ROLE OF CYCLIN-DEPENDENT KINASE INHIBITORS IN DIFFERENTIATION OF SATELLITE CELLS

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Abstract

Satellite cells are a rare population of stem cells residing in skeletal muscle. They are activated upon injury, enter the cell cycle, and proliferate. These activated satellite cells eventually stop their cell cycle, fuse with each other, and differentiate into multinucleated myotubes for muscle regeneration. Some small proportion of activated satellite cells undergo self-renewal to repopulate the satellite cell pool. For any cell, this transition occurs at the G1 phase, from where the cell can undergo cell cycle arrest, thereby moving towards differentiation, or it can proceed to S-phase and proliferate. The cyclin-dependent kinase inhibitors (CDKI) play a major role in this G1/S transition. In this study, we aim to investigate the roles of CDKIs such as p21, p27, and p57 in the differentiation process of satellite cells. We demonstrate that the overexpression of p21 reduces the Pax7(+)MyoD(-) reserve cell population, a population of *in vitro* quiescent satellite cells. We also show that the overexpression of p21, p27, and p57 increases the population of Myosin heavy chain (MHC)(+) differentiated cells, thus promoting the cells towards myogenic differentiation. Furthermore, we show that the overexpression of the N-terminal CDK inhibitory domain of p21 shows a higher number of MHC(+) differentiated cells compared with the control cells or C-terminal domain of p21, suggesting that the N-terminal domain of p21 is necessary in pushing the myoblasts towards differentiation. These data help in elucidating the molecular mechanisms involved in the differentiation of satellite cells, which could be used to better understand the muscle regeneration process.

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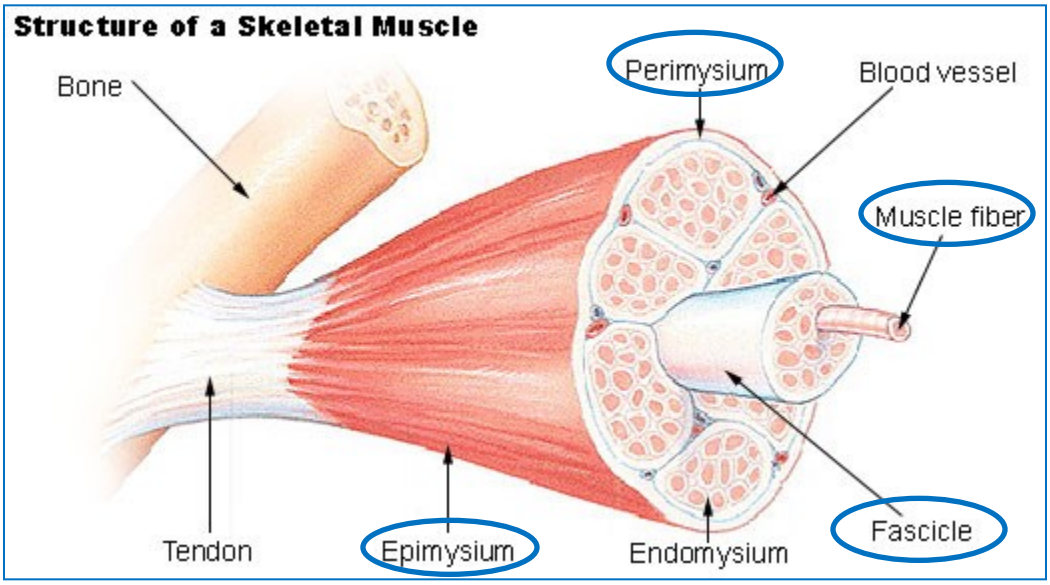
1. INTRODUCTION

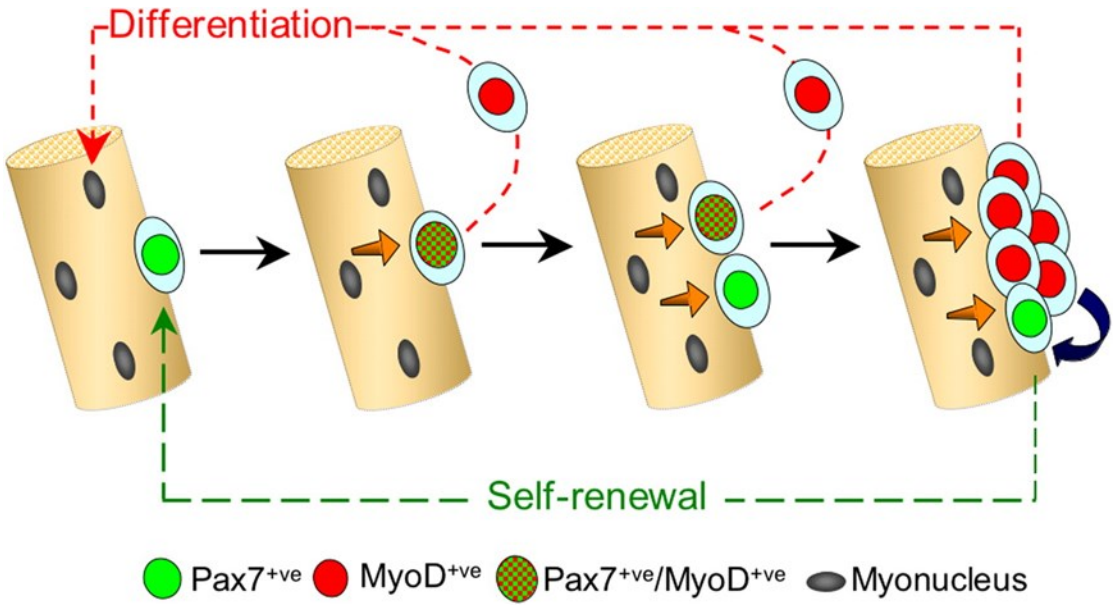
Skeletal muscles are one of the three major muscles that maintain the physique of the human body. These muscles consist of hundreds or thousands of muscle fibers, bundled together in the form of a connective tissue and referred as fascicles. Each bundle of muscle fiber is surrounded by a layer of connective called the perimysium and each muscle, in turn, is surrounded by a layer of tissue called the epimysium (Fig. 1). The muscle fibers consist of multiple myofibrils that control the contraction and relaxation of muscles during any physical activity.



Fi**igure 1. Structure of the skeletal muscle.** Skeletal muscle consists of bundles of muscle fibers called the fascicle. Each bundle of muscle is surrounded by a layer of connective tissue called the epimysium and each muscle is surrounded by a layer of tissue called the perimysium. Adopted and modified from <http://training.seer.cancer.gov/>

It is known that the skeletal muscle is one of the highly regenerative tissues in the body[1], even after being constantly subjected to the wear and tear of daily activities.

Muscle regeneration involves production of multinucleated myotubes, which help in reconstitution of the muscle fibers. These myotubes are terminally differentiated and hence cannot proliferate, which makes us wonder as to what cells might be promoting the regeneration of the skeletal muscle. This concern was addressed by the existence of stem cells in the muscle called the satellite cells[2]. Satellite cells are located beneath the basal lamina and normally remain in a quiescent state (Fig. 2)[3]. Upon muscle injury, these cells get activated and proliferate to form myogenic precursor cells called myoblasts[4]. The myoblasts proliferate and then stop proliferation to differentiate into myotubes. The different stages of the satellite cell activation and differentiation can be characterized by the induction of muscle-specific genes (Fig. 2)[5].



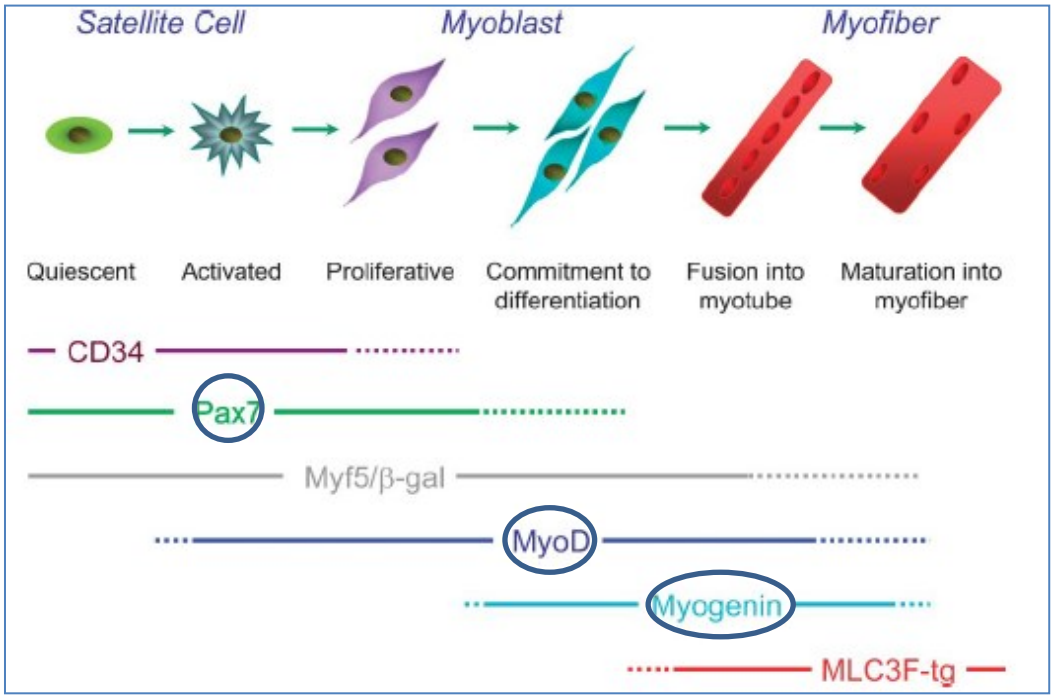


Figure 2. Schematic representation of muscle regeneration by satellite cells [3,5]. The quiescent satellite cells are Pax7(+) MyoD(-), that get activated upon muscle injury, and start to express MyoD. After several rounds of cell proliferation, MyoD(+) myoblasts exit cell cycle and differentiate into myocytes, marked by the onset of Myogenin, which is an early differentiation marker. A few Pax7(+)MyoD(+) myoblasts become Pax7(+)MyoD(-) quiescent satellite cells as a self-renewal process.

The quiescent satellite cells are marked by the presence of Pax7, which is a transcription factor belonging to the paired box family and is critical for the normal functioning of these cells[6]. The Pax7(+)MyoD(-) satellite cells get activated upon injury and turn on the expression of MyoD[7], which is termed as the master regulator of myogenesis. The Pax7(+)MyoD(-) cells, termed activated satellite cells (ASC) or muscle precursor cells or myoblasts, proliferate to expand their progeny and then stop cell cycle to start differentiation. The onset of Myogenin marks the process of differentiation in muscles, wherein the myoblasts fuse together to form multinucleated myotubes. Terminally

differentiated myotubes express Myosin heavy chain (MHC), which is a motor protein that is involved in muscle function and assembly of myofibrils. Studies also showed that expression of the correct isoform of MHC, is necessary for proper functioning of the muscle[8]. Apart from going towards differentiation, a minor population of myoblasts undergoes self-renewal to replenish the stem cell pool. The minor population of activated satellite cells stops cell cycle and down-regulates MyoD, and return to quiescent satellite cells. *In vitro*, upon serum deprivation, most of the proliferating myoblasts maintain MyoD expression and undergo differentiation into Myogenin(+) myocytes followed by cell fusion. A minor population of myoblasts stop cycling, down-regualte MyoD and maintain mononuclear cells under the serum depleted conditions. These mononuclear cells were termed as the "reserve cells", which serves as the *in vitro* equivalent of the quiescent satellite cells. Once these undifferentiated reserve cells were isolated and then subjected to growth conditions, they started to express MyoD, re-enter cell cycle and eventually undergo myogenic differentiation[9]. It has also been observed that these proliferating cells show distinct expression of MyoD and Myf-5 at different stages of cell cycle, correlating with the differentiation ability of the cell. It was observed that the cells lack expression of MyoD at the G0 phase, start demonstrating a high level of expression around mid-G1 phase, reduce the levels in G1/S phase and then again shown an increase in the MyoD expression from S through M phase[10]. On the other hand, Myf-5, which is another essential myogenic transcription factor, peaks at G0, decreases at G1 and then shows up again at the end of G1 phase and remains stable till the end of mitosis[10]. The

decision of a muscle cell to undergo differentiation involves cell cycle arrest, which occurs at the G1 phase, shown to be controlled by the growth factors, such as basic Fibroblast growth factor (FGF)[11].

It is very well known that the assembly of Cyclin-Cyclin dependent kinases (CDK) complexes, regulates the different stages of cell cycle (Fig. 3)[12,13]. As the cells enter into cell cycle from quiescent state upon mitogen stimulation, production of Cyclin-D protein begins. Cyclin D forms complexes with CDK4/6 and phosphorylate Retinoblastma protein (pRB), also known as the Restriction point. It has been shown that Cyclin D-CDK4/6 is able to only partially phosphorylate pRB, followed by complete phosphorylation and inactivation by Cyclin E-CDK2 complexes[14]. pRB exerts its inhibitory effects on cell cycle by binding to the transcription factor, E2F[15]. E2F is a transcription factor, that is involved in the promotion of cell cycle to S phase, by binding to the promoter regions of genes involved in cell proliferation such as DNA polymeraseα, Cyclin A, and CDC2[16]. Upon phosphorylation of pRB, the transcription factor E2F disassociates from pRB and induces the transcription of E2F-dependent genes, which promotes entry of cell to the S phase[17]. Cyclin A is induced next, which coincides with that of DNA synthesis in the S-phase. It has been shown to bind to both CDK2 and CDC2 and has functional roles in both S phase and Mitosis[18]. Cyclin A is followed by Cyclin B induction, which then associates with CDC2 to promote mitosis[19].

The antagonistic approach to cell proliferation is cell cycle arrest, which occurs at G1 phase and prevents the cells from proceeding to S phase. Cell cycle arrest is mediated by

the Cyclin-dependent kinase inhibitors (CDKI) that block the Cyclin-dependent kinase (CDK) activity by binding to them. There are two types of CDKI families based on their CDK specificity, structure and origin. One type consists of the INK4 family which consists of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$.The INK family CDKI bind to CDK4 and CDK6 subunits and inhibit their association with Cyclin D. The other type of CDKI family consists of the Cip/Kip family of proteins, consisting of $p21^{Cip1/Waf1/Sdi1}$, $p27^{Kip1}$ and $p57^{Kip\ 2}$, that binds to both the cyclins and the CDK and can modulate the activity of all the complexes[20]. p21 is a target of p53 gene and has been shown to induce cell cycle arrest in response to DNA damage[21]. Lack of p21 in mice caused defects in DNA-damage induced cell cycle arrest[22]. p27 binds to Cyclin E-CDK2 complexes and prevents their activation, thus arresting the cells at G1 phase[23]. p57, unlike the others, has a restricted expression only among certain tissues[24]. p57 is the only CDKI that is required during embryonic development and the absence of p57 in mice resulted in developmental abnormalities and death[25,26].

