IL-6, and tumor necrosis factor-α (TNF-α), which regulate muscle regeneration [14]. As an inducible element of the satellite cell niche, the inflammatory infiltrate provides an ideal target for selective interventions aimed at manipulating muscle regeneration [15]. However, because local inflammation regulates multiple events within the regeneration process, global anti-inflammatory interventions have both positive and negative effects on satellite cells [16]. Thus, it is important to elucidate the intracellular signaling by which inflammatory cytokines deliver information to individual genes in satellite cells.

Similarly to the embryonic stem cells that build organs, adult stem cells that regenerate organs are capable of symmetric and asymmetric division, self-renewal, and differentiation. This precise coordination of complex stem cell responses throughout adult life is regulated by evolutionally conserved signaling networks that cooperatively direct and control (1) the breakage of stem cell quiescence, (2) cell proliferation and self-renewal, (3) cell expansion and prevention of premature differentiation and finally, (4) the acquisition of terminal cell fate. This highly regulated process of tissue regeneration recapitulates embryogenic organogenesis with respect to the involvement of interactive signal transduction networks such as hepatocyte growth factor (HGF), Notch, MyoD, calcineurin, and SRF [17, 18]. This review aims to outline the molecular and cellular mechanisms of muscle regeneration.

2. Early immune response

Two distinct macrophage populations exist. Classically activated (or type I) macrophages are induced by interferon (IFN)-γ, alone or in concert with microbial stimuli (e.g. lipopolysaccharide) or selected cytokines (e.g. TNF-α and granulocyte macrophage colony-stimulating factor). They have pro-inflammatory functions: classically activated macrophages produce effector molecules (reactive oxygen and nitrogen intermediates) and inflammatory cytokines (IL-1β, TNF-α, IL-6), participate as inducer and effector cells in polarized Th1 responses, and mediate resistance against intracellular parasites and tumors. Type I macrophages characteristically and selectively express pro-inflammatory chemokines, in particular CCL [chemokine (C-C motif) ligand] 3. Alternatively activated (or type II) macrophages comprise cells exposed to IL-4 or IL-13, immune complexes, IL-10, and glucocorticoid; they participate in polarized Th2 reactions, promote killing and encapsulation of parasites, and are present in established tumors, where they promote progression. Moreover, alternatively activated macrophages are involved in wound healing and have immunoregulatory functions [18]. The expression of membrane receptors, like the hemoglobin scavenger receptor CD163, unambiguously identifies type II macrophages [19].

Studies in the rat have shown that type I macrophages are associated with muscle necrosis, whereas type II macrophages are associated with regenerative myofibers [20]. Of striking

interest, these cells, once within the muscle, apparently acquire a type II phenotype, revealing a previously ignored plasticity. What are the signals that trigger the shift? Recognition and phagocytosis of muscle cell debris is probably a critical event. Indeed while type I macrophages enhance the proliferation of local myogenic precursor cells, type II macrophages stimulate their fusion and differentiation [21]. Some molecular interactions are required for macrophage recruitment and function in damaged muscles. The muscle tissue of mice with a null mutation of CCR2, the CCL2 receptor, undergoes regenerating defects including fibrosis and calcification after muscle damage. In addition, uPA (urokinase-type plasminogen activator)-/- macrophages fail to infiltrate damaged muscle [22]. This failure is associated with defective muscle regeneration, demonstrating that uPA is required for the homeostatic response to injury. Mice lacking an inhibitor of uPA, PAI-1 (plasminogen activator inhibitor 1), exhibit increased uPA activity: injured muscle of PAI-1-/- mice shows evidence of increased macrophage accumulation, and of accelerated muscle repair [23]. Expression of uPA is apparently required for the expression of insulin-like growth factor-I (IGF-I), a central regulator of muscle regeneration [24]. IGF-I suppresses the expression and activity of macrophage migration inhibitory factor and the transcription factor NF-κB, possibly directly regulating the persistence of inflammatory responses [25, 26].

3. Hepatocyte growth factor and neuronal nitric oxide synthase

By 24 hours after muscle injury, satellite cells enter the G1/S phase of the cell cycle [27]. Two factors have been demonstrated to activate quiescent satellite cells. The first is HGF. Early experiments using single muscle fibers with associated quiescent satellite cells have shown that growth factors, such as IGF-I and fibroblast growth factor (FGFs), do not activate satellite cells in fibers [28, 29]. Although IGF-I and FGFs are reported to activate satellite cells, the studies involved typically used cultures of muscle cells that were not quiescent; IGF-I and FGFs increase the proliferative activity of satellite cells once they are activated, even when that activation results during the cell isolation process, i.e. prior to the plating of cells or fibers for culture. Moreover, platelet-derived growth factor BB, transforming growth factor-β (TGF-β), and epidermal growth factor do not stimulate quiescent cells to enter the cell cycle *in vitro* [30, 31]. Therefore, HGF is the only growth factor that has been established to have the ability to stimulate quiescent satellite cells to enter the cell cycle early in a culture assay and *in vivo* [32, 33]. HGF is localized to the extracellular domain of un-injured skeletal muscle fibers through a possible association with glycosaminoglycan chains of proteoglycans that are essential components of the extracellular matrix, and following injury, quickly associates with satellite cells [34] by binding to its receptor, c-Met [33].

The second component shown to be involved in satellite cell activation is nitric oxide (NO), possibly through activation of matrix metalloproteinases (MMP), which induce the release of HGF, from the extracellular matrix [34, 35]. Studies *in vitro* and *in vivo* using rodent muscle have shown HGF and NO to regulate the activity of many satellite cells [33, 34, 36, 37]. Intriguingly, inhibition of NO production inhibits HGF release, c-Met/HGF co-

localization, and satellite cell activation [34]. NO is a short-lived free radical that is well known as a freely diffusible and ubiquitous molecule produced by nitric oxide synthase (NOSs) from the L-arginine of substrates. In skeletal muscle, neuronal NOS (nNOS, also called NOS-1) is localized to the sarcolemma of muscle fibers by association at its amino terminus with alpha1-syntrophin linked to the dystrophin cytoskeleton [38]. The NO radical is normally produced in very low level pulses by muscles under conditions where satellite cells are quiescent [39], and the expression and activity of constitutive NOS (nNOS and eNOS) are up-regulated by exercise, loading injury, shear force, and mechanical stretch. NO also induces expression of follistatin [40], a fusigenic secreted molecule, known to antagonize myostatin, thus possibly contributing to the exit of satellite cells from quiescence.

