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CDK inhibitors for muscle stem cell differentiation and self-renewal

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Abstract Regeneration of muscle is undertaken by muscle stem cell populations named satellite cells which are normally quiescent or at the G0 phase of the cell cycle. However, upon signals from damaged muscle, satellite cells lose their quiescence, and enter the G1 cell cycle phase to expand the population of satellite cell progenies termed myogenic precursor cells (MPCs). Eventually, MPCs stop their cell cycle and undergo terminal differentiation to form skeletal muscle fibers. Some MPCs retract to quiescent satellite cells as a self-renewal process. Therefore, cell cycle regulation, consisting of satellite cell activation, proliferation, differentiation and self-renewal, is the key event of muscle regeneration. In this review, we summarize up-to-date progress on research about cell cycle regulation of myogenic progenitor cells and muscle stem cells during embryonic myogenesis and adult muscle regeneration, aging, exercise and muscle diseases including muscular dystrophy and muscle fiber atrophy, especially focusing on cyclin-dependent kinase inhibitors (CDKIs).

Keywords : muscle stem cell, satellite cell, CDK inhibitor, self-renewal, regeneration

Myogenic bHLH factors for muscle regeneration

Skeletal muscle is known to be a highly regenerative tissue in the human body[1], efficiently responding to the constant muscle damage incurred by everyday work. This muscle regeneration is prosecuted by muscle satellite cells, a stem cell population for skeletal muscle[2,3]. Existence of satellite cells was first established by Mauro et al. (1961), in the skeletal muscle fibers of a frog[4]. Satellite cells are normally mitotically quiescent, staying in the G0 phase of the cell cycle, and located beneath the basal lamina of muscle fibers[5,6]. These quiescent satellite cells (QSCs) are identified by the presence of Pax7, which is a paired box family of transcription factors. Pax7 is critical for the survival and native functioning of QSCs[7,8]. These QSCs normally do not express MyoD, a myogenic basic-helix-loop-helix (bHLH) transcription factor family which acts as a master regulator for myogenesis[9]. Upon activation due to muscle damage signals, these Pax7(+) MyoD(-) QSCs enter the G1 phase and initiate MyoD expression, which in turn promotes the process of myogenic differentiation programs. These Pax7(+)MyoD(+) cells are known as activated satellite sells (ASCs) or myogenic precursor cells (MPCs). MPCs initially expand their progeny and then exit their cell cycle to down-regulate Pax7 expression, activate myogenin and other terminal differentiation markers such as myosin heavy chain (MHC), and undergo terminal differentiation into myocytes. Finally, myocytes fuse together to form multinucleated myotubes, immature forms of muscle fibers. After accumulation of sarcomeric proteins, myotubes increase in size and start muscle contraction as mature muscle fibers. Other than MyoD, Myf5 is another myogenic bHLH transcription factor that also serves as a major regulator of this myogenic program during satellite cell activation, proliferation and differentiation[10]. Myogenin, which is the 3rd myogenic bHLH transcription factor, marks post mitotic myogenic cells and plays an essential role in the muscle differentiation process. MRF4, which is the 4th myogenic bHLH transcription factor, also works for the myogenic program. Terminally differentiated myocytes and myotubes can be identified by the presence of myogenin and MHC, a motor protein that regulates muscle function and myofibril assembly. Importantly, expression of the correct isoform of MHCs is necessary for the determination of skeletal muscle fiber types and the appropriate functioning of skeletal muscle[11].

Satellite cell self-renewal

Although most MPCs undergo terminal differentiation to create new muscle fibers, a small population of the Pax7(+)MyoD(+) MPCs exit the cell cycle, down-regulate MyoD, and then undergo self-renewal to repopulate the Pax7(+)MyoD(-) QSC pool. These QSCs stay beneath the basal lamina of muscle fibers as quiescent satellite cells. This self-renewal process is essential to maintain their stem cell pool and regeneration capacity in skeletal muscle during their life. *In vitro* experimentations revealed that upon serum deprivation, a large number of