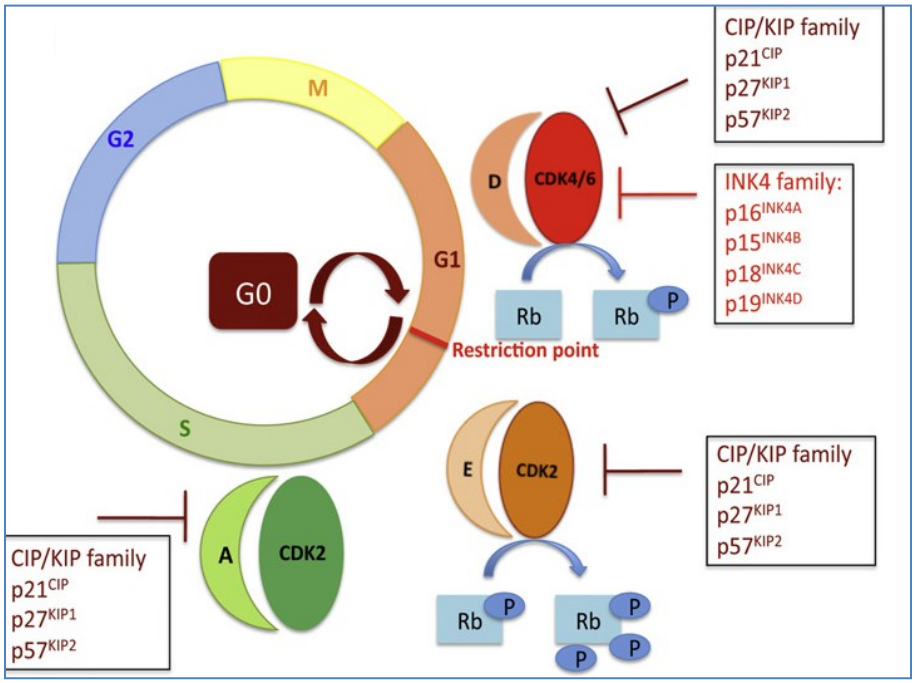


Figure 3. Phases of cell cycle and the genes involved[13]. Cell cycle consists of four phases: G0, G1, S and M phase. Each of the phase is regulated by Cyclins and Cyclin-dependent kinases (CDK). The cell cycle progression is halted by the Cyclin-dependent kinase inhibitors (CDKI), consisting of the Cip/Kip family and the INK family. Cyclin D-CDK4/6 initiates the cell cycle by phosphorylating pRB and the other cyclins follow through to advance the cell cycle. Cyclin D-CDK4/6 is inhibited by both Cip/Kip and the INK family of proteins.

In the context of skeletal muscle differentiation, it is known that the proliferating myoblasts undergo cell cycle arrest, to initiate differentiation[27]. For instance, it was observed that there was induction of p21 protein, followed by cell cycle arrest in the myoblasts, subjected to differentiation medium[28], indicating that the process of myogenic differentiation is controlled to a larger extent, by the CDKIs. Similarly, it has been previously indicated that dystrophin-deficient mice exhibit higher levels of p21 in their skeletal muscles, either due to a reduction in the number of undifferentiated non-p21

expressing satellite cells or increase in the p21 protein levels in the dystrophin-deficient cells[29].

Previous studies also indicate that p21 and p57, along with myogenin, are required for myoblast differentiation, which was observed to be drastically affected in the mice lacking both p21 and p57[30]. It has been also shown by previous studies that there is an increase in the expression pf p27 and cyclin-D3 expression and their association with the CDK2 complexes. This increased association seems to sequester the CDK2 from forming complexes with Cyclin A, thus inhibiting the Cyclin A-CDK2 activity required for DNA synthesis[31]. Other than the Cip/Kip family of CDKIs, the INK family (p15, p16 and p18) of proteins also seems to play a role in skeletal muscle differentiation. It was found that p18 was necessary for the initial cell cycle arrest along with p27. Myoblasts lacking both p18 and p27 were shown to reenter cell cycle at a higher frequency than the myotubes deficient only for p27. These results seemed to indicate that the both p18 and p27 were necessary for initiating cell cycle arrest, but the cells show capability of reentering cell cycle[32]. Furthermore, studies also indicated that MyoD controls the expression of p57 during C2C12 myoblasts differentiation. Using bioinformatics analaysis, it was found that p57 contains several putative sites for transcription factors such as MyoD. p57 expression was observed to be initially downregulated in MyoD silenced C2C12 myoblasts and then upregulated at a later day of differentiation, which was found to be corresponding to putative MyoD sites in the promoter regions of p57[33]. Previous tudies have also established the importance of CDKIs in the satellite cells, which are responsible

for the regeneration of muscle in the case of an injury. Supporting this, a recent study indicated that the expression of p27 was necessary for satellite cell self-renewal *in vivo*. Loss of p27 was shown to impair label retaining satellite cell (LRC) self-renewal *in vivo,* following muscle injury. p27 was also shown to promote proliferation of LRCs *in vitro* and also necessary for maintaining a more primitive state of satellite cells, which was observed upon isolation of LRCs from a regenerated adult muscle *in vitro*[34].

Moving forward in the same line, Asakura lab found that p21 protein was absent in the quiescent satellite cells (QSC), whereas the QSC exhibited positive levesl of p27 and p57 (unpublished data). On the contrary, p21 expression was not detected in the reserve cell[9] population (unpublished data), which is the *in vitro* equivalent of quiescent satellite cells observed *in vivo*. Asakura lab also observed that mice lacking p21 ($p21^{-/-}$) showed a significant increase in the number of quiescent satellite cells per myofiber (unpublished data). Previous studies had shown that p21 is essential for normal muscle regeneration and the lack of it causes impairment in muscle regeneration[35]. But in the case of the satellite cells, the deletion of p21 seemed to significantly increase the number of quiescent satellite cells, when compared to wild-type mice. On the contrary, overexpression of p21 in myoblasts *in vitro*, revealed a significant decrease in the Pax7(+)MyoD(-) reserve cell population in the p21-overexpressing myoblast culture (unpublished data), suggesting that the overexpression of p21 protein is detrimental to the self-renewal capability of reserve cells.

Taking into consideration the previous works on CDKIs in muscles, this study was performed to deduce the effects of overexpression of p21, p27 and p57, on the differentiation efficiency of myoblasts, particularly focusing on p21. In this study, we also compare the effects of presence and absence of Myc tag, on the function of p21, p27 and p57, in inducing myogenic differentiation. It has been known that the p21, p27 and p57 show similarities in the N-terminal region, containing the CDK inhibitory domain but have variations in their C-terminal region[36]. In this study, we also demonstrate the domain-specific effects of p21 on myoblast differentiation. Overall, in this study, we have made efforts to throw light on some of the molecular mechanisms that govern self-renewal or differentiation in a muscle precursor cell.

2. MATERIALS AND METHODS

2.1 Isolation of Myf5$^{+/nLacZ}$ myoblasts and culture

The Myf5$^{+/nLacZ}$ reporter mice[37] used in this study, carry β-galactosidase gene inserted into the Myf5-locus. Hind limb skeletal muscles were isolated from Myf5$^{+/nLacZ}$ mice of age 1-2 months. The muscles were then rinsed with Phosphate Buffer Saline (PBS) and minced rigorously into smaller pieces with the help of scissors. The tissues were further digested using an enzyme cocktail of collagenase type B and dispase II (Roche Applied Science) for about 20 minutes at 37°C. Further mincing was done and tissues were incubated at 37°C for another 20 minutes. Tissues were rinsed with 10ml of DMEM(Invitrogen) containing 2% FBS (Fisher Scientific). The digested tissue was split amongst two tubes and centrifuged at 800rpm for 30 seconds. Supernatant was collected in a new tube and centrifuged again for another wash. The tube was centrifuged again at 2000rpm for 5 minutes at 4°C to obtain cells. The cells were supplemented with new medium before subjecting to Magnetic-activated cell sorting (MACS). Cells were sorted using MiniMAC and MidiMAC kits (Miltenyi Biotec). Initially cells were negatively selected for Phycoerythrin (PE)-labeled CD31, PE-labeled CD45, PE-labeled Sca 1 antibodies (BD biosciences), and anti-PE micro beads (Miltenyi Biotec). Cells were then positively selected for Integrin α7 antibodies (MBL International) and anti-IgG1 micro beads (Miltenyi Biotec).These positively selected myoblasts were then utilised for all the experiments in this study.

$Myf5^{+/nLacZ}$ myoblasts were cultured on petridshes (100mm) coated with collagen (BD Biosciences) and supplemented with HAM's F10 (Invitrogen) media containing 20% fetal bovine serum (FBS, Fisher Scientific), basic Fibroblast Growth Factor (bFGF, R&D systems) at a concentration of 2.5ng/ml and 1% Penicillin/Streptomycin (Invitrogen) at a concentration of 50µg/ml. Myoblasts were incubated in the Tri Gas incubator (NuAire) at 37°C with 5% CO_2, 5% O_2 and 10% humidity until the cells become 70-80% confluent. Cells were supplemented with a change of media every alternate day. Myoblasts were induced towards differentiation using Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 5% Horse Serum (Invitrogen) along with 1% Penicillin/Streptomycin at a concentration of 50µg/ml.

2.2 Amplification of cyclin-dependent kinase inhibitor (CDKI) using PCR

Each of the murine cyclin-dependent kinase inhibitor (CDKI) (i.e.,) $p21^{WAF1/CIP1}$ (referred as p21), $p27^{CIP/KIP1}$(referred as p27) and $p57^{CIP/KIP2}$(referred as p57) was amplified, using PCR, from myoblast cDNAs containing full length cDNA of p21, p27 and p57 using specifically designed primers containing appropriate restriction sites (Table 1). Primers were also specifically designed to amplify the N-terminal and C-terminal regions of p21 using plasmid with full length p21 as the template (Table 1). The reaction mixture was prepared by adding Accuprime™ Pfx Supermix (Invitrogen), plasmids containing individual CDKI along with the forward and the reverse primers. PCR was set up using the following cycling parameters: 95°C for 5 minutes, 95°C for 15 seconds, 55-60°C for 30 seconds, 68°C for 3 minutes for 30 cycles, followed by 68°C for 7 minutes and the

reaction products were maintained at 4°C, in the Thermal Cycler (Eppendorf). The PCR products were checked for specific amplification, with 2% Agarose gel electrophoresis stained with Ethidium bromide.

Table 1. List of primers used for the amplification of CDKI

GENE	FORWARD PRIMER	REVERSE PRIMER
p21	**5'TTGAATTC**TATGTCCAATCC EcoRI **TGGT'3**	**5'TGAATTC**AGGGTTTTCTCTT EcoRI **GCAG'3**
p27	**5'CTCTCGAG**ATGTCAAACGTG XhoI **AGA'3**	**5'TAGCGGCCGC**TTACGTCT NotI **GGCGTC'3**
p57	**5'GCCTCGAG**ATGGAACGCTT XhoI **GGCCTCCAGCGAT'3**	**5'GCGCGGCCGC**TCATCTCAGA NotI **CGTTTGCGCG'3**
p21-N-terminal	**5'GCCTCGAG**ATGTCCAATCCT XhoI **GGTGATGTCCGA'3**	**5'GCGCGGCCGC**GGACCCAG NotI **GGCTCAGGTAGACCTTTAG'3**
p21-C-terminal	**5'GCCTCGAG**ATGTCGCTGTCT XhoI **TGCACTCTGGTGTCT'3**	**5'GCGCGGCCGC**TCAGGGTTTT NotI **CTCTTGCAGAAGAC'3**

2.3 Construction of pMX vectors

To analyse the potential of Cyclin-dependant kinase inhibitor (CDKI) in promoting myogenic differentiation in myoblasts, each of the three CDKI i.e., p21, p27 and p57 were cloned into pMX-puromycin (pMX-puro) plasmid (Cell Biolabs, INC) vectors with or without Myc tag (MT).

Initially, the 6x Myc tag epitope (MT) was obtained from pCS2+MT plasmid and cloned into pMX-puro plasmid using BamHI/NotI restriction sites to produce pMX-MT-puro vector. Next, p21, p27 and p57 was cloned into the pMX-MT-puro vectors using specific restriction sites present in the vector backbone. Finally, Myc tag was removed from these vectors to synthesise non-MT vectors.

2.4 Plat-E cell culture

Platinum-E (Plat-E) Retroviral Packaging Cell Line (Cell Biolabs, INC) tubes were thawed in a water bath at 37°C and immediately transferred to a 15ml tube containing 5ml of DMEM supplemented with 2% FBS and 1% Pencillin/Streptomycin (50µg/ml). The tube was spun down at 1000rpm for 5minutes. The supernatant was discarded and the cells were resuspended in 4ml of DMEM supplemented with 10% FBS and 1% Pencillin/Streptomycin (50µg/ml). The cell suspension was transferred into a collagen-coated petridish (100mm) containing 4ml of DMEM supplemented with 10% FBS and 1% Pencillin/Streptomycin (50µg/ml). The cells were incubated at 37°C with 5% CO_2. Expansion of Plat-E cells was performed once the cells reach about 80% confluence.

2.5 Production of retroviral supernatant

Plat-E cells were seeded onto 100mm petridishes and cultured using DMEM supplemented with 10% FBS and 1% Penicillin/Streptomycin (50µg/ml) until the cells become 70-80% confluent. The day before transfection of the pMX vectors, the media was changed to DMEM with 10% FBS and without Pencillin/Streptomycin. For the transfection of the pMX vectors containing CDKI with or without Myc tag (MT), 25µg of plasmid DNA (pMX vectors) was added into an Eppendorf tube containing 1ml of Opti-MEM media (Invitrogen), followed by 30µl of TransIT-2020 transfection reagent (Mirus Bio LLC). The tube was inverted gently to mix the contents and incubated for 15 minutes at room temperature, for the formation of DNA-TransIT-2020 complexes. After incubation, the mixture was added onto Plat-E cells in the petridishes, followed by 5ml of culture media (DMEM with 10% FBS lacking the antibiotics). The plates were incubated at 37°C for 24 hours and change of media (10ml) of DMEM with 10% FBS lacking antibiotics) was given the following day. After 24 hours, the retroviral supernatant was harvested into 2.0ml cryotubes and stored at -80°C for later use (Fig. 4).