More recently, Tatsumi and Allen [37] proposed the intriguing hypothesis that HGF has another role in satellite cells. Although, in culture, a low level of HGF (2.5 ng/ml) optimally stimulates the activation of satellite cells, high levels of HGF (10-500 ng/ml) promote the re-entering of quiescence through a concentration-dependent negative feedback mechanism. Such a role seems to be regulated by the induction of the cyclin-dependent kinase (CDK) inhibitor p21 in a myostatin-dependent manner. Further descriptive analysis is needed to elucidate whether HGF and myostatin really do interact in skeletal muscle *in vivo*. Tatsumi and Allen [37] suggested the importance and difficulty of monitoring whether or not extracellular HGF concentrations reach a threshold (over 10 ng/ml) in muscle of living animals.

4. The proliferating process of satellite cells

4.1. Leukemia inhibitory factor

Leukemia inhibitory factor (LIF) is a newly discovered myokine [41], originally identified by its ability to induce the terminal differentiation of myeloid leukemic cells. Today, LIF is known to have a wide array of functions, including acting as a stimulus for platelet formation, the proliferation of hematopoietic cells, bone formation, neural survival and formation, muscle satellite cell proliferation and acute phase production by hepatocytes [42]. LIF is a long chain four α-helix bundle cytokine, which is highly glycosylated and may be present with a weight of 38-67 kDa, which can be deglycosylated to ~20 kDa [43, 44]. Several tissues, including skeletal muscle, express LIF. LIF is constitutively expressed at a low level in type I muscle fibers [45, 46] and is implicated in conditions affecting skeletal muscle growth and regeneration [45-47]. Indeed, LIF knockout mice showed a decrease in the area occupied by regenerating myofibers after crush injury compared to wild-type mice, which was restored by administration of exogenous LIF [48]. Administration of LIF to the site of crush injury in wild-type mice increased the area occupied by regenerating fibers with an associated increase in average myofiber diameter [48, 49]. These original studies suggested that enhanced regeneration and increases in fiber size occurred, at least in part via stimulation of the proliferation of muscle-forming myoblast cells, thus providing more cells to fuse to and increase the size of regenerating fibers.

In 1991, Austin and co-workers demonstrated that LIF stimulated myoblast proliferation in culture [50], thereby showing that LIF functions as a mitogenic growth factor when added to muscle precursor cells *in vitro*. To date, different groups have confirmed this finding and shown that LIF induces satellite cell and myoblast proliferation, while preventing premature differentiation, by activating a signaling cascade involving Janus kinase 1 (JAK1), signal transducer and activator of transcription (STAT) 1, and STAT3 [51, 52]. In line with this, the specific LIF receptor is primarily expressed by satellite cells and not by mature muscle fibers [53]. Thus, it seems that LIF has the potential to affect satellite cells rather than mature muscle fibers.

Earliest descriptions of LIF as a possible mitogen for myoblasts suggested that LIF treatment increased the number of human and mouse-derived primary myoblast cells in a dose-dependent manner after several days of culture, with the earliest increases noticeable after 6 days [50, 54]. There is evidence to suggest that LIF promotes survival of myoblasts and other cell types [55, 56]. Hunt et al. [57] found that LIF treatment significantly reduced staurosporine-induced apoptotic DNA fragmentation by 37% and also reduced the proteolytic activation of caspase-3 by 40% compared to controls. This apoptosis-inhibiting role of LIF was completely abolished by a PI3-K (phosphatidylinositol 3-kinase) inhibitor (wortmannin). Therefore, LIF appears to increase the number of satellite cells by promoting proliferation and blocking apoptosis.

4.2. Insulin-like growth factor-I and MAPK (proliferation phase)

The anabolic effects of IGF-I have been demonstrated in both muscle cell lines and animal models [58-60]. For example, the addition of IGF-I to cultured myotubes results in an enlargement of myotube diameters and a higher protein content, while the delivery of IGF-I either through osmotic pumps or genetic overexpression results in increased muscular mass in rodents [24, 58]. Mechanical loading also results in skeletal muscle synthesis of IGF-I [61, 62] *in vivo*, which stimulates gene expression, DNA and protein synthesis, different transport mechanisms, migration, proliferation, and differentiation [63]. Therefore, investigators conclude that IGF-I is a critical factor involved in skeletal muscle hypertrophy in vivo as well as in cultured myotube enlargement in vitro.

IGF-I is thought to induce muscle growth through the increased proliferation of satellite cells and the enhancement of protein translation resulting in an increase in the rate of protein synthesis [63, 64]. In addition to stimulating myoblast proliferation, IGF-I stimulates myoblast differentiation [65]. For example, IGF-I inhibits production of myogenin, a protein that stimulates muscle cell differentiation, thus allowing increased myoblast proliferation. It is known that the binding of IGF-I to its receptor, after tyrosine (auto)phosphorylation of the receptor, results in the initiation of intracellular cascades of various kinase systems. However, the interplay between the elements of these intracellular signaling pathways has been described based on results of experiments with skeletal muscle cell types of different species and under various conditions. Namely, in mouse and rat skeletal muscle preparations, the involvement of both the MAPK (mitogen-activated protein kinase)

pathway and MAPK-independent signaling mechanisms, including PI3-K/Akt and protein kinase C (PKC), was equally documented [66-68]. In primary cultured human skeletal muscle cells, Czifra et al. [69] demonstrated that the proliferation-enhancing effect of IGF-I was completely inhibited by the PKCδ-specific inhibitor Rottlerin but not by inhibitors of the "conventional" PKCα and γ isoforms or by inhibitors of the MAPK or PI3-K pathway. In addition, overexpression of a kinase inactive mutant of PKCδ prevented the proliferating action of IGF-I. Furthermore, they showed, in mouse C2C12 cells, that the MAPK inhibitor PD098059 partially inhibited the action of IGF-I. Taken together, these results demonstrate a novel, central and exclusive involvement of PKCδ in mediating the action of IGF-I in human skeletal muscle cells, with an additional yet PKCδ-dependent contribution of the MAPK pathway in C2C12 myoblasts.