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proliferating primary MPCs (myoblasts) isolated from adult skeletal muscle maintained MyoD expression and underwent myogenic differentiation to form multinucleated myotubes, recapitulating *in vivo* muscle regeneration programs. However, in the same derived conditions, a small population of myoblasts down-regulate MyoD and up-regulate Pax7 expression to form non-cell cycling mononuclear cells. These Pax7(+)MyoD(-) mononuclear cells termed "reserve cells" are able to re-enter the cell cycle, start expressing MyoD and undergo muscle differentiation once they are re-exposed to growth media[12]. This property of the reserve cells make them an equivalent of the QSCs present *in vivo*. MyoD down-regulation is important for satellite cell self-renewal. Therefore, satellite cells isolated from MyoD gene knockout mice display increased self-renewal capacity, and this down-regulation of MyoD is regulated by the Notch signaling cascade during satellite cell self-renewal[13,14]. Satellite cells possesses multi-differentiation capability at least *in vitro*, since they can differentiate into adipogenic and osteogenic cell types in addition to myogenic cells[15].

Interestingly, the proliferating myoblasts have been found to exhibit distinct expression of MyoD and Myf5 at different stages of the cell cycle, which correlates with their differentiation ability. Cell cycle stage-specific expression of MyoD starts with null expression at the G0 phase, which increases by the mid-G1 phase, then decreases during the G1/S phase, and finally increases again during the S through M phase[16]. In contrast, Myf5 shows higher expression at G0, followed by a decrease in levels at G1; and then further up-regulation at the end of G1, thereafter maintaining a stable level of expression until mitosis is complete[16]. Terminal differentiation events in muscle cells requires cell cycle exit at the G1 phase, which is shown to be repressed by the presence of basic fibroblast growth factor (bFGF). Once the myoblasts are deprived of FGF, the cells exit the cell cycle after completing just one round of final cell division and starting to fuse to form myotubes[17].

Cell cycle regulation by Cip/Kip type CDK inhibitors (CDKIs)

The cell cycle process can be separated into four phases (G1/S/G2/M). Among these phases, G1 is the only phase which can be determined by external stimuli including growth factors for the progression to the S phase. Without such stimuli, cells undergo a quiescent G0 phase. Importantly, the G1 phase consists of early and late G1 phases which are separated by the "restriction point" or "G1/S checkpoint." Once past this point, cell cycle progression goes through complete cell division in a growth factor-independent manner[18]. It has been established that the cyclins and cylin dependent kinases (CDKs) play a key role in the initiation of the cell cycle[19]. The different cyclins such as Cyclin D/E/A and B bind with specific complimentary CDK complexes such as CDK4/6, CDK2 and CDC2, respectively. Upon growth factor stimulation, the cells enter into the cell cycle mediated by the Cyclin D-CDK4/6 and Cyclin E-CDK2 complexes, which execute phosphorylation of Retinoblastoma protein (pRB) in a sequential manner[20]. Phosphorylated RB (pRB) is added to counteract its inhibitory effects on the cell-cycle promoting transcription factor E2F[21,22]. E2F specifically promotes the cell to enter the S-phase, after which the cyclin-CDK complexes interact to initiate mitosis[23-25].

Going deeper into the discussion about the cell cycle, it is known that cell cycle arrest occurs at the G1 phase and inhibits progression into the S phase. This arrest is regulated by the Cyclin-dependent kinase inhibitors (CDKIs), which inhibit CDK activity by binding to them. Based on their CDK specificity, structural organization and origin, there are two classes of CDKIs: INK4 family ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d/ARF}$, referred as p16, p15, p18 and p19) and the Cip/Kip family ($p21^{Cip1/Waf1/Sdi1}$, $p27^{Kip1}$ and $p57^{Kip2}$, referred as p21, p27 and p57). The INK4 family binds to CDK4/CDK6 subunits and blocks their interaction with Cyclin D, whereas the Cip/Kip family interacts with all the cyclin/CDK complexes and blocks their activity[26,27]. Structurally, p21, p27 and p57 are highly conserved during evolution, especially concerning a conserved CDK binding-inhibitory domain in their N-terminal regions[28]. These three CDKIs are able to substitute each other for the basal functions such as cell cycle arrest[29]. Among these three CDKIs, p27 and p57 are more closely related, and in addition to the conserved CDK binding-inhibitory domain, both CDKIs share a conserved QT domain in their C-terminal regions and exhibit similar biochemical characteristics[30]. By contrast, p21, but not p27 or p57, possesses strong affinity binding to proliferating cell nuclear antigenS (PCNA). This association interferes with PCNA-dependent enzyme activities involved in DNA synthesis[31]. p57 also binds in vitro with PCNA with a much lower affinity than p21[32]. Therefore, there are common and specific roles for each CDKI. Previous studies have shown that the CDKIs interact to induce a stress response. For instance, following anti-mitogenic signals, p21 and p27 bind to cyclin-CDK complexes to induce cell cycle arrest (G1 arrest) by inhibiting CDK activity. The DNA damage response (DDR) pathway induces the transcription factor p53 to upregulate p21 as a major pathway of DRR, which induces G1-phase arrest or prevents S phase entry followed by DNA replication, and thus sustains the G2 arrest checkpoint[33]. DDR also induces p27 to induce G1-phase arrest in a p53 independent manner. However, p57 is not part of the DNA damage response. p57 is a target of p73β, a p53 related DRR protein, so it is possible that p57 may be an additional downstream DDR effector[32]. Interestingly, the human p57 gene is located on chromosome 11p15.5, an imprinted region implicated in both Beckwith-Wiedemann syndrome and sporadic cancers[34]. Similarly, the mouse