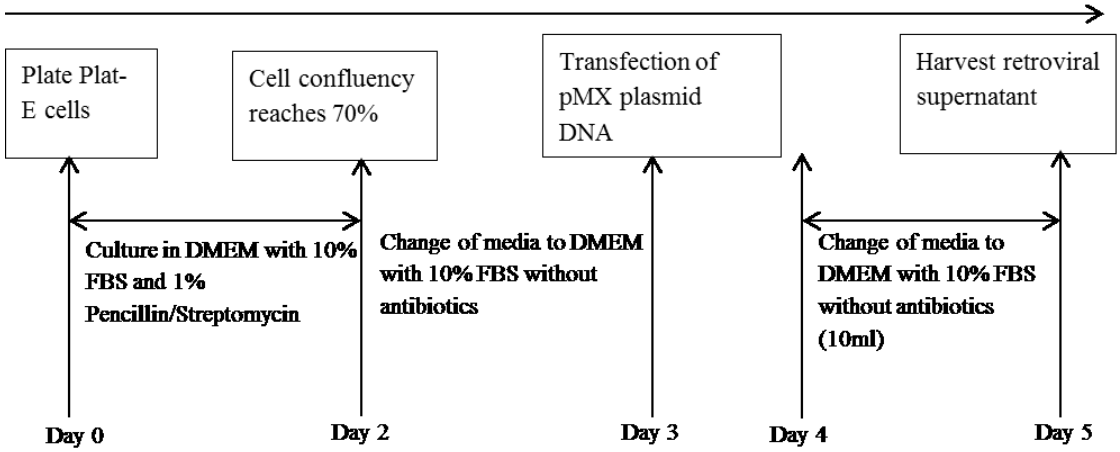


Figure 4. Schematic depicting the production of retroviral supernatant using Plat-E cells

2.6 Infection of Myf5$^{+/nLacZ}$ myoblasts using the retroviral supernatant

Myf5$^{+/nLacZ}$ myoblasts were initially plated on 100mm petridishes and cultured using HAM's F10 media with 20% FBS and 1% Penicillin/Streptomycin until they become about 70-80% confluent. The cells are then split into 35mm petridishes, containing 1x10^5 cells each and supplemented with HAM's F10 containing 20% FBS and 1% Penicillin/Streptomycin. Once the cells are ready, 750µl of the viral supernatant is added onto the cells followed by 0.75µl of polybrene (10µg/ml, Polyplus-transfection, Millipore) and are incubated for 4 hours at 37°C. After incubation, the viral supernatant is removed and the cells are supplemented with HAM's F10 media containing 20% FBS and 1% Penicillin/Streptomycin. Cells are allowed to grow and fixed using 2% paraformaldehyde (PFA) at appropriate time for immunocytochemistry (Fig. 5).

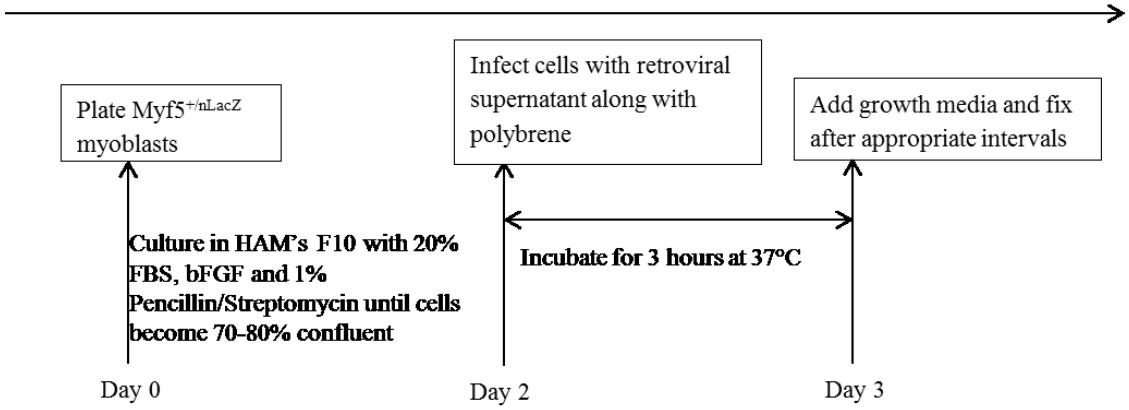


Figure 5. Schematic depicting the retroviral infection into myoblasts

2.7 Immunocytochemistry of myoblasts

Immunocytochemistry of Myf5$^{+/nLacZ}$ myoblasts were performed after fixing the cells at growth (Day 0) and differentiation (Day 1, Day 2 and Day 3) conditions (DMEM supplemented with 5% horse serum (Invitrogen) and 1% Penicillin/Streptomycin), with or without retroviral infection. Cells, with or without retroviral infection, were fixed using 2% paraformaldehyde (PFA), for 5 minutes at room temperature. After fixation, cells were rinsed, twice, with 0.01% Triton-X 100 in Phosphate buffer saline (PBS). Cells were permeabilised using 0.2% Triton in PBS for 5-10 minutes, followed by rinsing with 0.01% Triton-X 100 in PBS once. To avoid loss of antibodies, PAP-pen (Sigma-Aldrich) was used to define the area for blocking. Cells were blocked with 1% BSA for 30 minutes at room temperature(RT). After blocking, 200µl of primary antibodies (Table 2), dissolved in 1% BSA, was added and stored at 4°C in a moisture box.

After 24 hours of primary antibody incubation, the cells were rinsed with 0.01% Triton-X 100 in PBS, twice. Next, the cells were incubated with 200µl fluorophore-conjugated

secondary antibodies (Table 2), dissolved in 1% BSA, for an hour at RT. After incubation, cells were rinsed with 0.01% Triton-X 100 in PBS twice. To counterstain the nuclei, cells were then incubated with DAPI dye (diluted to 1:10,000 with PBS containing 0.01% Triton) for 20 minutes at room temperature. Cells were then viewed under a fluorescent microscope after a placing a coverslip, with the help of DAKO mounting media. After imaging, plates were stored at 4°C in a moisture box.

Table 2. List of antibodies used for immunocytochemistry

Primary Antibody	Dilution	Company
p21 HUGO 291	**1:1000**	**Abcam**
p27 (C-19)	**1:1000**	**Santa Cruz**
p57 (H-91)	**1:1000**	**Santa Cruz**
Myosin heavy chain (MHC) MF20	**1:20**	**Developmental Studies Hybridoma Bank (DSHB)**
Myc-tag (MT)	**1:50,000**	**Abcam**

Secondary Antibody	Dilution	Company
Alexa Fluor 488-labeled anti-mouse IgG	1:1000	Invitrogen
Alexa Fluor 568-labeled anti-rabbit IgG	1:1000	Invitrogen
Alexa Fluor 488-labeled anti-rat IgG	1:1000	Invitrogen

2.8 Fluorescence Imaging

Fluorescent images were captured using an Olympus BX51 fluorescence microscope with 4X, 10X, 20X and 40X UPlanFLN objectives (Olympus America). Images were captured DP controller software and were processed with the help of Adobe Photoshop CS3 (Adobe Systems).

2.9 Preparation of cell extracts from myoblasts

Cell pellets were obtained from wild-type and infected Myf5$^{+/nLacZ}$ myoblasts, exposed to growth and differentiation media, by initial rinsing of cells with PBS (to remove media), followed by trypsin digestion (0.25% trypsin) and washing of cells with DMEM containing 2%FBS and then stored at -80°C. The frozen pellets were thawed on ice and washed with PBS initially and centrifuged at 1500 rpm for 3 minutes, to remove proteins present in wash media. The cells were then lysed using Mammalian-Protein Extraction Reagent (M-PER, Pierce), containing a proprietary detergent dissolved in 25mM bicine buffer (p^H 7.6) plus 1 tablet of cOmplete protease inhibitor cocktail, EDTA free (Roche).

After gentle agitation to dissolve the pellet in M-PER, the solution was centrifuged at 13,500 rpm for 10 minutes. The supernatant was obtained and aliquoted in Eppendorf tubes and stored at -80°C, for immunoblotting.

2.10 Immunoblotting

Total protein from wild-type and differentiating myoblasts, obtained using M-PER, was taken from -80°C and spun at 14,000rpm for 10 minutes at 4°C, to pellet out denatured proteins formed during freeze-thaw procedure. The concentration of the protein was measured using Quick Start™ Bradford protein assay (Bio-Rad). Protein equal to 10µg was separated by SDS-Polyacrylamide gel electrophoresis (PAGE) (Bio-Rad Mini Protease Tetra system). The bands were then transferred onto a polyvinyldiene fluoride (PVDF) membrane using wet transfer set-up (PAGEgel Dual transfer setup). The transfer was performed at 200V for 1.5 hours at 4°C. After transfer, the membrane was subjected to blocking using Blocking One (Nacalai tesque) for 1 hour at room temperature, to prevent non-specific binding reactions. After blocking, the membrane was incubated with primary antibodies (Table 3), dissolved in 10% Blocking One in PBS and stored at 4°C, on a rocker. After 24 hours of primary antibody incubation, the membrane was washed with wash buffer (1X TBS with 0.05% Tween-20), thrice, each for 5 minutes and then 10 minutes on a rocker. The membrane was then incubated with HRP-conjugated secondary antibodies (Table 3), dissolved in 10% Blocking One in PBS for 1 hour at room temperature. After incubation, the membrane was washed with wash buffer (1X TBS with 0.05% Tween-20), thrice, each for 5 minutes and 10 minutes, on a rocker. The

membrane was then incubated with enhanced chemiluminescent substrate solution (Supersignal West Femto Maximum sensitivity substrate, Thermo scientific), which contains 1:1 volume of Luminol/Enhancer solution and stable peroxide solution for 5 minutes at room temperature. Once the incubation was over, the membrane was developed (Konica SRX-101A) by exposing the membrane at different time points.

Table 3. List of antibodies utilised for Western blot analysis

Primary Antibody	Dilution	Company
p27 (sc-528)	**1:1000**	**Santa Cruz**
p57 (sc-8298)	**1:1000**	**Santa Cruz**
Anti-myc tag	**1:5,000**	**Abcam**

Secondary Antibody	Dilution	Company
Goat anti-Rabbit IgG (H+L), HRP conjugate	**1:50,000**	**Pierce**

2.11 Statistical Analysis

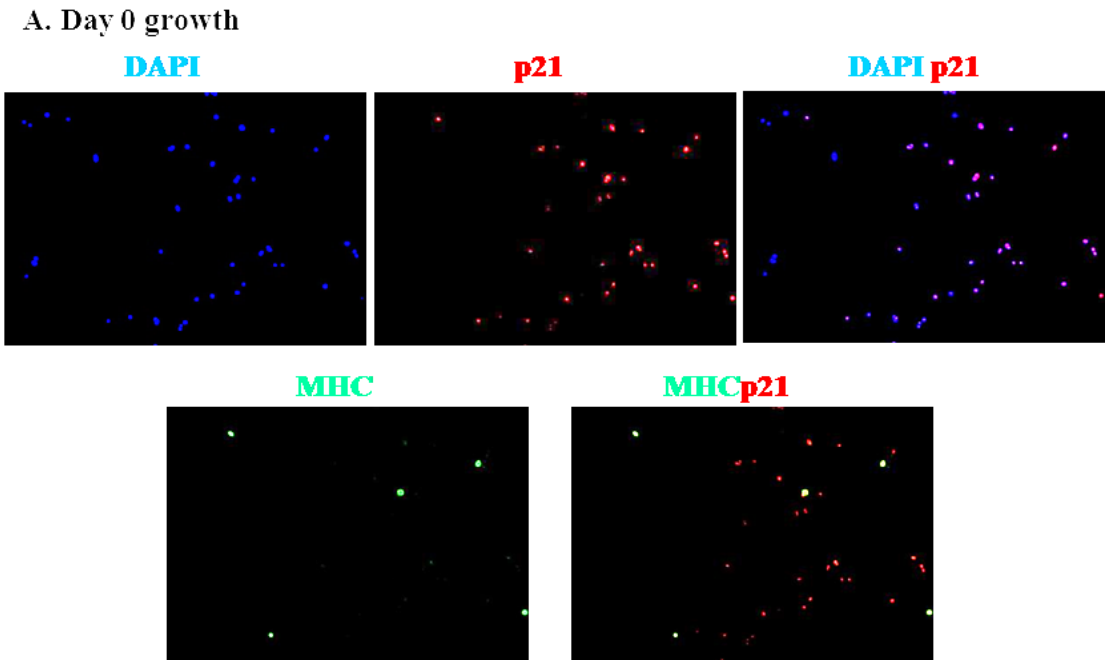
All the quantification was calculated as the average of three randomly chosen fields, in a single petridish, ± standard error (STDEV/SQRT (3)). All the experiments were performed once (n=1).

3. RESULTS

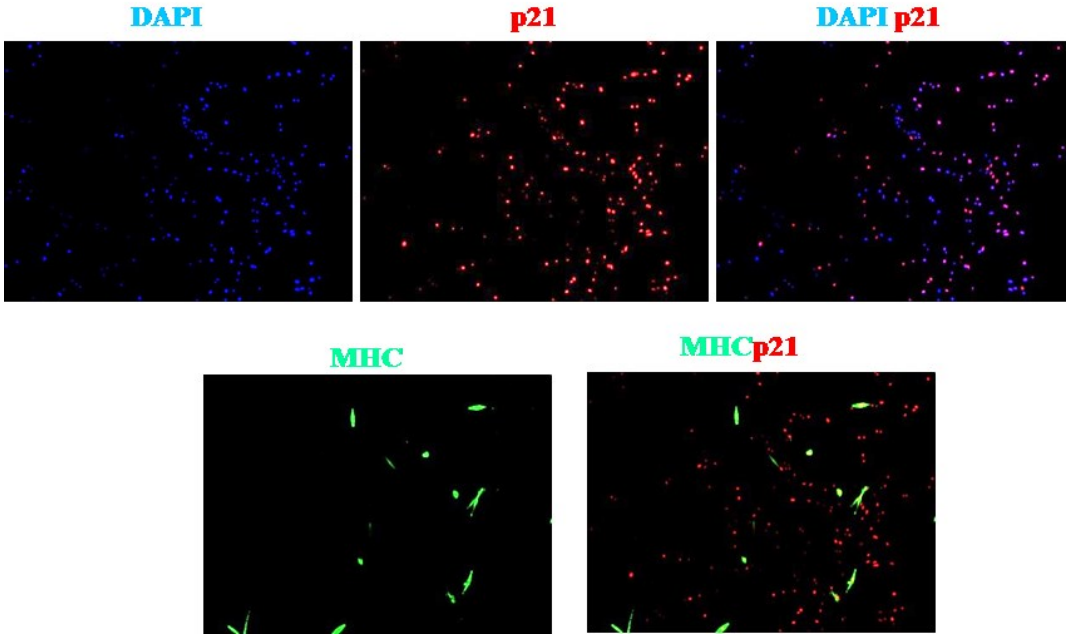
3.1 Endogenous expression of CDKI in the myoblast culture