4.3. Notch-dependent signaling

The proliferating process in satellite cells appears to be controlled by Notch signaling during muscle regeneration [70]. Within hours to days following muscle injury, there is increased expression of Notch signaling components (Delta-1, Notch-1 and active Notch) in activated satellite cells and neighboring muscle fibers [8, 70]. Up-regulation of Notch signaling promotes the transition from activated satellite cells to highly proliferative myogenic precursor cells and myoblasts, as well as prevents differentiation to form myotubes [8, 71, 72]. Proliferation was decreased and differentiation was promoted when Notch activity was inhibited in myoblasts with a Notch antagonist, Numb, a gamma-secretase inhibitor, or with small-interfering RNA (siRNA) knockdown of presenilin-1 [70, 71, 73]. In addition, mutations in Delta-like 1 or CSL result in excessive premature muscle differentiation and defective muscle growth [74]. Apparent impairment of Notch signaling occurs in aged muscle, because expression of the Notch ligand, Delta, is not upregulated following injury in this muscle. Forced activation of this pathway with a Notch-activating antibody can restore the regenerative potential by inducing the expression of several positive regulators (PCNA, Cyclin D1) of cell cycle progression [8, 9].

A recent study revealed that levels of TGF-β are higher in aged than young satellite cell niches [10]. Further analysis showed greater activation of the TGF-β pathway in old satellite cells, and physical competition between Notch and pSmad3 at the promoters of multiple CDK inhibitors [10, 75]. Furthermore, the decline of Notch1 signaling with age is thought to be another cause of the decreased regenerative potential of aged skeletal muscle. Indeed, enhancement of Notch-1 signaling promotes muscle regeneration in old skeletal muscle [8, 9]. Although these experiments suggest a crucial role for Notch1 signaling in satellite cell function, much remains to be determined, especially regarding the role of Notch3 signaling during muscle regeneration. Notch3 was expressed in satellite cells, and various structural and functional differences between Notch3 and Notch1/Notch2 have been reported [76]. More recently, Kitamoto and Hanaoka [77] conducted two very intriguing experiments. They analyzed muscle after repeated injuries, by generating mice deficit in Notch3 and also by repetitive intramuscular injections of cardiotoxin (CTX) into the Notch3-deficient mice.

They found a remarkable overgrowth of muscle mass in the Notch3-deficient mice but only when they suffered repetitive muscle injuries. Analysis of cultured myofibers revealed that the number of self-renewing Pax7-positive satellite cells attached to myofibers was increased in the Notch3-deficient mice compared to control mice. Given these findings, the Notch3 pathway might act as a Notch1 repressor by activating Nrarp, a negative feedback regulator of Notch signaling.

5. The differentiation of satellite cells

5.1. MyoD family

Satellite cell myogenic potential mostly relies on the expression of Pax genes and myogenic regulatory factors (MRFs: MyoD, Myf5, myogenin, and MRF4). Sequential activation and expression of Pax3/7 and MRFs is required for the progression of skeletal myoblasts through myogenesis. Pax7 is expressed by all satellite cells and essential to their postnatal maintenance and self-renewal [78]. Pax7 induces myoblast proliferation and delays their differentiation not by blocking myogenin expression [79] but by regulating MyoD [80]. In parallel, myogenin directly down-regulates Pax7 protein expression during differentiation [80]. MyoD is required for the differentiation of skeletal myoblasts [81, 82]. In addition, MyoD null satellite cells showed reduced myogenin expression and absolutely no MRF4 expresion, and displayed a dramatic differentiation deficit [82]. Indeed, muscle regeneration *in vivo* is markedly impaired in MyoD null mice [83]. In contrast, Myf5 regulates the proliferation rate and homeostasis [84]. MyoD can compensate for Myf5 in adults. Myf5 deficiency leading to a lack of myoblast amplification and loss of MyoD induced an increased propensity for self-renewal rather than progression through myogenic differentiation. The differentiation factors myogenin and MRF4 are not involved in satellite cell development or maintenance [84] but induction of myogenin is necessary and sufficient for the formation of myotubes and fibers.

5.2. IGF-I and calcineurin-dependent signaling

IGF-I positively regulated not only the proliferation but also the differentiation of satellite cells/myoblasts *in vitro* possibly through a calcineurin-dependent pathway. Since activated calcineurin promotes the transcription and activation of myocyte enhance factor 2 (MEF2), myogenin, and MyoD [85-87], calcineurin seems to control satellite cell differentiation and myofiber growth and maturation, all of which are involved in muscle regeneration [88, 89]. In fact, our previous study [88] showed a marked increase in the amount of calcineurin protein and the clear colocalization of calcineurin and MyoD or myogenin in many myoblasts and myotubes during muscle regeneration. In addition, we showed that the inhibition of calcineurin by cyclosporine A (CsA) induced extensive inflammation, marked fiber atrophy, and the appearance of immature myotubes in regenerating muscle compared with placebo-treated mice [88]. Several other studies indicated such defects in skeletal muscle regeneration when calcineurin was inhibited [90, 91], whereas transgenic activation

of calcineurin is known to markedly promote the remodeling of muscle fibers after damage [92, 93].

Many researchers have utilized CsA, though in different amounts, to determine the downstream modulators of calcineurin signaling. We found that intraperitoneal CsA treatment daily at 25 mg/Kg/day enhanced the expression of myostatin and Smad3 mRNA in regeneration-defective tibialis anterior muscle after an injection of bupivacaine [89]. The possibility that myostatin is a downstream mediator of calcineurin signaling has been indicated by experiments with two different transgenic mice [94]. In addition, calcineurin's pharmacological inhibition caused a decline in the transcription and activation of myogenin and MyoD during myogenic differentiation by a downregulation of MyoD expression [95]. Considering these findings, calcineurin seems to block the myostatin-Smad3 pathway to enhance the expression of myogenic differentiation factor (MyoD) during muscle regeneration *in vivo*. Using CsA treatment *in vivo*, recent evidence including that obtained by our group has also identified Id1 [87, 89], Id3 [87], and Egr-1 [87] as a possible downstream negative hypertrophic effector target of the calcineurin-NFAT (nuclear factor of activated T-cells) pathway.