p57 gene is genomically imprinted, and paternally inherited alleles are transcriptionally repressed by methylation. Mouse p27 is mapped within a cluster of imprinted genes, including Insulin-2, Insulin-like growth factor-2, H19 and Mash2 on chromosome 7[35].

Gene knockout mice for p21, p27 and p57

p21 gene knockout (KO) mice display viable, fertile, and normal body size phenotypes. Interestingly, actively dividing cells in p21 KO mice are not arrested in the G1 phase of the cell cycle in response to DNA damage[36]. However, earlier studies demonstrated that p21 KO mice do not develop tumors, while p53 KO mice do spontaneously produce tumors with 100% penetrance and normally die within half a year after birth. By contrast, later studies demonstrated that p21 KO mice are more susceptible to chemically- and irradiation-induced tumorigenesis and metastases in the skin and colon[37-40]. Finally, p21 KO mice develop spontaneous tumors on a 129Sv/C57BL6 mix background (at an average age of 16 months)[41], and when crossed with other gene KO mice, such as mucin 2 $(Muc2)^{-/-}$ and adenomatosis polyposis coli $(Apc1638)^{+/-}$[40-42].

Mice carrying gene KO for p27 (p27 KO) and p57 (p57 KO) display quite different phenotypes, despite the conserved protein similarities between p27 and p57. p27 KO mice are viable and display a hyperproliferative phenotype characterized by enlarged organs as organ hyperplasia (including thymic hyperplasia and a higher cell count), and higher incidence of tumorigenesis. Consequently, p27 KO mice grow to be 25-40% larger than their littermate controls[43-45]. In the spleen, p27 KO mice display enhanced proliferation of hematopoietic progenitor cells. p27 KO mice also display an ovulatory defect and female sterility. Consequently, these KO mice have a shortened lifespan due to the growth of benign intermediate lobe pituitary tumors[30,46]. When p27 KO mice are crossed with mice carrying gene KO of p53, PTEN, pRB, p16 and Apc, they display varying degrees of increased tumorigenesis[40,47-51]. By contrast, p57 KO mice display neonatal lethality and developmental defects in multiple tissues[52-54]. For example, mice without p57 normally display embryonic lethality with several defects such as abdominal muscle, cleft palate, endochondral bone ossification defects with incomplete differentiation of hypertrophic chondrocytes, renal medullary dysplasia, adrenal cortical hyperplasia and cytomegaly, lens cell hyperproliferation, and increases in body weight[52,54,55]. Many of these characteristics are also detected in Beckwith-Wiedemann syndrome patients, a pleiotropic hereditary disorder characterized by overgrowth and predisposition to cancer[52]. These phenotypical differences are possibly attributable to differences in the spatiotemporal p27 and p57 expression patterns or to protein structural differences of p57 not shared by p27, which might cause differences in the interacting protein partners for these two CDKIs[5,30,56,57].