In our aim of investigating the roles of cyclin-dependent kinase inhibitors (CDKIs) in the myoblast culture, as a first step, we detected the endogenous levels of $p21^{WAF1/CIP1}$ (referred as p21), $p27^{CIP/KIP1}$ (referred as p27) and $p57^{CIP/KIP2}$ (referred as p57) in the myoblast culture, isolated from Myf5nLacZ reporter mice, using immunofluorescence (Figs. 6, 7, 8). The cells were stained with anti-p21 antibody and anti-Myosin Heavy Chain (MHC) antibody, which is a terminal differentiation marker, under the growth conditions, containing higher percentage of serum (20% FBS) and differentiation conditions, containing lower amount of serum (5% horse serum). We observed an increase in the percentage of p21(+) myoblasts from under the growth conditions (32.6%) to almost 75% upon introduction of the differentiation conditions by day 1 (Fig. 6A, 6B). By the second day of differentiation (day 2), the percentage of p21(+) myoblasts increased to 82%, suggesting that the cells promote the expression of p21 during myogenic differentiation (Fig. 6C, 6D). This was consistent with the previous studies, which demonstrated that up-regulation of p21 was a necessary event for the induction of differentiation and commitment in C2C12 skeletal muscle cells[38]. We also found that the levels of MHC were about the same, 5-7% between the growth (day 0) and differentiation (day 1) conditions. However, by day 2, the percentage of MHC(+) cells increased to more than 59%, which is an indication of myoblasts undergoing differentiation to form myocytes and myotubes. Among the MHC(+) cells, almost all the cells also stained

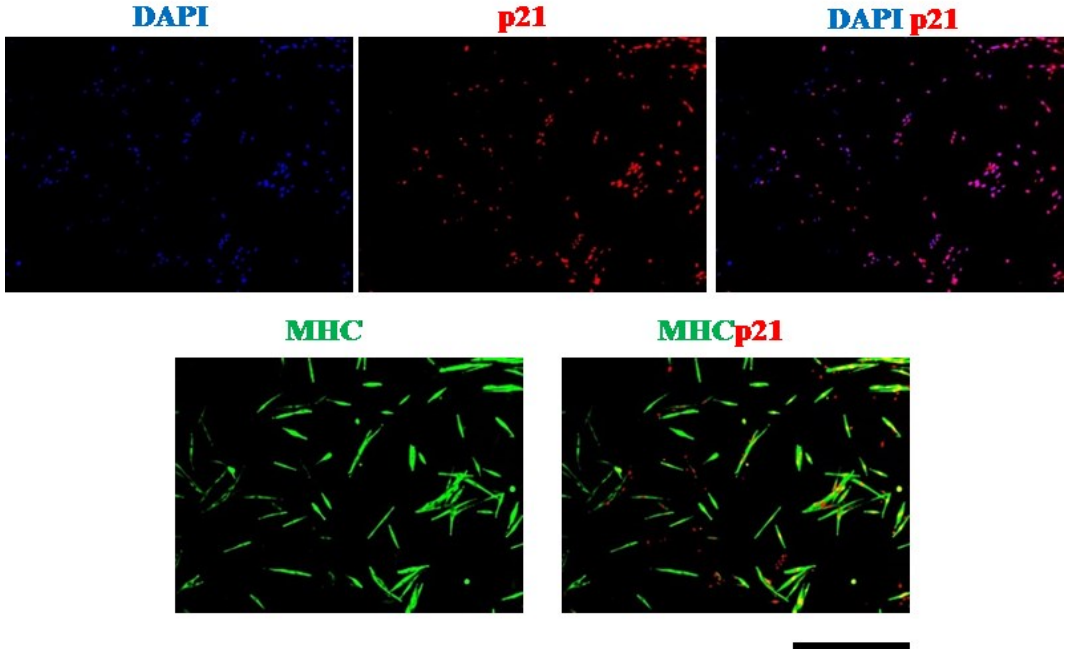
positive for p21, suggesting that p21 expression induces myogenic differentiation. These data suggest that the p21 up-regulation might be a part of the myogenic differentiation, for promoting cells to exit cell cycle and thus augment myogenic cell fusion and differentiation.



B. Day 1 differentiation



C. Day 2 differentiation



D.

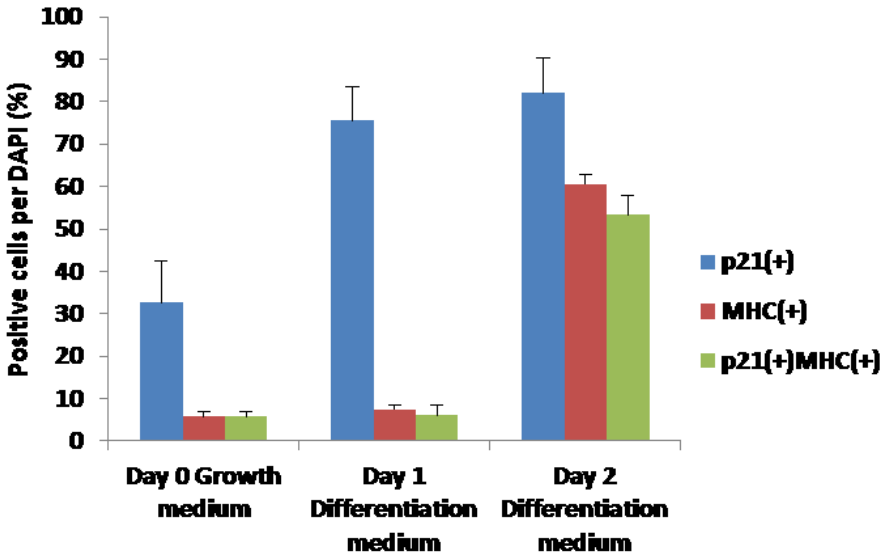
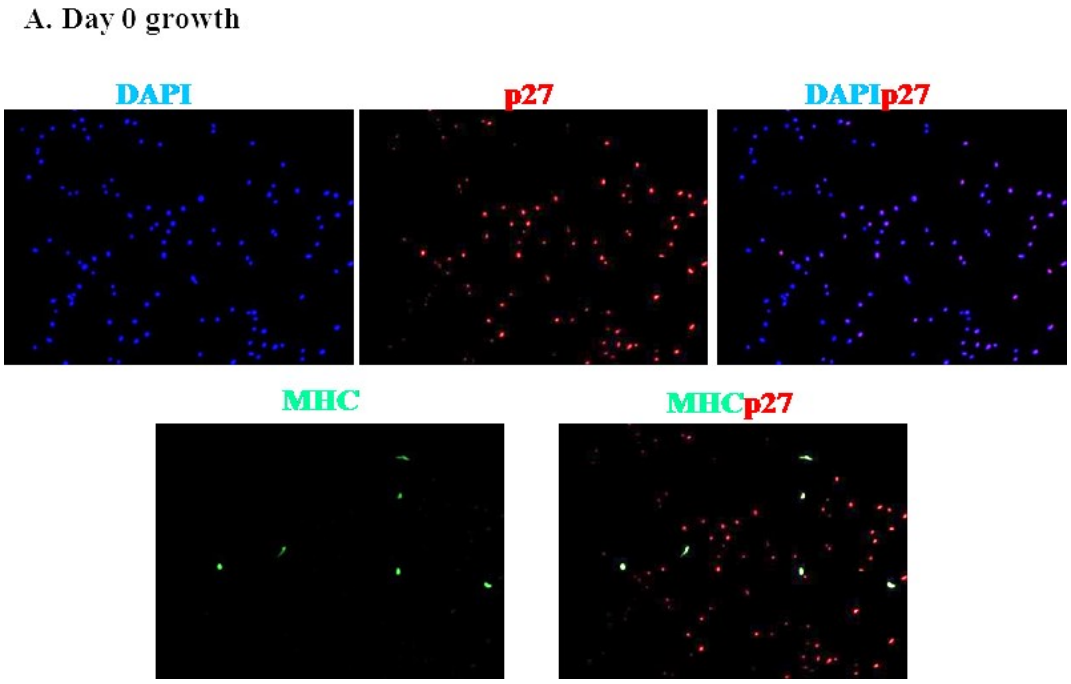


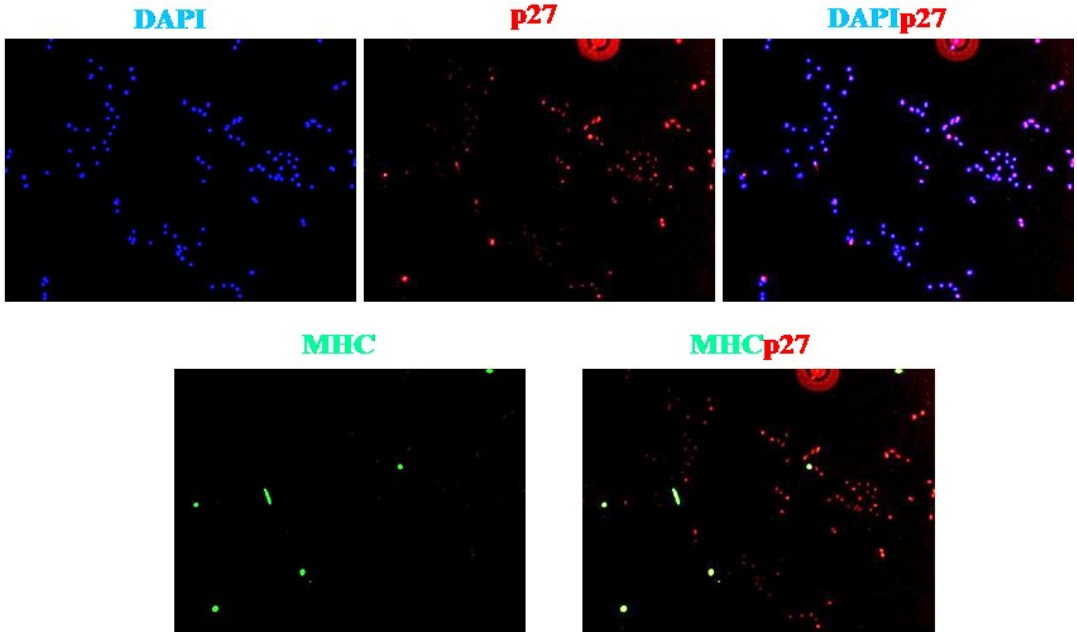
Figure 6. Endogenous p21(+) cells were increased during myogenic differentiation. Myoblasts were stained with antibodies for MHC (green) and p21 (red) under A) the growth condition at day 0, B) & C) differentiation conditions at day 1 and day 2, respectively (n=1). DAPI was used as a nuclear stain (blue).Scale bar = 100 µm. D) Histograms representing the percentage of p21(+), MHC(+) and p21(+)MHC(+) on all three days. Error bars= standard error mean.

Next, we investigated the endogenous expression of p27 under the growth and differentiation conditions and found that the expression pattern was similar to that of p21. There was an up-regulation of p27 protein, when the myoblasts start myogenic differentiation. The induction of p27 expression was evident from the percentage of p27(+) nuclei that coincides with the DAPI(+) nuclei, among the growth (day 0) (32%) and differentiation conditions (day 1) (75%) (Fig. 7A, 7B). Upon investigating further, we found no increase between day 1 (88%) and day 2 (90%) under the differentiation conditions (Fig. 11B, 11C, 11D). These data suggest that cells induce the expression of p27 to initiate the wave of MHC (+) myogenic differentiation, through cell cycle arrest.

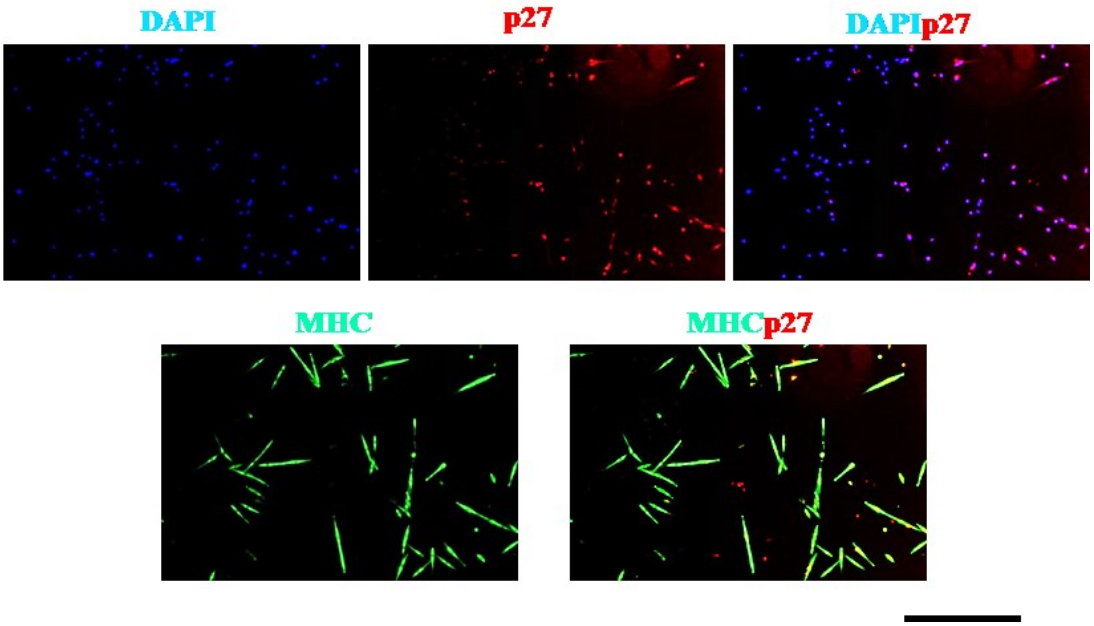
MHC staining revealed no large difference amongst the growth (day 0, 3%) and differentiation (day 1, 7%) conditions (Figs. 7A, 7B, 7D). However, by day 2, the percentage of MHC(+) cells increased to 61%, with almost 50% of them being positive for p27, suggesting that p27 was also involved in promoting differentiation of myoblasts towards formation of myotubes (Fig.7C, 7D).