FOXO (forkhead box O)-induced expression of Atrogin-1 has been shown to inhibit calcineurin activity [96]. More recently, the calcineurin variant CnAβ1 was suggested to block the nuclear localization of the FOXO protein and the expression of several genes targeted by FOXO [the muscle ring finger-1 (MuRF1), Gadd45a, Pmaip1, and atrogin genes] in C2C12 myoblasts [93]. In addition, transgenic up-regulation of CnAβ1 expression promotes the remodeling of cardiotoxin-treated muscle fibers [93]. In cardiomyocytes, calcineurin directly binds and dephosphorylates (inactivates) Akt; FOXO indirectly activates Akt by inhibiting calcineurin phosphatase activity [97]. In murine C2C12 myotubes, Akt was shown to antagonize calcineurin signaling by causing hyperphosphorylation of NFATc1 [60]. Interaction between CnAβ1 and FOXO during muscle regeneration is a very attractive idea, although it has not been demonstrated in adult skeletal muscle *in vivo*.

5.3. Serum response factor

SRF is an ubiquitously expressed member of the MADS box transcription factor family, sharing a highly conserved DNA-binding/dimerization domain, which binds the core sequence of SRE/CArG boxes [CC (A/T)6 GG] as homodimers [98]. Functional CArG boxes have been found in the cis-regulatory regions of various muscle-specific genes, such as the skeletal α-actin [99], muscle creatine kinase, dystrophin, tropomyosin, and myosin light chain 1/3 genes. The majority of SRF's targets are genes involved in cell growth, migration, cytoskeletal organization, and myogenesis [100, 101]. SRF was first shown to be essential for both skeletal muscle cell growth and differentiation in experiments performed with C2C12 myogenic cells. In this model, SRF inactivation abolished MyoD and myogenin expression, preventing cell fusion in differentiated myotubes [102]. SRF also enhances the hypertrophic process in muscle fibers after mechanical overloading [103]. For example, we showed that, in mechanically overloaded muscles of rats, SRF protein is co-localized with MyoD and

myogenin in myoblast-like cells during the active differentiation phase [104]. Recent results obtained with specific SRF knock-out models, by the Cre-LoxP system, emphasize a crucial role for SRF in postnatal skeletal muscle growth and regeneration [105], by direct binding of IL-4 and IGF-I promoters *in vivo*. These lines of evidence appear to indicate that SRF modulates the differentiating process of satellite cells in adult mature muscle.

The expression and cellular localization of SRF and myocardin-related transcription factor-A (MRTF-A) appear to be regulated by several upstream factors including β1-integrin, RhoA, striated muscle activators of Rho signaling (STARS) [106], and MuRF2 [107]. For instance, Lange et al. [107] demonstrated that SRF is blocked and relocalized by the nuclear translocation of MuRF2, which regulates a signaling pathway composed of titin-Nbr1-p62/SQSTM1 at the position of the sarcomere depending on mechanical activity. To date, there has been no attempt to investigate whether titin-Nbr1-p62/SQSTM1 and MuRF2 affect muscle regeneration. In addition, the mutation of SRF delineated the translocational action of MRTF-A induced *in vitro* by STARS, a muscle-specific actin-binding protein [106].

5.4. Wnt-dependent signaling

Similar to Notch signaling, canonical Wnt signaling is critical for muscle repair [108-111]. The canonical Wnt signaling cascade requires soluble Wnt ligands to interact with Frizzled receptors and low-density lipoprotein receptor-related protein co-receptors (LRP). This coordination stimulates phosphorylation of Disheveled and inactivates GSK3β's phosphorylation of β-catenin. In the nucleus, the de-phosphorylated β-catenin binds to T-cell factor/Lymphoid enhancer factor-1 transcription factors [112], which may directly activate Myf5 and MyoD or may upregualte MRF co-activators such as c-Jun N-terminal kinases [113, 114]. It is suggested that Notch activity presides during myoblast proliferation after which there is a temporal switch to Wnt signaling and subsequent myoblast differentiation and fusion into myotubes [108]. Inhibiting Notch (with soluble Jagged ligand or with a γ-secretase inhibitor) or activating Wnt (by inhibiting GSK3β or adding Wnt3a) decreases Myf5 expression and promotes muscle differentiation providing evidence that Notch signaling needs to be turned off and Wnt turned on for differentiation to ensue [108, 115].

This hypothesis was supported by the finding that aberrant activation of the Wnt pathway can lead to fibrogenic conversion of cells in different lineages [116-118]. In fact, Wnt signaling was shown to be enhanced in aged muscle and in myogenic progenitors exposed to aged serum [116]. To directly test the effects of Wnt on cell fate and muscle regeneration, Brack et al. [116] altered Wnt signaling *in vitro* and *in vivo.* Addition of Wnt3A protein to young serum resulted in increased myogenic-to-fibrogenic conversion of progenitors *in vitro* [116]. Conversely, the myogenic-to-fibrogenic conversion of aged serum was abrogated by Wnt inhibitors [116]. *In vivo*, the injection of Wnt3A into young regenerating muscle 1 day after injury resulted in increased connective tissue deposition and a reduction in satellite cell proliferation [116]. The authors therefore tested whether inhibiting Wnt signaling in aged muscle would reduce fibrosis and enhance muscle regeneration.