For hematopoiesis, p27 KO mice do not show any phenotypes for the number, cycling, or self-renewal of HSCs[58], while experiments using mice lacking p57, a most abundant CDKI family member in quiescent HSCs, have revealed the importance of p57 for maintenance of HSC "stemness"[59-61]. p57 KO mice display a severe defect in the self-renewal capacity of HSCs and a reduction of the proportion of cells in the G0 phase[62]. Double knockout (DKO) mice for p21 and p57 genes display further decreased self-renewal capacity of HSCs. These hematopoietic phenotypes in p57 KO mice can be corrected by knocking in the p27 gene at the p57 locus, indicating the partial functional redundancy of these two CDKIs. While p21 single KO mice display normal self-renewal phenotypes of HSCs, it was found that self-renewal of HSCs is impaired under stressful conditions in which DNA is damaged by exposure to 5-fluorouracil or γ-irradiation[62-64].

Experiments with combined knockout mice for these three genes have been conducted[65]: p21/p27 DKO mice survive until adulthood[66,67], while p21/p57 DKO mice display lethality between embryonic day 16.5 (E16.5) and postnatal day 0 due to a placental defect[65,68]. By contrast, p27/p57 DKO mice display the most severe developmental phonotypes and die between E12 and E16.5 due to the placental defects and hypoplasia of many organs in association with cell apoptosis[69]. p21p27/p57 triple knockout (TKO) mouse embryos die at E13.5 similar to p27/p57 DKO mice, and display the placental defects associated with increased proliferation and apoptosis, which essentially resemble those of p27/p57 DKO mice. These results strongly suggest that p27 and p57 play an essential role during embryonic development, while p21 plays only an auxiliary role, such as DNA-damage response during development[65].

Cell cycle regulation by INK type CDKIs

The INK family proteins, in comparison with the Cip/Kip family of CDKIs, which can inhibit the cyclin/CDK complexes, can only inhibit cyclinD-CDK4/6 interaction. $p16^{INK4a}$ (referred to as p16) functions as a suppressor by inhibiting pRB, thus facilitating cell cycle arrest at the G1 phase. p16 has also been shown to play an important role in cellular senescence. Up-regulation of both p21 and p16 levels is observed in high passage human fibroblasts; but p16 is exclusively up-regulated in senescent cells and not p21, making it the major contributor of senescence[70,71]. Over-expression of p16 in young human fibroblasts, has been shown to induce senescent-like phenotypes[72,73]. Interestingly, p16 levels are also induced upon introduction of the oncogenic form of the Ras gene, leading to premature senescence[74].

The INK4a gene locus codes for another CDKI, termed as $p19^{ARF}$ (referred to as p19 for mice), through the

mechanism of alternative splicing. p19 shares the same gene locus with the p16 gene, but is encoded by two different transcripts[75,76]. The human $p19^{ARF}$ is referred to as $p14^{ARF}$ (referred to as p14). p19/p14, like p16, exhibits cell growth inhibition[75]. Interestingly, p19/p14-mediated growth arrest involves interaction of p19 with MDM2, and, in turn, blocks MDM2-mediated p53 degradation, inducing growth arrest through p53-mediated cell cycle arrest[77-80]. Oncogenes such as Myc, E2F1 and E1A seem to induce p19 expression and, henceforth, activate the p53-mediated apoptotic response[81,82]. INK4a-ARF KO mice lose their ability to undergo cell cycle arrest through either the p16-CDK4/6-RB or p19-MDM2-p53 pathway[80]. Similar to the involvement of p16 in many human cancers, p19/p14 has been observed to control the function of Stat3 in the case of hepatocellular carcinoma (HCC)[83]. However, p19 KO mice display no tumorigenic phonotype or proliferative disorders, suggesting that p19 is not a tumor suppressor gene[84,85]. By contrast, these KO mice only display testicular atrophy due to increased apoptosis of germ cells where p19 shows higher expression[86-88].