B. Day 1 differentiation



C. Day 2 differentiation



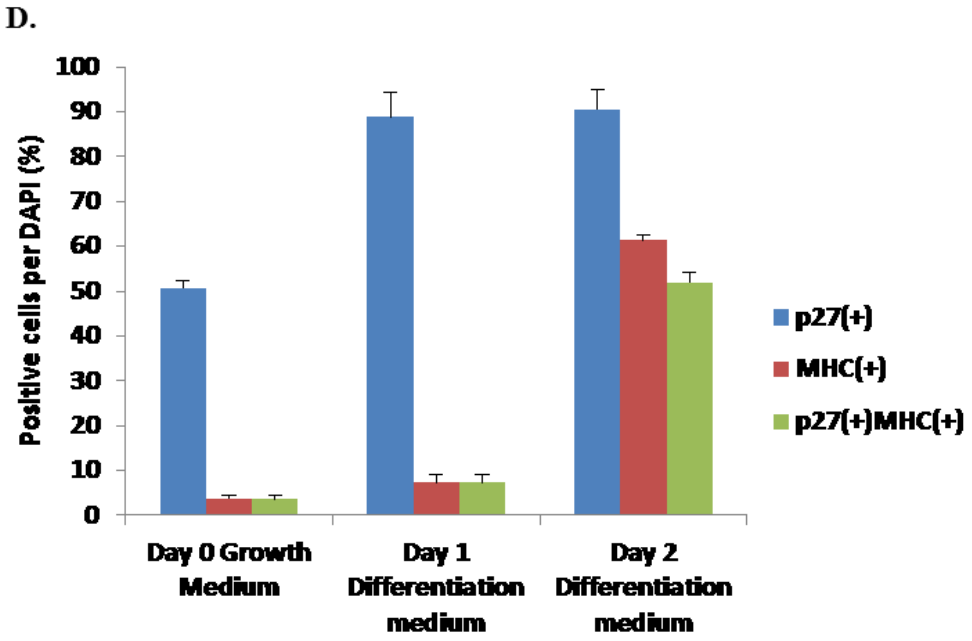
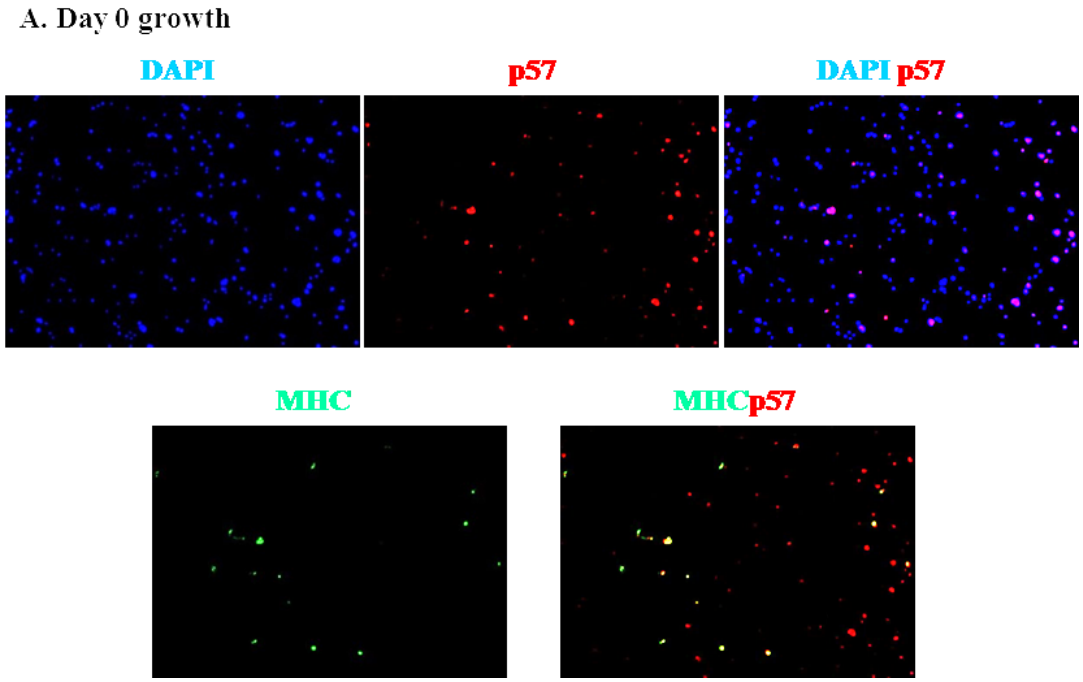


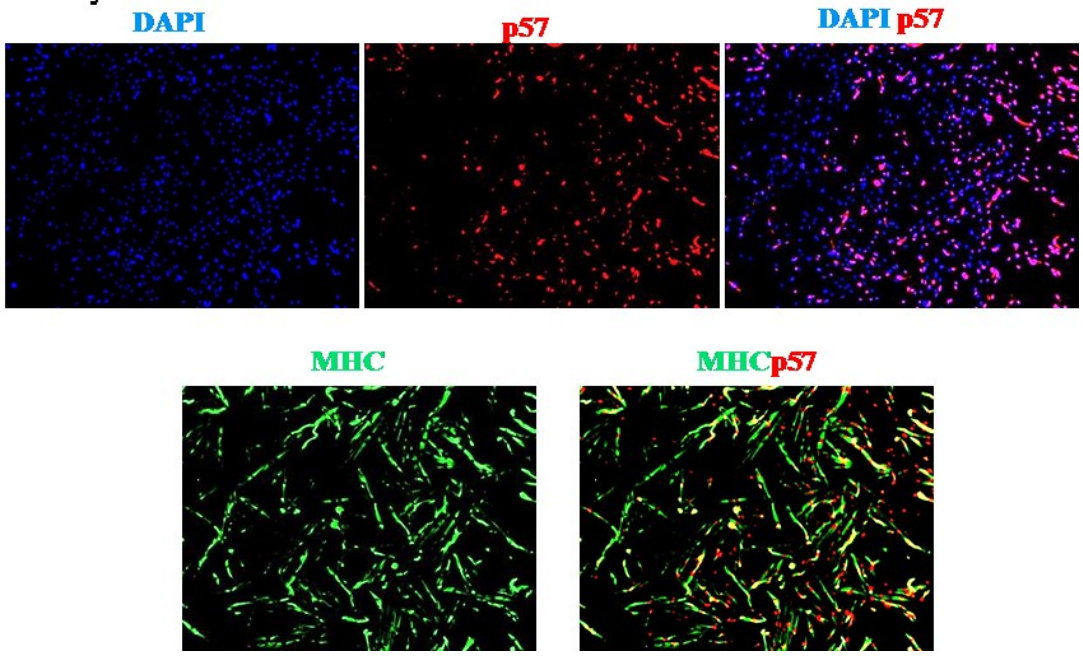
Figure 7. Increase in p27(+) cells during myogenic differentiation. Myoblasts were stained using antibodies for MHC (green) and p27 (red) under A) the growth condition at day 0, B) & C) differentiation conditions at day 1 and day 2 respectively(n=1). DAPI was used as a nuclear stain (blue). Scale bar = 100µm. D) Histograms representing the percentage of p27(+), MHC(+) and p27(+)MHC(+) on all three days. Error bars = standard error mean.

Next, we sought to investigate the levels of p57 protein during myogenic differentiation. We could observe an increase in the levels of p57 protein between the growth and differentiation conditions (Fig. 12). The up-regulation of p57 protein was clearly evident from the percentage of p57(+) cells, that was increased from 19% in the growth medium to about 61% in the differentiation medium (day 1) and finally to 99% at day 2 in the differentiation condition (Fig. 8). These data reveal that the p57 protein expression is enhanced during myogenic differentiation. This was consistent with the previous studies involving C2C12 myoblasts that showed an increase in p57 protein during myogenic

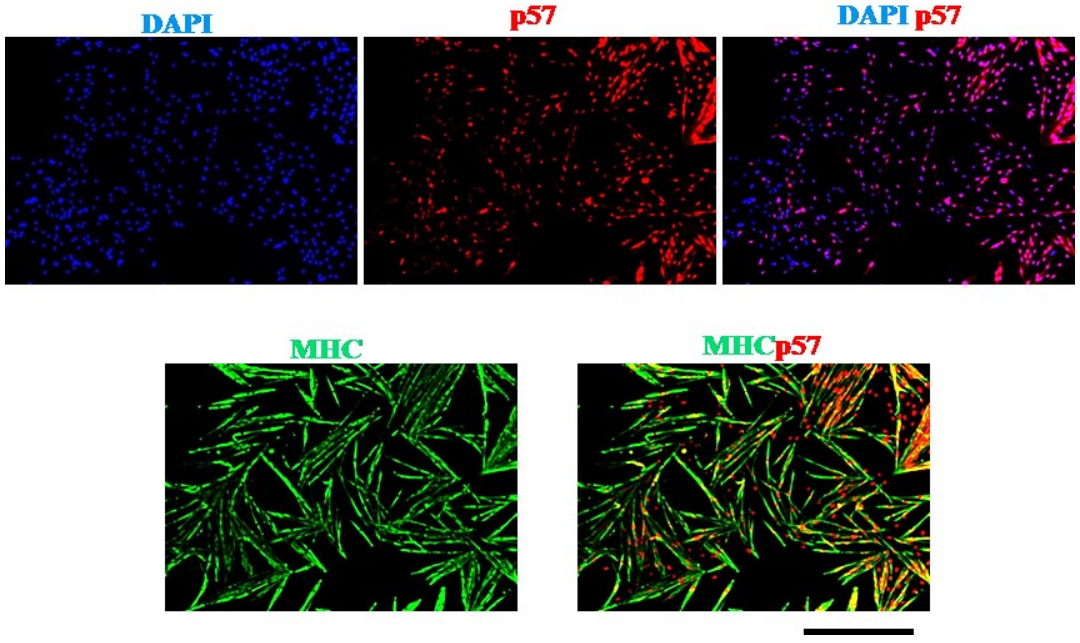
differentiation[39]. Consistent with the p57 levels, the cells showed enhanced expression of MHC during myogenic differentiation. This was clearly evident from the percentage of MHC(+) cells that was increased from 6% in the growth condition (day 0) to 50% at day 1 and finally to 91% at day 2 in the differentiation medium. This higher efficiency of myogenic differentiation at day 1 for p57 experiments compared to those for p21 and p27 experiments at day 1 (50% vs. 7% and 3% for MHC positive, respectively) can be attributed to the higher cell density for p57 experiments. Since all CDKIs (p21, p27 and p57) protein expressions are up-regulated during myogenic differentiation, these CDKIs may play essential roles in promoting the myoblasts towards differentiation and commitment.



B. Day 1 differentiation



C. Day 2 differentiation



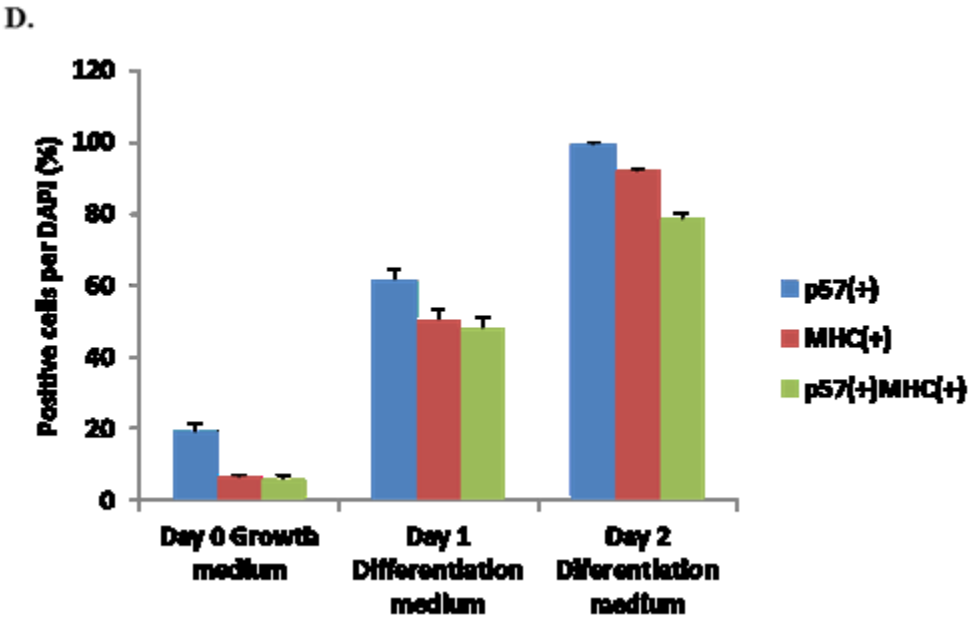


Figure 8. Increase in the number of p57(+) cells in myoblast culture during myogenic differentiation. Myoblast cultures were stained using antibodies for MHC (green) and p57(red) under A) the growth condition at day 0, B) and C) differentiation conditions at day 1 and day 2 respectively (n=1). DAPI was used as a nuclear stain (blue). Scale bar = 100 µm. D) Histogram representing the percentage of p57(+), MHC(+) and p57(+)MHC(+) on all three days. Error bars = standard error mean.

3.2 Western blotting revealed an increase in the expression of CDKIs during myogenic differentiation

To complement our immunostaining analysis of the endogenous levels of CDKIs, we also investigated the protein levels of p21, p27 and p57 using western blotting. β-tubulin, which is ubiquitously expressed in both myoblasts and differentiating cells was also tested using anti- β-tubulin antibody. Cell lysates were prepared from Myf5nLacZ myoblast cultures and total protein was prepared using M-PER mammalian-protein specific extraction reagent (Thermo Scientific). Ten µg protein was used for SDS-PAGE

and tested for p21, p27 and p57 protein expression under growth and differentiation conditions. First, we tried to detect p21 levels by using three different antibodies. However, none of them showed any positive bands, indicating that those antibodies did not work for the western blotting (data not shown) since we clearly showed p21 expression during myogenic differentiation in immunofluorescence (Fig. 6). Next, we investigated the expression of p27 protein in the myoblast culture, under growth (day 0) and differentiation (day 1, day 3 and day 5) conditions using anti-p27 antibody. The western blotting analysis revealed a steady increase in the levels of p27 protein from day 0 to day 3, after which this expression level was maintained at day 5 as the almost all the cells become terminally differentiated (Fig.9).These data were consistent with the result observed in immunostaining, suggesting that p27 seemed to be up-regulated during myogenic differentiation and may be required for myogenic commitment. Next, we investigated the expression of p57 protein in the myoblast culture under the same conditions as mentioned above using anti-p57 antibody. The western blotting revealed an increase in the expression of p57 protein from day 0 to day 3 as seen in immunostaining (Fig 9). The level was decreased by day 5 as almost all cells become terminally differentiated. Taken together, expression of all the CDKIs (p21, p27 and p57) were up-regulated during myogenic differentiation.

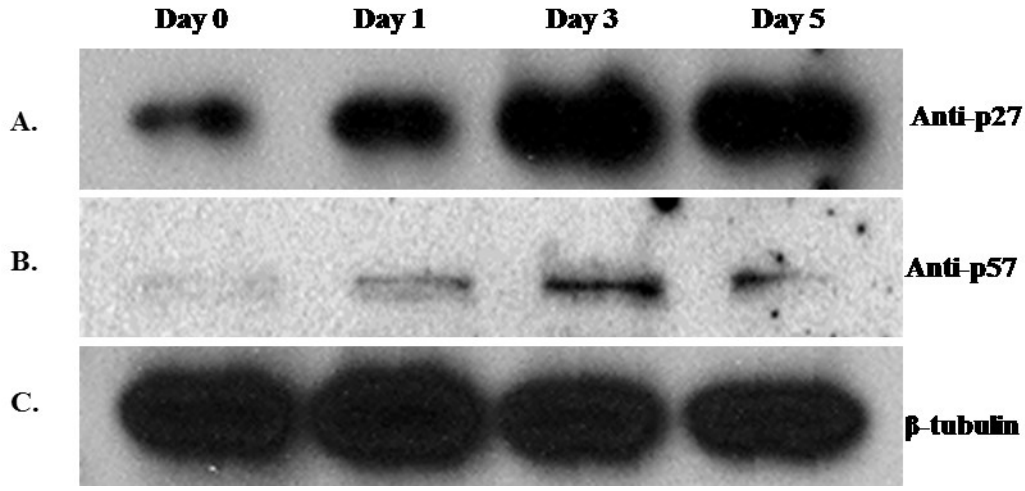


Figure 9. Endogenous expression of in A) p27, B) p57 and C) β-tubulin in Myf5LacZ myoblast culture under the growth (Day 0) and differentiation (Day 1, Day 3 and Day 5) conditions. Cell lysates were obtained from Myf5LacZ myoblast culture, under the growth and differentiation conditions and total protein (10 μg) was separated using 12% SDS-PAGE for western blotting with antibodies for p27 and p57. Protein expression was detected using HRP conjugated secondary antibody on addition of luminol and an enhancer solution. Each horizontal lane represents an individual SDS-gel, that was then utilised for specific protein detection using antibodies.