6. Second-stage fusion (late differentiation)

Growth and maturation of the muscle cells are achieved through a second-stage fusion, which occurs between the nascent myofibers/myotubes and myoblasts. Although many regulators of this fusion process have been revealed in recent years [119], a better understanding of the regulation is still needed. mTOR (mammalian target of rapamycin) is one of the candidates regulating the fusion. mTOR signaling regulates a wide range of biological processes, including cell growth, various types of cellular differentiation, and metabolism [120, 121]. mTOR assembles two biochemically and functionally distinct protein complexes, mTORC1 (mTOR complex 1) and mTORC2, which are sensitive and insensitive to rapamycin, respectively [120]. Rapamycin-sensitive mTORC1 signaling has emerged as a key regulator of skeletal muscle differentiation and remodeling. Rapamycin inhibits myoblast differentiation *in vitro* [122, 123], compensatory myofiber hypertrophy *in vivo*, and regrowth of myofibers after atrophy [124]. The regulation of skeletal myocyte differentiation by mTORC1 occurs at two stages via distinct mechanisms. mTORC1 controls the initiation of myoblast differentiation by regulating IGF-II expression [123], whereas late-stage myocyte fusion leading to myotube maturation is regulated by mTORC1 through a yet to be identified secreted factor [125]. More recent findings pointed out that the fusion factor targeting mTORC1 is follistatin during the late differentiation phase. Sun et al. [126] have found that, in C2C12 cells differentiating for 24-72h, miR-1 luciferase (enhancer) activity was markedly downregulated after treatment with rapamycin but not wortmannin (PI3-K inhibitor) or SB203580 (MAPK inhibitor). In addition, rapamycin increased the amount of histone deacetylase 4 (HDAC4) protein and reduced follistatin mRNA and MyoD protein levels in C2C12 and C3H10T1/2 cells. Furthermore, daily administration of trichostatin A and a single dose of adenovirus expressing follistatin rescued the defective muscle regeneration caused by treatment with rapamycin. Sun et al. [126] proposed the intriguing hypothesis that mTOR-miR-1 promotes myocyte fusion by recruiting HDAC4-follistatin during myoblast differentiation *in vitro* and skeletal muscle regeneration *in vivo.*

7. Satellite cell self-renewal

A hallmark of stem cells is their ability to self-renew. In skeletal muscle, asymmetric cell division takes place in a subset of the satellite cell population to generate a self-renewing progenitor and hyperplastic daughter cell which later contributes to *de novo* muscle formation [127]. Several extrinsic pathways have been implicated in mediating this phenomenon [108, 127, 128]. One family of candidate peptides is the Wnt family of signaling molecules which consists of over 19 cysteine-rich secreted glycoproteins that in part bind the Frizzled (Fzd) receptors [129].

In a non-canonical Wnt cascade, Wnt7a has been characterized for its role as the extracellular ligand mediating asymmetric cell division which is thought to be the mechanism by which satellite cells are able to self-renew [128]. Lineage tracing of satellite cell populations indicates ~90% of cells to have at some point expressed Myf5 (Pax7+Myf5+) [127]. The Myf5+ cells have a reduced potential to self-renew as the majority undergo

symmetrical cell divisions and later contribute to muscle syncitia [127]. The remaining ~10% of satellite cells divide asymmetrically and give rise to Pax7+Myf5- as well as Pax7+Myf5+ progeny thereby maintaining the stem cell pool of muscle progenitors [127]. The capacity of Pax7+Myf5- cells to self-renew is explained by expression of the Wnt receptor Fzd7 on these cells but not on Pax7+Myf5+ cells, thus allowing induction of asymmetrical cell division via Wnt7a-induced signaling [128]. Importantly, stimulation of satellite cells with Wnt7a leads to an increase in the symmetrical expansion of satellite cells, while muscle from Wnt7a knockout mice displays a dramatic reduction in satellite cell numbers following regeneration [128].

The Notch inhibitor, Numb is also asymmetrically expressed on the activated satellite cells and may regulate cell fate choices by promoting progression down the myogenic lineage [130]. Self renewal may also occur through symmetrical division in which both daughter cells maintain stem-cell properties [131, 132]. Cells that do not express MyoD but continue to express Pax7 are suggested to be refrained from self-renewal [133].

8. Other regulators of the muscle regenerating process

8.1. Myostatin and TGF-β

The TGF-β superfamily plays a crucial role in normal physiology and pathogenesis in a number of tissues. Myostatin was first discovered during screening for novel members of the TGF-β superfamily, and shown to be a potent negative regulator of muscle growth [134]. Like other TGF-β family members, myostatin is synthesized as a precursor protein that is cleaved by furin proteases to generate the active C-terminal dimer. When produced in Chinese hamster ovary cells, the C-terminal dimer remains bound to the N-terminal propeptide, which remains in a latent, inactive state [135]. Most, if not all, of the myostatin protein that circulates in blood also appears to exist in an inactive complex with a variety of proteins, including the propeptide [136]. Myostatin binds to and signals through a combination of Activin IIA/B receptors on the cell membrane, but has higher affinity for ActRIIB. On binding to ActRIIB, myostatin forms a complex with a second surface type I receptor, either activin receptor-like kinase (ALK4 or ActRIB) or ALK5 to stimulate the phosphorylation of receptor Smad and the Smad2/3 transcription factors in the cytoplasm. This leads to the assembly of Smad2/3 with Smad4 to form a heterodimer that is able to translocate to the nucleus and activate the transcription of target genes [137].

Studies indicate that myostatin inhibits the activation, differentiation, and self-renewal of satellite cells [138-140] and the expression of the muscle regulatory factors crucial for the regeneration and differentiation of myofibers [138, 141]. One of the known downstream targets of Smad signaling is MyoD. Interestingly, myostatin downregulates MyoD expression in an NF-κB-independent manner [142]. Myostatin also inhibits Pax3 expression, which is possibly an upstream target of MyoD [142]. Recently, it was found that FOXO1 and Smad synergistically increase the expression of myostatin mRNA and its promoter activity

in C2C12 myotubes [95]. Taken together, myostatin-mediated signaling activates FOXO, and this leads to the expression of ubiquitin ligases.

TGF-β1 is expressed during myogenesis, and its spatial and temporal expression in the developing connective tissue is correlated with the fiber-type composition of the surrounding myotubes. Myotubes formed before the expression of TGF-β1 develop into slow fibers, whereas fast fibers form when myoblasts are adjacent to connective tissue expressing TGF-β1 [143]. TGF-β1 has been shown to inhibit the differentiation of fetal myoblasts but does not affect embryonic myoblasts [144]. In mature adult muscle, TGF-β negatively affects skeletal muscle regeneration by inhibiting satellite cell proliferation, myoblast fusion, and expression of some muscle specific-genes [145]. Furthermore, TGF-β1 induced the transformantion of myogenic cells into fibrotic cells after injury [146].