$p15^{INK4b}$ (referred to as p15), another member of the INK family, is encoded by the INK4b locus (also known as the multiple tumor suppressor 2 (MTS2) gene). p15 shares high homology with p16, but also has a different set of amino acid sequences[89]. Similar to p16, p15 only associates with CDK4/6. p15 and its association with the CDKs is specifically up-regulated upon treatment of cells with TGF-β, indicating that p15 may play an important role in the TGF-β-mediated cell cycle arrest by inhibiting the function of the CDKs[89]. The p15 locus has been shown to be in most of the cancers similar to the p16 locus. Hypermethylation is commonly observed in both p15 and p16 gene loci in non-Hodgkin's lymphomas (NHL) and other types of cancers as well as myelodysplastic syndrome (MDS)[90]. Up-regulation of p15 has been observed in hematopoietic progenitors during megakaryocyte or granulocyte differentiation, mediated by autocrine TGF-β signaling.

p18 was found to only associate with CDK4/6 followed by pRB inhibition[91] and to be highly expressed in acute leukemic cells compared to nonleukemic or lymphoid or myeloid leukemic cells; and was expressed more in human brains compared to other organs[92]. p18 was mapped to chromosome 1, band p35-36. This region is most frequently deleted in cases of neuroblastomas and melanomas, indicating that p18 might have a role as a tumor suppressor[93]. Point mutations were also found in the p18 gene in human breast tumors, causing cell cycle arrest, and hence may be a factor in promoting tumors[94].

CDKIs for myogenesis and muscle regeneration

As discussed in previous sections, CDKIs play important roles in cell growth arrest, and mutation or deletion of any of these CDKIs could potentially lead to disastrous consequences in cells and organism bodies. Growth arrest is seen to modulate uncontrolled proliferation of cells and in some processes this turns out to be necessary. After looking at the differentiation processes in skeletal muscle, it was found that cell cycle arrest is a necessary event seen in proliferating myoblasts, which then go onto differentiate into post mitotic myocytes and fuse with each other to form multinucleated myotubes[95]. It was observed that myoblasts show up-regulation of p21, p27 and p57 proteins, followed by cell cycle arrest, upon subjection to a differentiation medium[96,97].

A study looking at p21 KO mice revealed that the p21 KO mice develop normally and have no obvious skeletal muscle phenotype. However, upon cardiotoxin (CTX) injury to the muscles, which induces muscle degeneration followed by regeneration, muscle regeneration in the p21 KO mice was greatly decreased compared to the wild-type muscle. There was the case of decreased differentiation of myoblasts as well as increases in cell proliferation, immune cell infiltration and increases in TUNEL-positive apoptotic cells in the p21 KO mice[97,98]. The ultimate regeneration that was detected in the p21 KO mice might be due to possible contributions from p27 and/or p57, indicating that p21 is required for proper skeletal muscle regeneration, and that the lack of the gene abrogates muscle differentiation[97]. Interestingly, p21/p57 DKO mice display perinatal lethality and significantly reduced myotube formation accompanied with increased proliferation and apoptotic rates of myoblasts; and also display endoreplication in residual myotubes, which are very similar phenotypes to mice lacking myogenin. Since myogenin is shown to be necessary for skeletal muscle terminal differentiation, these results indicate that p21 and p57 work together to induce cell cycle arrest and trigger terminal differentiation of myoblasts during fetal myogenesis[68].

The contribution of INK type CDKIs has also been significant in the process of skeletal muscle differentiation. A study aimed at deducing the levels of CDKIs and their association with CDKs was performed in a murine C2C12 myoblast cell line. It was found that while there was a gradual increase in p21 and p27 expression, the levels of p18 rose drastically to 50-times the initial levels 8-12 hours post induction. The increased levels also corresponded to increased association with CDK6, which was maintained at constant low levels[99]. Surprisingly, the p18 gene encodes for two mRNA transcripts, p18(L) encoding 2.4 kb mRNA and p18(S) encoding 1.2-kb mRNA. During myogenic differentiation in C2C12 myoblasts, the shorter transcript, p18(S), was transcribed from a downstream promoter leading to differentiation. The larger transcript, p18(L) was transcribed from an upstream promoter in the proliferating myoblasts[100]. Along the same line, it was observed that myoblasts deficient in p18 and p27 were able to efficiently reenter the cell cycle upon exposure to differentiating conditions when compared to mice deficient only in p27, indicating the

importance of a combinatorial role. Upon introduction to mitogenic stimuli, p18/p27 DKO cells proliferated more, whereas both p18/p21 DKO and p21/p27 DKO cells were not able to revert growth arrest, thus indicating that the presence of p18 and p27 might be needed for cell cycle arrest in myoblasts[101]. Studies have indicated that there is up-regulation of p27 and cyclin-D3 expression, and, thus, their subsequent interaction with CDK2 complexes. The increased interaction leads to sequestration of CDK2 complexes, thus inhibiting its interaction with Cyclin-A, which eventually abrogates DNA synthesis[68].