3.3 Over-expression of Myc-tagged CDKIs in myoblast culture reduces the number of MHC(+) cells during myogenic differentiation

3.3.1. Construction of retroviral expression vectors for Myc-tagged (MT)-CDKIs.

Asakura lab had already demonstrated that retroviral over-expression of p21 in myoblast culture reduces the Pax7(+)MyoD(-) reserve cells, which is an *in vitro* population of quiescent satellite cells (unpublished data). We wanted to investigate whether over-expression of CDKIs in myoblast culture promotes myogenic differentiation. For this, we first constructed pMX-puromycin (puro) retroviral vector with 6x Myc-tag (MT) gene corresponding to EQKLISEEDL amino acid sequences (termed pMX-MT-puro), serving

as a tag to identify cells that have taken up the retroviral vector by Immunostaining (Fig. 10A). Next, we amplified cDNAs for mouse p21, p21-N-terminal region (p21-N), p21-C-terminal region (p21-C), p27 and p57 genes from template cDNA of differentiated muscle cells by RT-PCR using Pfx polymerase (Invitrogen), followed by cloning each cDNA into TOPO cloning vector (Invitrogen). Next, we inserted p21, p21-N, p21-C, p27 and p57 cDNA fragments into the pMX-MT-puro after MT with inframe sequences to be translated as fusion proteins (termed pMX-MTp21-puro, pMX-MTp21-C-puro, pMXMT-p21-C-puro, pMX-MTp27-puro and pMX-MTp57-puro). Using the Plat-E retroviral packaging cell line, we generated viral supernatant of the three CDKI which were used for infection to the myoblasts. We then harvested the cell lysates from cells at day 0, under the growth condition, and at day 1 and day 3, under the differentiation conditions. We checked for the presence of MT fusion proteins by an anti-Myc tag (MT) antibody in the infected myoblasts versus non-infected myoblasts as the control. We detected the presence of MT-fusion proteins, MTp21, MTp27 and MTp57 in the myoblasts infected with the retroviral supernatant and not in the control myoblasts (Fig. 10B). These data indicated that the retroviral vectors have successfully integrated with the host genome and resulted in the production of Myc-fusion proteins.

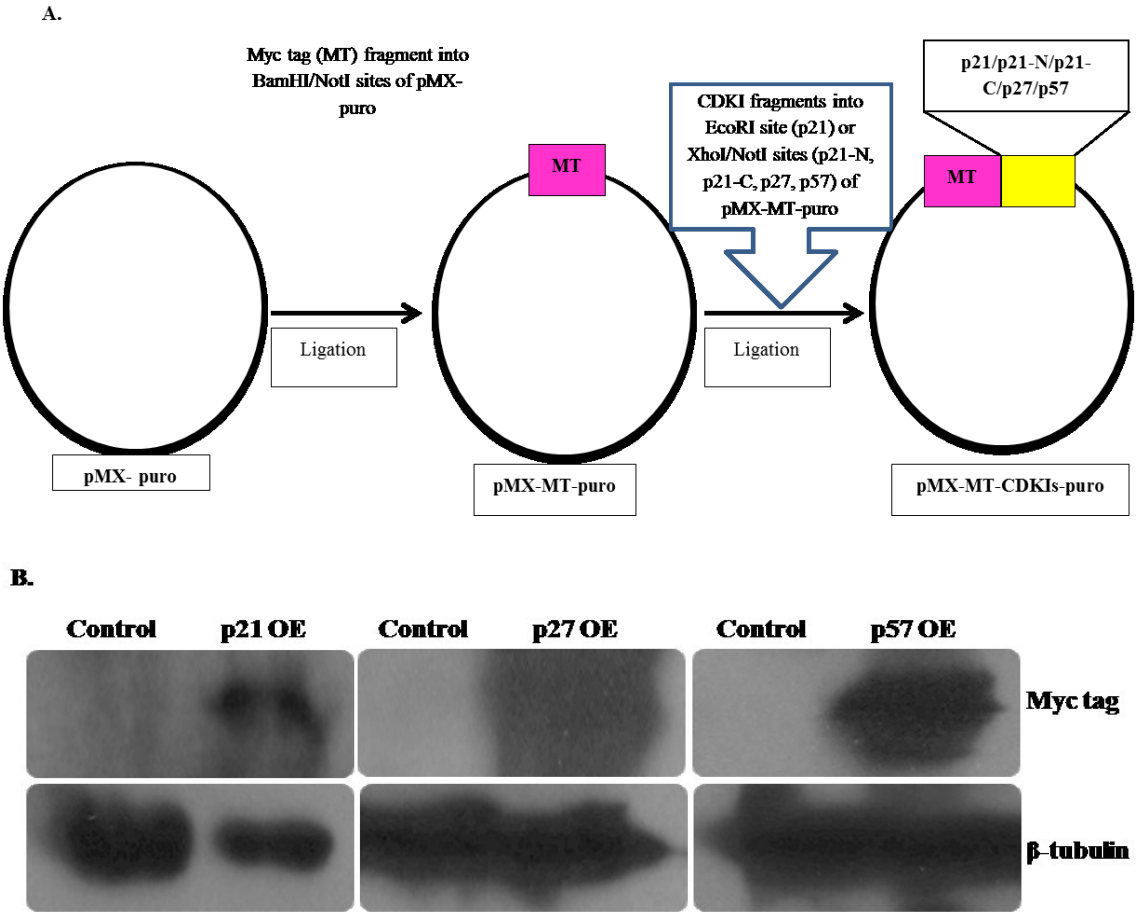
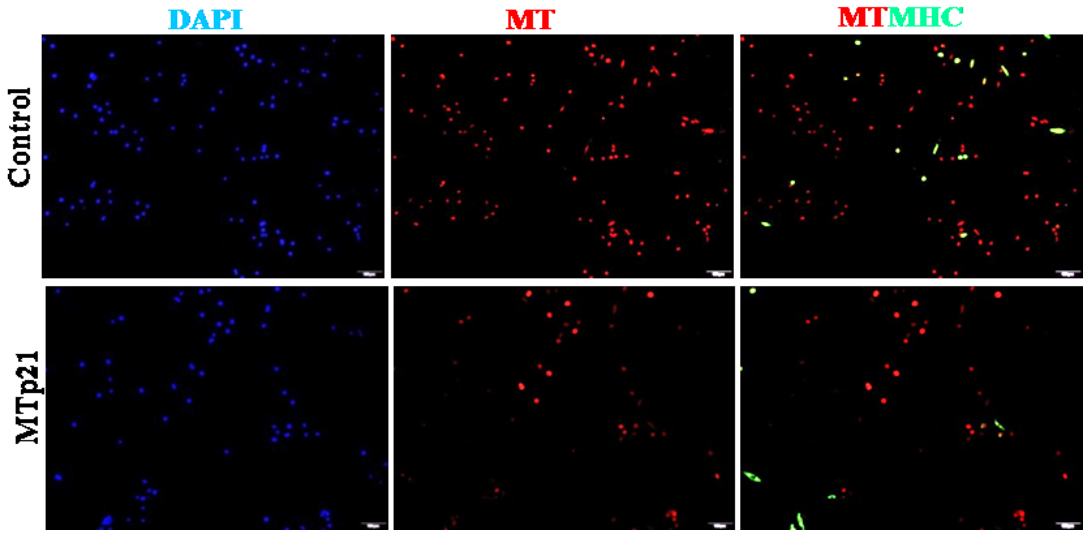


Figure 10. Schematic showing the construction of Myc tagged CDKI retroviral vectors and presence of Myc-tagged protein in myoblasts after over-expression. A) PCR-amplified Myc tag fragment was ligated into BamHI/EcoRI sites of pMX-puro retroviral vector, followed by ligation of PCR-amplified CDKI fragments into appropriate restriction sites of pMX-MT-puro to generate pMX-MTp21-puro, pMX-MTp21-N-puro, pMX-MTp21-C-puro, pMX-MTp27-puro and pMX-MTp57-puro. B) Cell lysate was obtained from myoblasts infected with MTp21, MTp27 and MTp57 retrovirus vectors under growth condition for overexpression (OE). Total protein (10 µg) was separated using 12% SDS-PAGE stained using antibodies for Myc-tag (MT). Protein expression was detected using HRP conjugated secondary antibody on addition of luminol and an enhancer solution. β-tubulin was used as loading control.

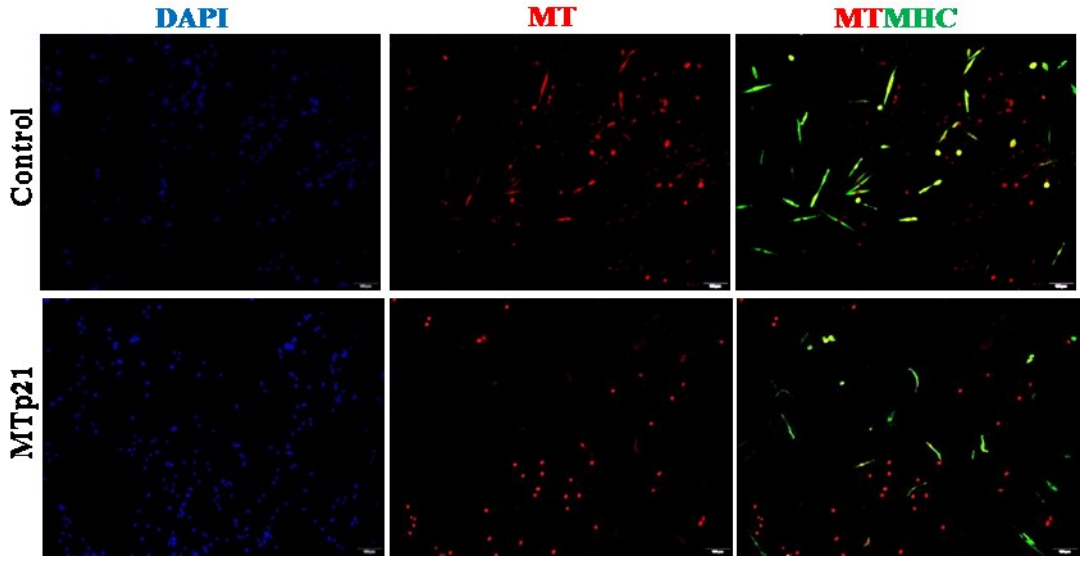
3.3.2 Over-expression of MTp21, MTp27 and MTp57 causes reduction in MHC(+) cell population.

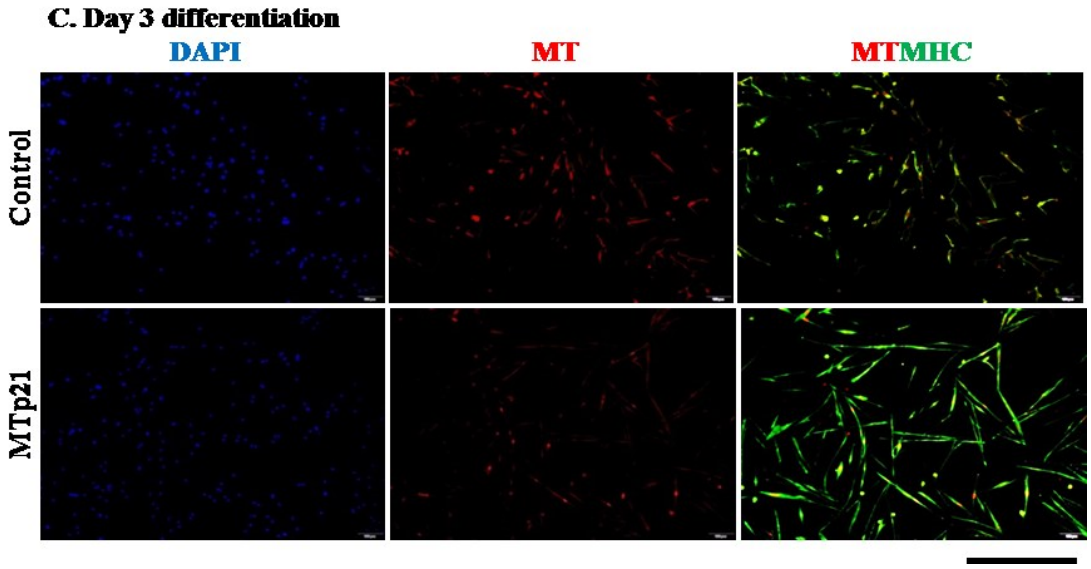
To elucidate the effects of over-expression of CDKI on the myogenic differentiation efficiency, we infected the cells with the retroviral supernatant of each of the MT-CDKI individually, fixed the cells at growth condition (day 0) and differentiation conditions (day 1, day 3 and day 5) and then stained for MT and MHC) using specific antibodies respectively. Our aim was to specifically look at cells that are over-expressing MT-CDKI cells that have taken up the vector and hence would be positive for MT. Hence we stained the cells for MT and MHC and counted the number of MHC(+) cells amongst the MT(+) cells. We observed that the percentage of MHC(+) amongst the MT(+) population decreased in the MTp21-over-expressed cells, compared with the control cells infected with empty vector (pMX-MT-puro). The decrease in the MHC(+) cells was observed in both growth condition (day 0) and differentiation conditions (day 1) but by Day 3, the MHC(+) cells caught up to the levels observed in the control (Fig. 11A, 11B, 11C, 11D). This was expected as by day 3, almost all cells differentiated and started to express MHC. We also observed a decrease in the infection efficiency by counting the number of MT(+) cells, compared with the control cells (Fig. 11E). The decrease in infection efficiency might be due to the toxicity of p21 over-expression since p21 has been reported to be also involved in cell death[40]. These data suggest that over-expression of MTp21 seemed to reduce the myogenic differentiation efficiency of myoblasts when compared with the myoblasts infected with empty vector.