TGF-β1, a potent regulator of tissue wound healing and fibrosis, is physiologically upregulated in regenerating skeletal muscle after injury and exercise and is thought to participate in a transient inflammatory response to muscle damage [147, 148]. Persistent exposure to the inflammatory response leads to an altered extracellular matrix and increased levels of growth factors and cytokines, including TGF-β1, which contribute to the formation of fibrotic tissue [147, 148]. Increased levels of TGF-β1 inhibit satellite cell activation and impair myocyte differentiation [145, 149]. Figure 1 summarizes the calcineurin-, myostatin-, and Akt-dependent signaling in muscle regeneration.

8.2. TNF-α signaling

TNF-α has long been viewed as the quintessential proinflammatory cytokine, capable of classical activation of macrophages to the M1 phenotype, and thereby inducing the production of other proinflammatory, Th1 cytokines. Following muscle injury, the early invading neutrophil and macrophage populations express TNF-α [152], suggesting that the cytokine may contribute to the early inflammatory stages that precede muscle regeneration. TNF-α levels in muscle following acute injury peak at 24h postinjury, which indicates that TNF-α production is most tightly coupled with the Th1 inflammatory response in injured muscle [153]. Because findings show that TNF-α induces iNOS expression in myeloid cells and that myeloid cell-derived NO can cause muscle fiber damage early on, Th1 inflammatory cells have been associated with muscle damage. However, TNF-α levels remain elevated for nearly 2 weeks following acute injury, indicating that TNF-α may also modulate the regenerative process [153]. Intriguingly, the expression of TNF-α receptors by muscle cells themselves is elevated as a later consequence of injury, during the regenerative process, and enables TNF-α to act directly on muscle cells to modulate their proliferation and differentiation [152].

Numerous experimental observations indicate that TNF-α acts directly on muscle cells in affecting muscle regeneration. For example, TNF-α null mutants and TNF-α receptor mutants show lower levels of MyoD and MEF2 expression than wild-type controls following acute injury [153, 154]. The application of exogenous TNF-α to myoblasts *in vitro*