It is an established fact that MyoD is the master regulator of myogenesis. Although MyoD is detected in both proliferating myoblasts and differentiating myocytes, MyoD activity is increased during differentiation. In addition, MyoD is able to induce cell cycle arrest in addition to activating genes for muscle-specific proteins including muscle structural proteins during differentiation. Studies aimed at identifying genes that are downstream of MyoD during skeletal myogenesis have been underway for quite some time. By concentrating on CDKIs, it was first found that p21 is a direct target gene of MyoD during myogenic differentiation in inducing cell cycle arrest[102-104]. This MyoD-mediated induction of p21 during differentiation does not require p53. p300 is required for this MyoD-dependent cell cycle arrest via p21 transactivation and muscle-specific gene transcription[105]. Loss of MyoD activation of p21 transcription in rhabdomyosarcomas, skeletal muscle tumors, is correlated with failure to arrest in the G1 phase, supporting a role for p21 in MyoD-induced cell cycle arrest[106]. During myogenic differentiation, while most myoblasts become MHC-positive mononuclear myocytes and multinucleated myotubes, minor populations of myoblasts become MyoD(-)p21(-)Pax7(+) mononuclear reserve cells, which are in vitro generated QSC-like cell populations[12]. During myogenic differentiation, apoptotic cell death increases, and this apoptosis is positively regulated by the MyoD-p21 axis. Interestingly, p21 KO myoblasts fail to undergo apoptosis during differentiation, indicating the essential role for p21 in differentiation-associated cell apoptosis[107]. However, this p21-dependent cell apoptosis also requires MyoD. This MyoD-dependent cell apoptosis is mediated by MyoD-target microRNAs, microRNA-1 and microRNA-206, which can down-regulate Pax3 gene expression via binding Pax3 3'UTR[108], resulting in transcriptional down-regulation of anti-apoptotic factors Bcl-2 and Bcl-xL. Therefore, these data suggest that MyoD not only regulates terminal differentiation, but also apoptosis through activation of p21 and miRNA-mediated down-regulation of Pax3.

It is also reported that p57 is a downstream target of MyoD, and its transcription is controlled by MyoD. Interestingly, this MyoD-dependent p57 transactivation is restricted to cells lacking p21, suggesting that the two CDKIs play a similar role in cell cycle arrest during MyoD-induced differentiation. p57 cannot be replaced by p21 in MyoD-induced cell arrest and apoptosis[109]. Further analysis revealed the presence of three MyoD-regulated regions in the p57 gene. The transcriptional regulation of p57 by MyoD was further confirmed by testing the levels of p57 in MyoD-knockdown C2C12 myoblasts, which show an expected decrease in p57 levels during differentiation[110]. p57 requires MyoD binding to a long-distance element located within the imprinting control region KvDMR1, which is located 150 kb downstream of the p57 gene[111]. Unlike p21 transactivation, which is p53-independent and positively regulated by MyoD, p57 transactivation is induced by MyoD in combination with Erg1 and Sp1 through a p73 (a p53 family of protein)-dependent pathway[112,113].

CDKIs for muscle aging and disease

The role of satellite cells in the regeneration of injured muscle is well known. Studies have been undertaken to deduce the role of CDKIs in the satellite cells. Along the same line, Sousa-Victor et al. established that geriatric satellite cells attain their state of irreversible senescence due to the up-regulation of p16. The group was able to single out the cause of senescence using K-means clustering analysis of the various genes expressed in young, old and geriatric mice, which indicated the involvement of p16. Inhibition of p16 through conditional ablation led to reduced expression of senescence-associated genes and restoration of regenerative properties of the satellite cells[114]. Other ground-breaking research revealed that expression of p27 was a requirement for the self-renewal process of satellite cells in vivo. Using a TetO-H2B-GFP inducible reporter system induced by doxycycline, the group was able to specifically label the satellite cells in vivo. These cells were termed label-retaining cells (LRC). The LRC and nonLRC go on to form different subsets of cells, where the former is involved in self-renewal and the latter in differentiation. Higher levels of p21 and p27 were detected in LRC. Accordingly, deletion of p27 resulted in inhibition of the self-renewing capability of LRC, whereas deletion of p21 resulted in inhibition of differentiation in the nonLRC in vitro[115]. A recent study by Zalc et al., revealed that there exists a muscle-specific enhancer region in the p57 gene, which is regulated by both Notch signaling (Hes1/Hey1) and myogenic regulatory factors (MRFs). The study goes on to show that Notch signaling is required for the suppression of the cell cycle inhibitor p57 in the progenitor cells, and that the lack of this inhibitor causes up-regulation in p57 levels, thereby inducing cell cycle exit in these cells[116].