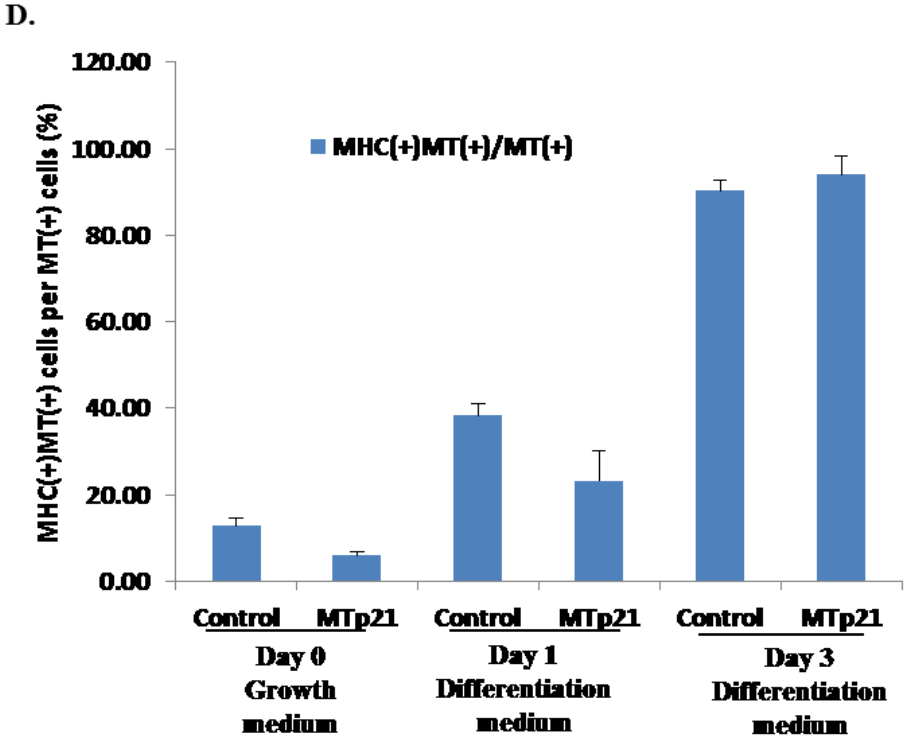
A. Day 0 growth



B. Day1 differentiation




C. Day 3 differentiation
DAPI
MT
MTMHC
Control
MTp21


D.
MHC(+)MT(+)/MT(+)
MHC(+)MT(+) cells per MT(+) cells (%)
120.00
100.00
80.00
60.00
40.00
20.00
0.00
Control
MTp21
Control
MTp21
Control
MTp21
Day 0
Growth
medium
Day 1
Differentiation
medium
Day 3
Differentiation
medium

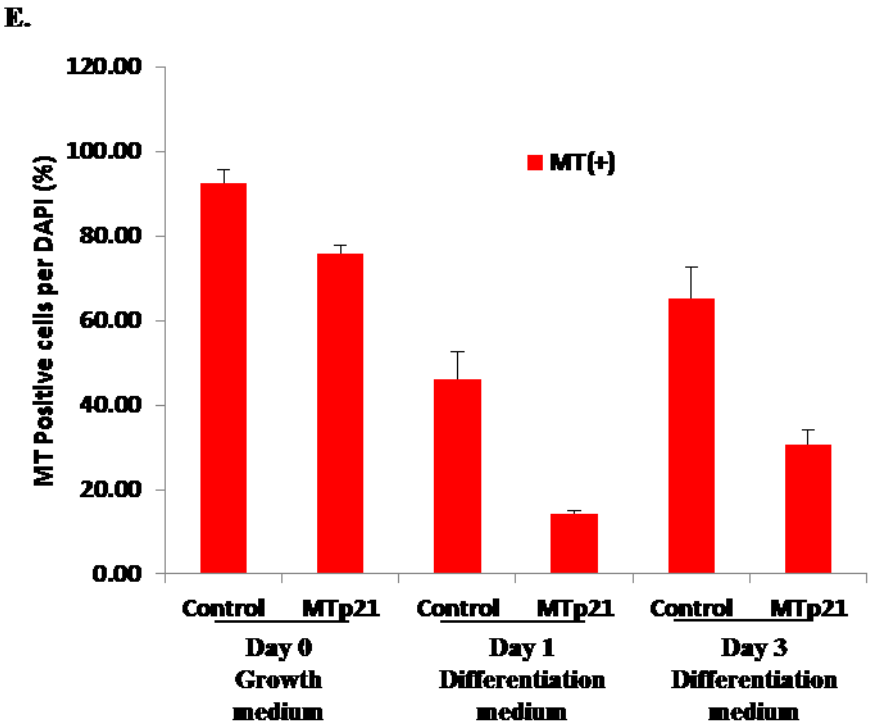
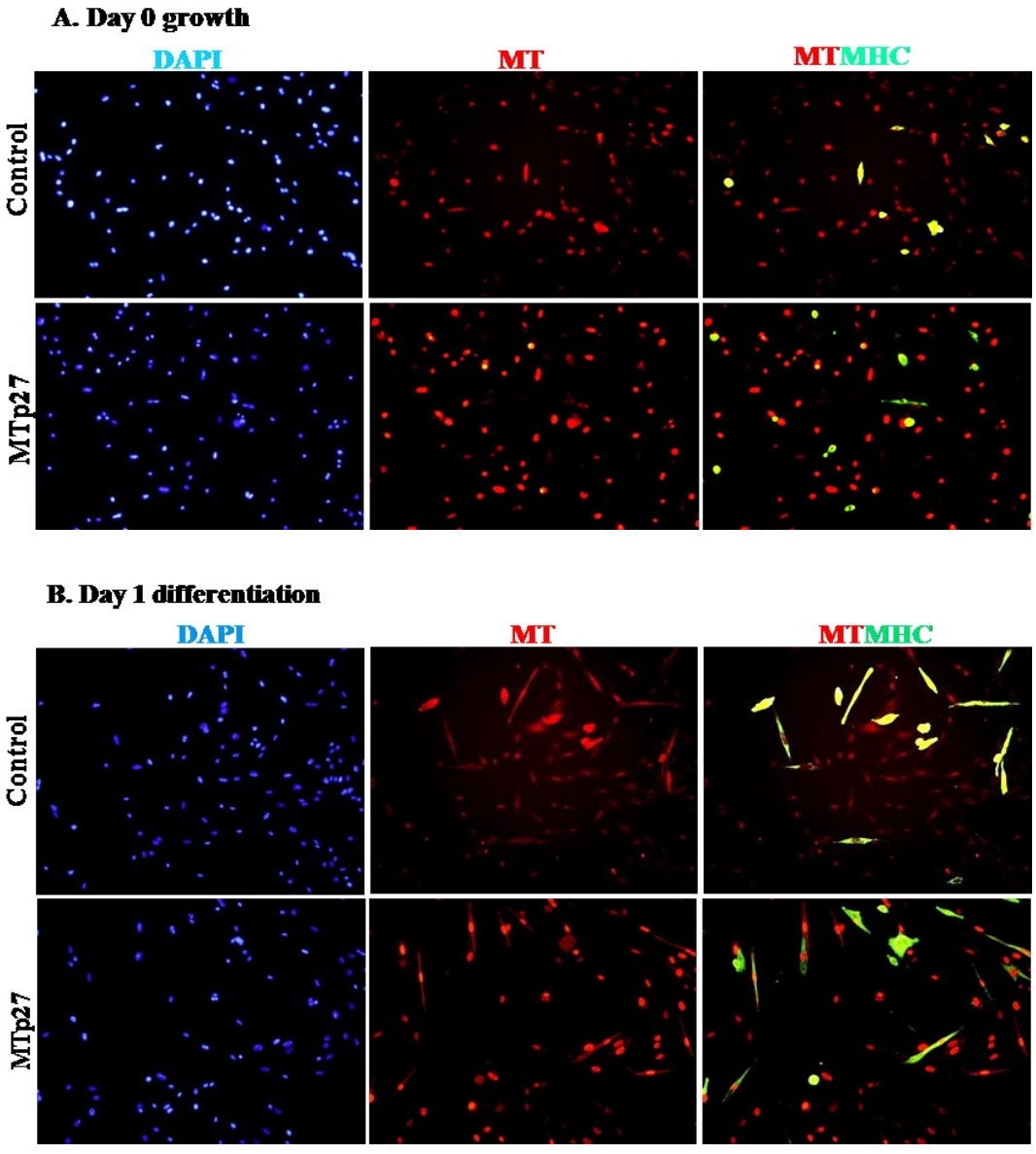
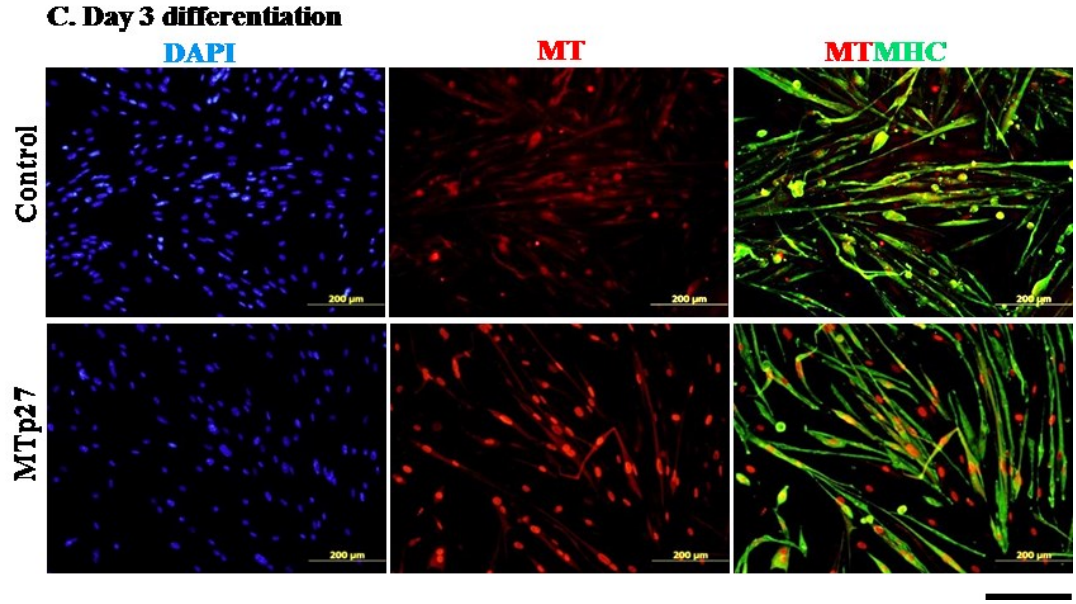


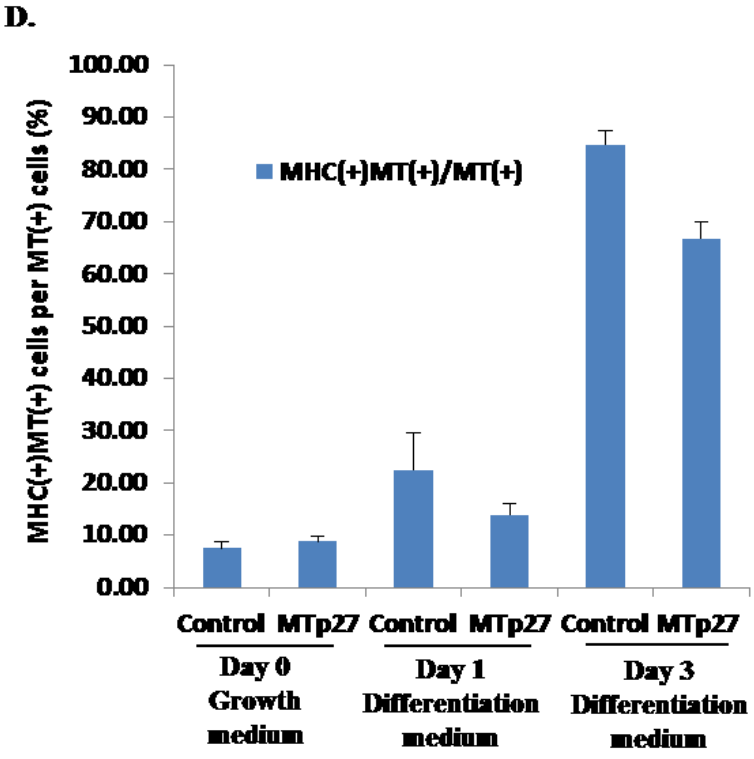
Figure 11. Over-expression of pMX-MTp21-puro in myoblast culture reduced the number of MHC(+) cells during myogenic differentiation. Myoblasts were infected with retroviral supernatant of pMX-MTp21-puro and of pMX-MT-puro as a control. Cells were fixed and stained using antibodies for MHC (green) and MT (red) under A) growth condition at day 0, and B) and C) differentiation conditions at day 1 and day 2 (n=1). DAPI was used as a nuclear stain (blue). Scale bar = 100 µm. D) Histogram representing the percentage of MHC(+)MT(+)/MT(+) on all three days. E) Percentage of MT(+) cells per DAPI (+) nuclei. Error bars = standard error mean.

Next, we investigated the effects of over-expression of MTp27 protein in the myoblast culture. We followed the same approach that was used for p21 gene and infected the cells with MTp27 retroviral vector. Looking for the percentage of MHC(+) cells amongst the MT(+) cells, we found a decrease in the MHC(+) cells, when compared with the control cells (Fig. 12A, 12B, 12C, 12D). We also looked at the infection efficiency by counting the number of MT(+) cells, and found a higher percentage of infection efficiency

compared to MTp21 fusion protein (Fig. 12E). The percentage of MT(+) cells were comparable between control cells and p27-over-expressing cells at day 0 (growth) and day 1 (Differentiation) but decreased at day 3. On the whole, similar to the result from over-expression of MTp21, over-expression of MTp27 also decreased the number of MHC(+) cells, thus reducing the percentage of myogenic differentiation.