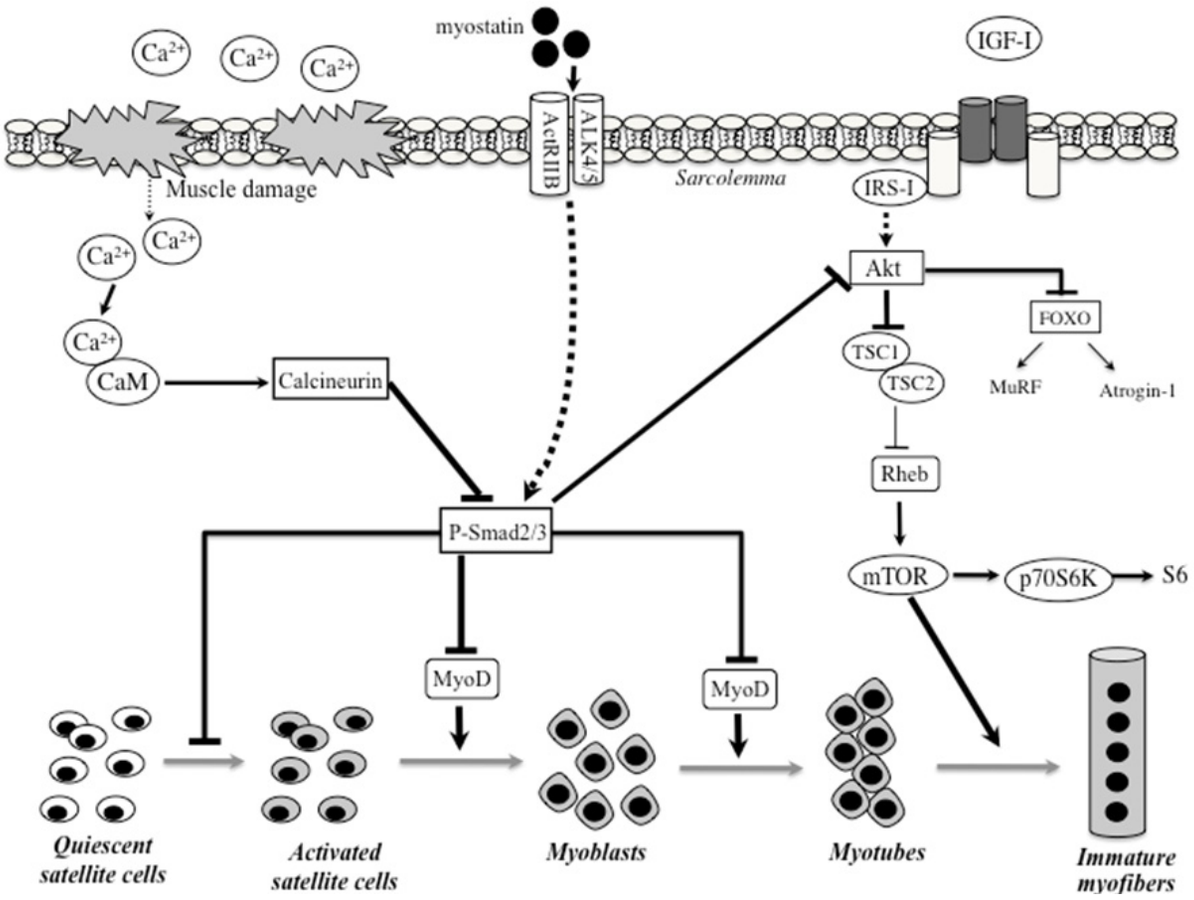

Figure 1. Schematic diagram of calcineurin-, myostatin-, and Akt-dependent signaling in muscle regeneration. Myostatin acts through activin receptor IIB (ActRIIB). The ALK4/5 heterodimer activates Smad2/3 with blocking of MyoD transactivation in an autoregulatory feedback loop. In addition, Smad3 sequesters MyoD in the cytoplasm to prevent it from entering the nucleus and activating the stem cell population. In proliferating myoblasts, this pathway arrests cell proliferation and differentiation. Moreover, recent findings [150, 151] suggest that the myostatin-Smad pathway inhibits protein synthesis probably by blocking the functional role of Akt. Damage the muscle fiber membranes after treatment with myotoxin elicits an increase in intracellular Ca^{2+} levels via the influx of Ca^{2+} from the extracellular space. Binding of the Ca^{2+}/CaM complex to the calcinuerin regulatory subunit leads to its activation. Activated calcineurin dephosphorylates a range of transcription factors (including MEF2 and NFAT). Activated calcineurin inhibit the functional role of Egr-1 and Smad2/3 [87, 89], and promotes myogenic differentiation. Calcineurin signaling is markedly inhibited by myostatain [94] and FOXO [96, 97]. IGF-I produced by the regenerating muscle activates PI3-K-Akt-mTOR signaling resulting in a positive protein balance. One part of mTOR (mTORC1) enhances myotube differentiation at later stages probably through the induction of follistatin expression. CaM; calmodulin, IRS-1; insulin receptor substrate-1

increases their proliferation, and inhibited the process of early differentiation to terminal differentiation [155-157]. Experiments *in vivo* using a lung-specific TNF-α transgene also showed a differentiation-inhibiting role [158]. These TNF-α abundant mice exhibited attenuated expression of developmental myosin heavy chain (MHC) in reloaded soleus muscle after hindlimb suspension [158]. TNF-α affects several intracellular signaling pathways leading to the activation of NF-κB, caspase 8, and stress-induced factors like c-Jun N-terminal kinase (JNK) and p38 MAPK [159]. Activation of NF-κB can inhibit myogenesis

through several processes. NF-κB can promote the expression and stability of cyclin D1 in muscle [155], leading to increased cell proliferation and inhibition of differentiation. Furthermore, NF-κB can cause destabilization of MyoD mRNA and degradation of MyoD protein [155, 156]. The role of JNK in the effect of TNF-α on myogenesis has been less investigated. A recent study suggested that activation of JNK by TNF-α blocks IGF-I signaling necessary for the differentiation of myoblasts [160].

TNF-α can activate signaling through other pathways independent of NF-κB to promote muscle differentiation. Both IL-1 and TNF-α can activate p38 kinase [161], promoting the differentiation. In particular, inhibition of p38 in skeletal muscle cells *in vitro* inhibits myocytes from fusing to form myotubes and reduces the expression of MEF2, myogenin, and myosin light chain kinase [162], all of which indicate that p38 activation can promote muscle differentiation. Furthermore, p38 activation can also increase the activity of MyoD [162, 163]. The ability of p38 to promote myogenesis relies, in part, on its ability to phosphorylate and increase the transcriptional activity of MEF2 [162, 164]. In contrast, p38 activation can also inhibit myogenesis by the phosphorylation of other MyoD family members (MRF4). The elevated expression and activity of p38 late in muscle differentiation leads to increased MRF4 phosphorylation and, as a consequence, a decline in desmin and skeletal α-actin expression [165]. In fact, overexpression of MRF4 in a transgenic mouse line caused defective muscle regeneration following injury [166]. Therefore, TNF-α-dependent signaling regulates various aspects of the muscle regenerating process (immune response, and proliferation and differentiation of satellite cells) through different downstream mediators (NF-κB, JNK, and p38) [Figure 2].

8.3. TWEAK

TNF-like weak inducer of apoptosis (TWEAK) is a pro-inflammatory cytokine belonging to the TNF superfamily of ligands. Initially synthesized as a type II transmembrane protein, TWEAK is cleaved to its soluble form, and signals as a trimerized molecule [167]. Generally, TWEAK signaling occurs through binding to Fn14, a type I transmembrane receptor belonging to the TNF receptor superfamily. TWEAK has been found to promote the regeneration and growth of myofibers after injury [168-170]. Dogra et al. [168] reported that TWEAK inhibits the differentiation of cultured C2C12 or primary myoblasts into multinucleated myotubes. More recently, a transgenic model of TWEAK also suggested a differentiation-promoting role in muscle regeneration *in vivo*. In fact, mRNA levels of TNF-α, IL-6 and CCL-2 and protein levels of embryonic MHC were significantly reduced in cardiotoxin (CTX)-injected TA muscle of TWEAK-KO mice compared to that of wild-type mice [171]. In addition, these parameters were found to be significantly inceased in regenerating TA muscle of TWEAK-Tg mice compared to that of control mice. Since such a modulation of the TWEAK gene caused no apparent differences in levels of phospho-Akt and phospho p38MAPK in the regenerating muscle among each mouse model, TWEAK seems to function independently of Akt- and p38-linked signaling [171]. Intriguingly, electromobility shift asay by Mittal et al. [171] indicated the possibility of TWEAK-NF-κB signaling, although further descriptive analysis needs to be done.