Along the same line, dystrophin-deficient myogenic cells obtained from Duchenne muscular dystrophy (DMD) patients express a higher level of p21 compared to healthy control cells. The same result was observed in mdx mice, an animal model for DMD, indicating elevation in p21

levels in myogenic cells compared to wild-type cells, which pushes the mdx cells towards early differentiation. It has not yet been made clear whether the elevation in p21 levels is due to the exhaustion of p21-negative satellite cells or the dystrophin-deficient cells exhibiting higher levels of p21 because of the cell autonomous event[117].

During tissue aging, it is well known that both stem cell number and function are reduced[118]. The number and function of stem cells is regulated by both cell autonomous and non-cell autonomous manners such as the local microenvironment (niche) and systemic environment (circulation)[119]. p16 and p21 have been shown to increase dramatically as tissue ages, which affects tissue regeneration capacity[120]. Therefore, expression of p16 or p21 could a potential marker for aging cells in tissues, and targeting p16 or p21 is an attractive therapy for protection from aging and age-related diseases. In muscle, p16 and p21 are epigenetically repressed in adult satellite cells, but this repression is relieved in aged cells. Consequently, p16 and p21 become active even in QSCs in aged muscle, causing G0 and/or G2 arrest in QSCs, and thus these QSCs lose ASC transition ability, regeneration capacity, and self-renewal[114]. Interestingly, in the BubR1 progeroid aging model mice carrying a transgene, which expresses a drug-induced toxic protein under the p16 promoter, lifelong removal of p16-expressing cells delayed the onset of aging phenotypes[121]. Therefore, targeting of p16 or p21 is a potential therapeutic approach for delaying skeletal muscle aging.

Aging or disease-induced muscle atrophy can increase morbidity and mortality if prolonged. Muscle atrophy occurs as a consequence of the balance between muscle protein synthesis (MPS) and breakdown (MPB), as well as cross-talk between carbohydrate and protein metabolism. Recent work demonstrated that the molecular pathways of ATF4, p53 and p21 axis regulate disuse muscle atrophy. The unfavorable metabolic effects of muscle immobilization may be counteracted by the positive effects of exercise training associated with the beneficial metabolic effects. ATF4 and p53 promote immobilization-induced skeletal muscle atrophy[122,123]. p21 has been identified as a downstream gene of ATF4 and p53 in immobilization-induced skeletal muscle atrophy. While p21 gene expression is very low in healthy active muscle, its expression is markedly increased during immobilization due to activation of ATF4 and p53. Overexpression of p21 sufficiently reduces muscle fiber size and induces muscle fiber atrophy. p21-mediated muscle fiber atrophy occurs in ATF4/p53 double-knockout mice, indicating that p21 is a downstream effector of the ATF4 and p53 axis. In addition, p21-knockdown by RNA interference reduces skeletal muscle fiber atrophy. This p21-knockdown-mediated muscle fiber atrophy reduction also occurs even if ATF4 or p53 is overexpressed. Therefore, p21 plays central roles in skeletal muscle fiber atrophy as a downstream gene of ATF4 and p53.

Conclusions

Current work has demonstrated that CDKIs play central and essential roles in cell cycle regulation of myogenic progenitor cells and muscle stem cells during embryonic myogenesis, adult muscle regeneration, muscle aging, exercise, and muscle diseases including DMD and muscle atrophy. Studies about elucidating detailed expression patterns and functions of CDKIs in myogenic progenitor cells and muscle stem cells will help further understanding of embryogenic myogenesis and regeneration processes including stem cell differentiation, self-renewal and maintenance. In addition, future applications involving the modulation of CDKI expression and function can be potential therapeutic approaches for age-related muscle fiber atrophy and muscle diseases.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this article.

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