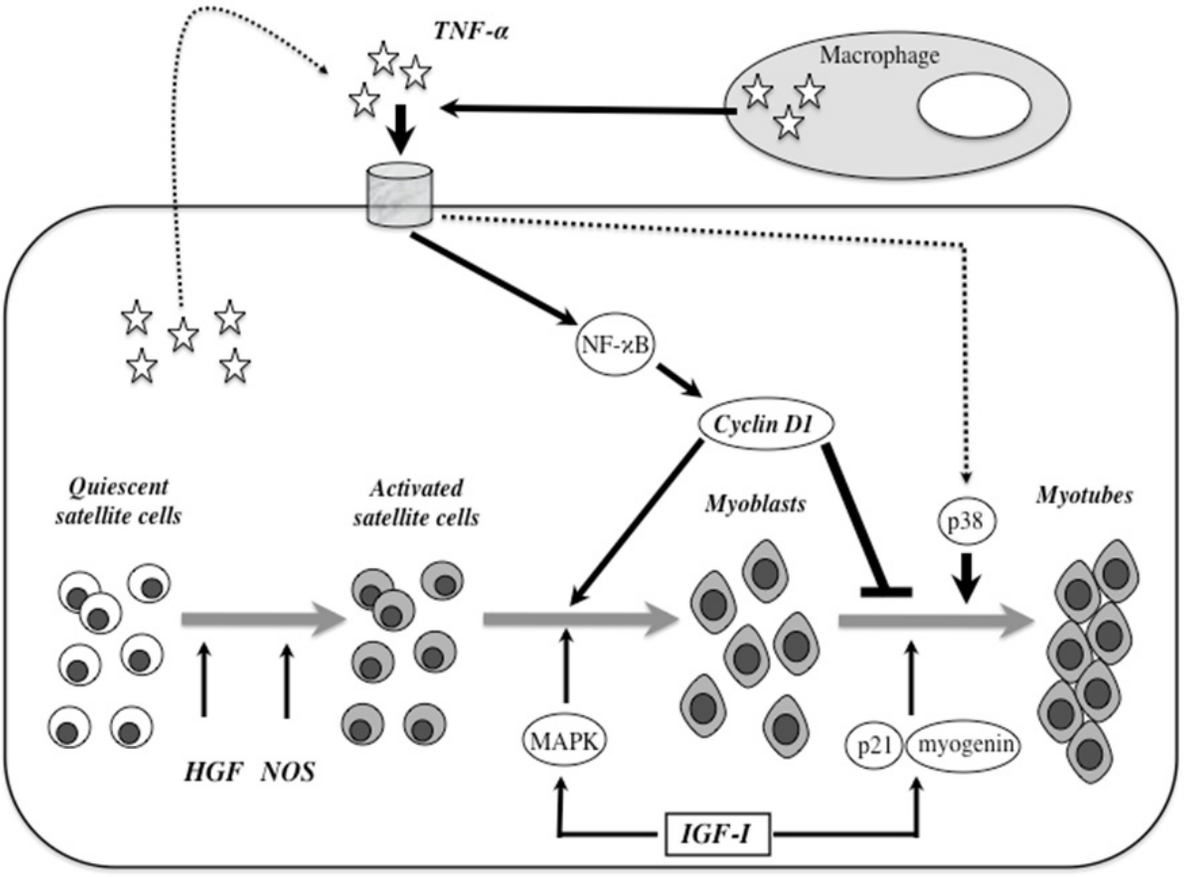

Figure 2. The functional role of TNF-α signaling in the regenerating muscle. HGF and nNOS co-ordinate the switch from quiescence to activation in satellite cells. IGF-I enhances the proliferation of satellite cells via a MAPK-dependent pathway. IGF-I also promotes myogenic differentiation via p21 and myogenin. In regenerating muscle after treatment with myotoxin, the differentiating myotubes seem to be fused together and/or incorporated into the existing muscle fibers. TNF-α, which is produced by the damaged muscle and macrophages, stimulates TNFR. TNFR activates NF-κB-signaling, in turn cyclin D1 activate the proliferation, but not differentiation, of satellite cells. In addition, TNF-α activates p38-dependent signaling leading to the differentiation of myoblasts.

8.4. MicroRNAs

The human genome contains thousands of non-coding RNAs, the best-studied class of which are microRNAs (miRNAs)[172], which regulate gene expression at the transcriptional and post-transcriptional levels. miRNAs suppress gene expression through their complementarity to the sequence of one or more RNAs, usually at a site in the 3′ untranslated region. The formation of a miRNA-target complex results either in inhibition of protein translation or in degradation of the mRNA transcript through a process similar to RNA interference [173]. There is no doubt that the formation, maintenance, and physiological and pathophysiological responses of skeletal muscles, with all their complex regulatory circuits, are subject to regulation by non-coding RNAs.

Many miRNAs are expressed in skeletal and cardiac muscle. Some of them are found specifically, or at least are highly concentrated, in skeletal and/or cardiac muscle, suggesting

specific roles in myogenesis [174]. The expression of the muscle-specific miRNAs miR-1, miR-133, miR-206, and miR-208 seems to be under the control of a core muscle transcriptional network, which involves the pleiotropic SRF, MyoD, and the bHLH transcription factor Twist in cooperation with MEF2 [175-177]. Chromatin immunoprecipitation followed by a microarray analysis indicated that MyoD and myogenin bind sequences upstream of miR-1 and miR-133 [176]. miR-133a increases myoblast proliferation, via its repression of SRF [178], while miR-1 stimulates myoblast differentiation via its inhibition of histone deacetylase 4 (HDAC4) [178]. In addition, MyoD has been demonstrated to utilize miRNAs, including miR-1 and miR-206, to suppress downstream gene expression [178, 179]. More recently, Hirai et al. [180] have demonstrated that miR-1 and miR-206 bind to two miR-1/miR-206-binding sequences within the Pax3-3'UTR and suppress Pax3 expression. Since Pax3 expression increases cell survival and suppresses myogenic differentiation in myoblasts, down-regulation of Pax3 has been shown to elicit proper myogenic differentiation along with an increase in apoptosis [180]. An analogus role was described for the regulation of Pax7, which is repressed by miR-1 and miR-206 [178, 181]. In contrast, miR-221 and miR-222 are downregulated during the transition from proliferation to differentiation [182]. Decreases in these miRNAs are associated with increased expression of the cell cycle inhibitor p27. Overexpression of miR-221 and miR-222 in differentiating myotubes delays cell cycle withdrawal and differentiation, a response associated with a reduction in sarcomeric protein [182].

9. Conclusions and perspectives

In normal, skeletal muscle possesses a robust capacity to repair itself, the ability to augment and enhance this process would significantly advance the treatment of congenital muscle disorders and severe muscle trauma for which, even with the best of present-day treatments, physical handicap or amputation are the most likely outcomes. Sarcopenia seems to include the defect of muscle regeneration probably due to the repetitive muscular damage [8-10]. Currently available data show that resistance training combined with amino acid-containing supplements would be the best way to prevent age-related muscle wasting and weakness. Therefore, for these endogenous repair therapies to advance, it is essential that an understanding exists of the biochemical, cellular and mechanical cues that promote skeletal muscle repair.

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Acknowledgement

This work was supported by a research Grant-in-Aid for Scientific Research C (No. 23500578) from the Ministry of Eduction, Cultute, Sports, Science and Technology of Japan.

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