C. Day 3 differentiation
DAPI
MT
MTMHC
Control
MTp27
200 μm


D.
MHC(+)MT(+) cells per MT(+) cells (%)
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
MHC(+)MT(+)/MT(+)
Control
MTp27
Control
MTp27
Control
MTp27
Day 0
Growth
medium
Day 1
Differentiation
medium
Day 3
Differentiation
medium

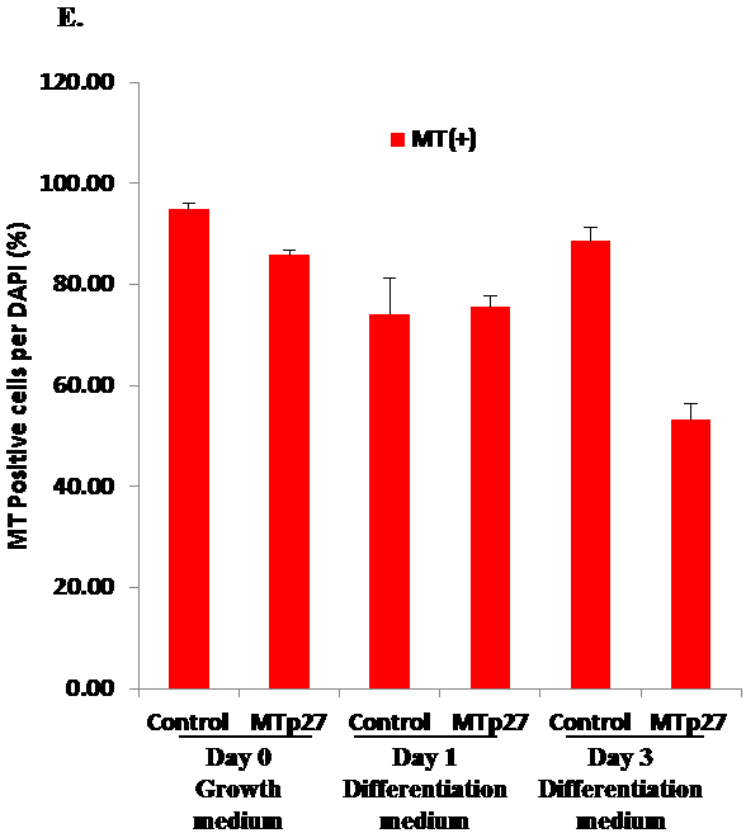
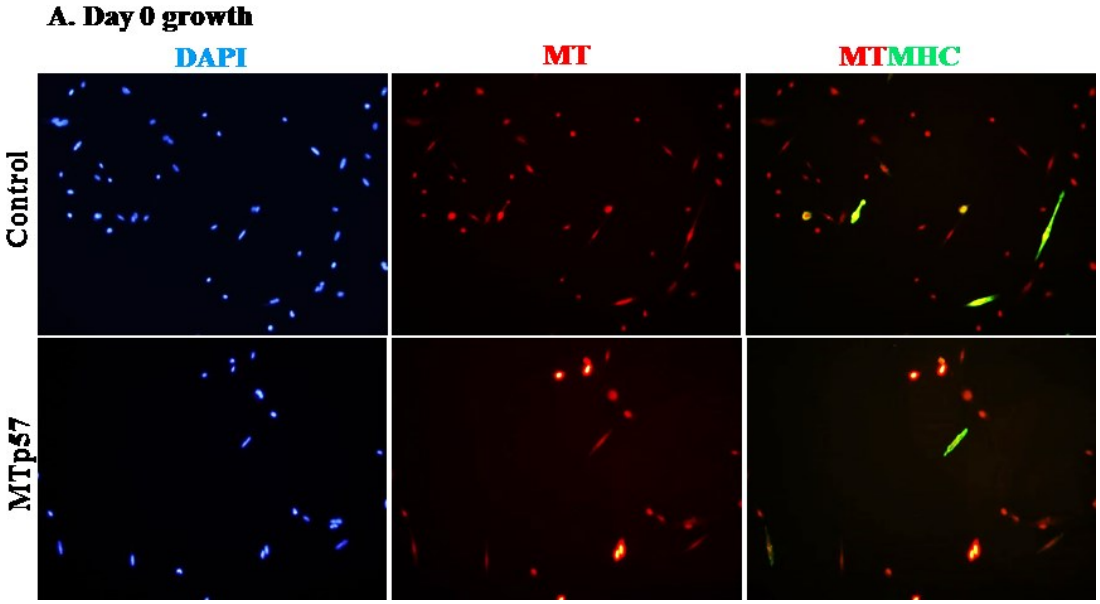
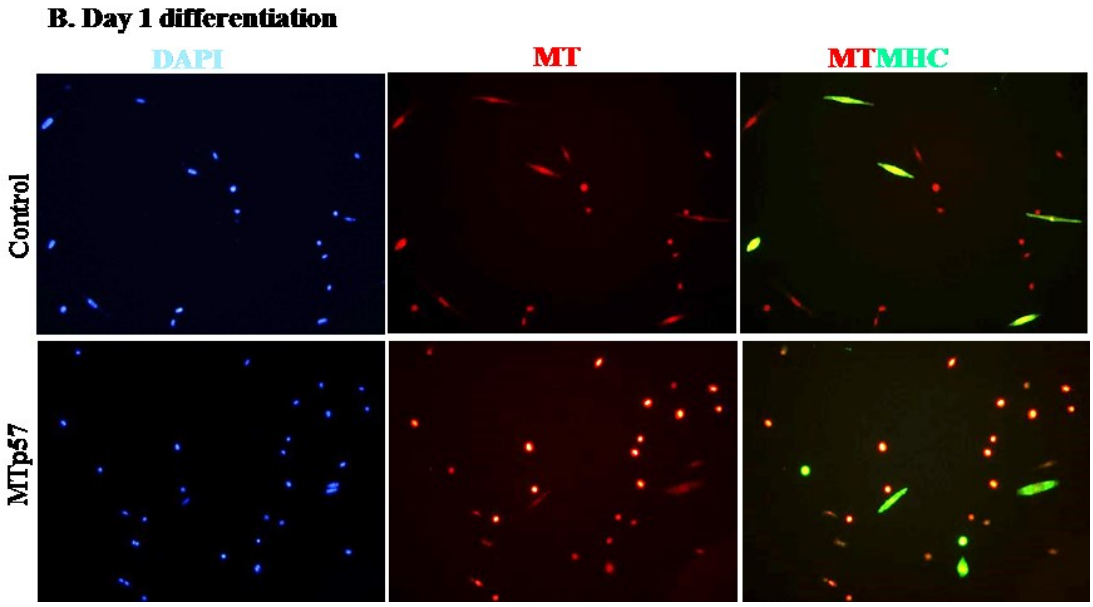


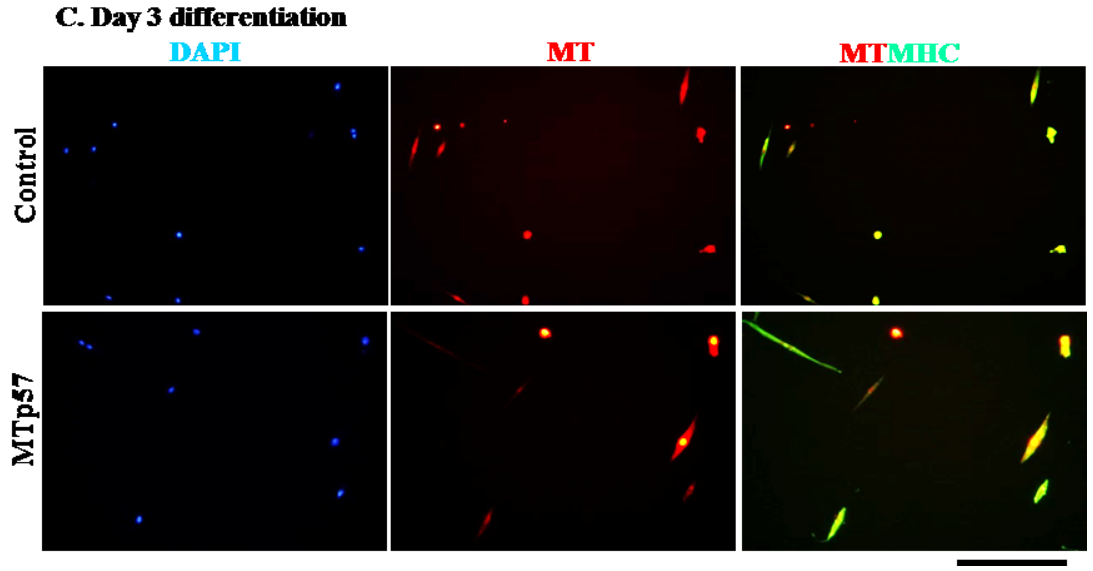
Figure 12.Over-expression of pMX-MTp27-puro in myoblast culture decreased ithe number of MHC(+) cells during myogenic differentiation. Myoblasts were infected with retroviral supernatant of pMX-MTp27-puro and pMX-MT-puro (control). Cells were fixed and stained using antibodies for MHC (green) and MT (red) under A) growth condition at day 0, and B) and C) differentiation conditions at day 1 and day 2 (n=1). DAPI was used as a nuclear stain (Blue). Scale bar = 200 µm. D) Histogram representing the percentage of MHC(+)MT(+)/MT(+)on all three days. E) Percentage of MT(+) cells per DAPI(+) nuclei. Error bars = standard error mean.

Finally, we investigated the effects of over-expressing MTp57 in the myoblast culture, using the same approach utilised for p21 and p27. Upon over-expression of MTp57, we found a decrease in the percentage of MHC(+) cells among all time points (Fig. 13A, 13B, 13C, 13D), indicating that over-expression of MTp57 reduces the differentiation

efficiency in myoblasts. We found that the percentage of MT(+) cells is initially decreased under growth condition but then reaches to a comparable level as that of control cells (Fig. 13E).Taken together, these results indicate that over-expression of MTp21, MTp27 and MTp57 decreases the number of MHC(+) cells, thus reducing the percentage of myogenic differentiation, which contradicts previously reported works.




B. Day 1 differentiation
DAPI
MT
MTMHC
Control
MTp57


C. Day 3 differentiation
DAPI
MT
MTMHC
Control
MTp57

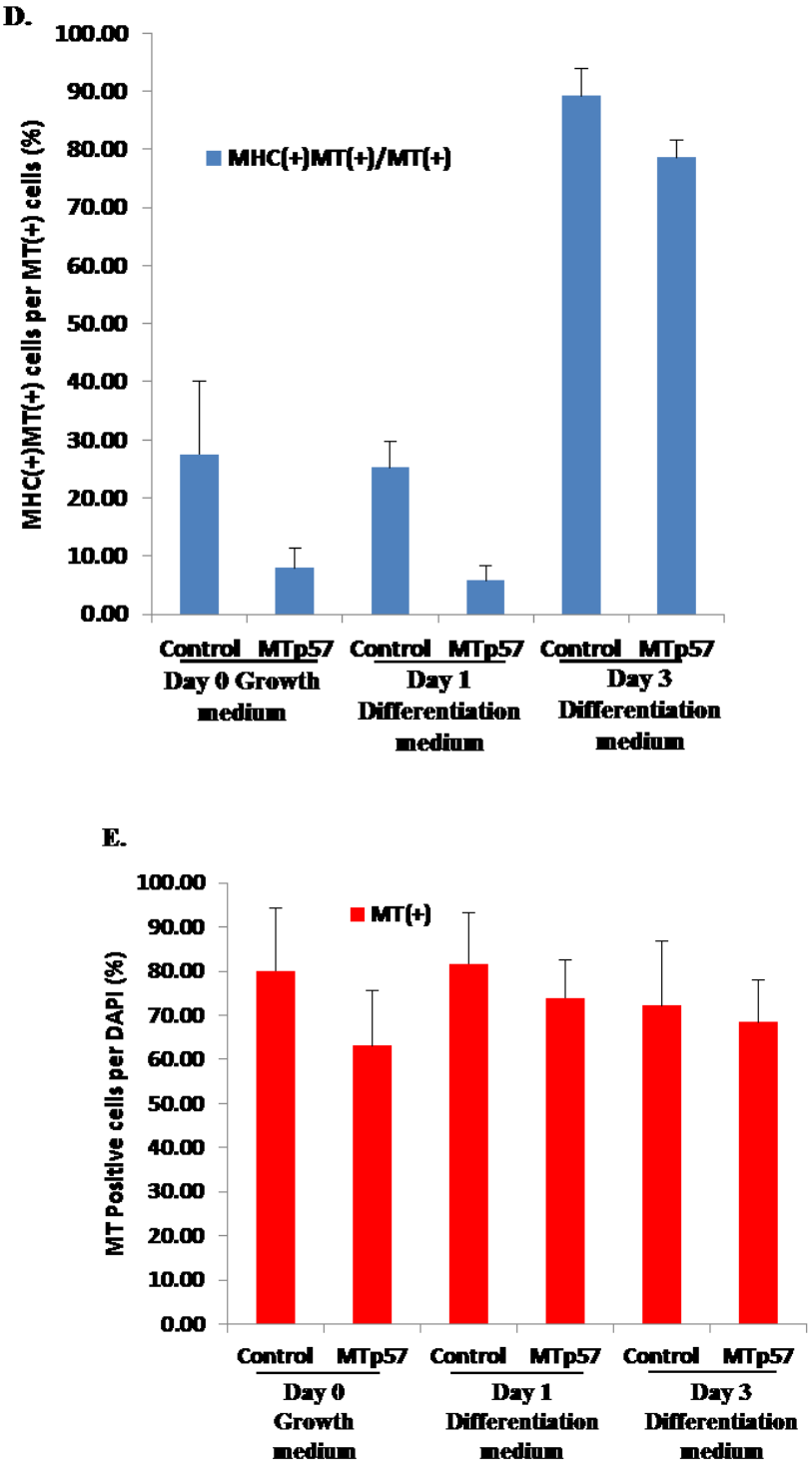


Figure 13. Over-expression of pMX-MTp57-puro in myoblast culture decreased the number of MHC(+) cells during myogenic differentiation. Myoblasts were infected

with retroviral supernatant of pMX-MTp57-puro and pMX-MT-puro (control). Cells were fixed and stained using antibodies for MHC (green) and MT (red) under A) growth condition at day 0, and B) and C) differentiation conditions at day 1 and day 2 (n=1). DAPI was used as a nuclear stain (blue). Scale bar = 100 µm. D) Histogram representing the percentage of MHC(+)MT(+)/MT(+)on all three days. E) Percentage of MT(+) cells per DAPI(+) nuclei. Error bars = standard error mean.

3.3 N-terminal domain-specific induction of differentiation by p21

Previous works has revealed the existence of similarities and differences amongst p21, p27 and p57, in terms of their protein structure (Fig. 14)[36]. All the three proteins have a CDK inhibitory domain in their N-terminal region, which is involved in the inhibition of cyclin-CDK activity[36]. The study also revealed that there were stark differences among the C-terminal domain of p21, p27 and p57, where the *Proliferating cell nuclear antigen* (PCNA) binding region was shown to be unique to the C-terminal region of the p21 protein[36]. *PCNA* is a processivity factor for DNA polymerase δ and essential for DNA replication. Thus, p21 binding to PCNA might be involved in suppression of DNA replication. Using this knowledge, we wanted to investigate the individual contribution of each domain in the process of myogenic differentiation in murine myoblasts.

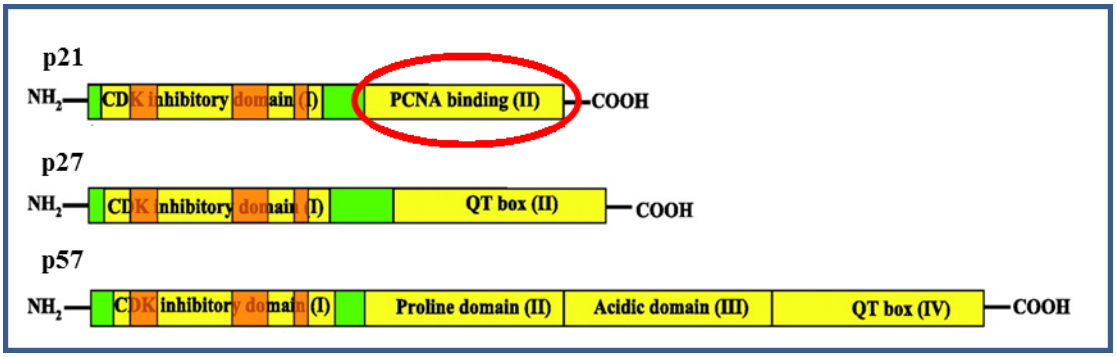
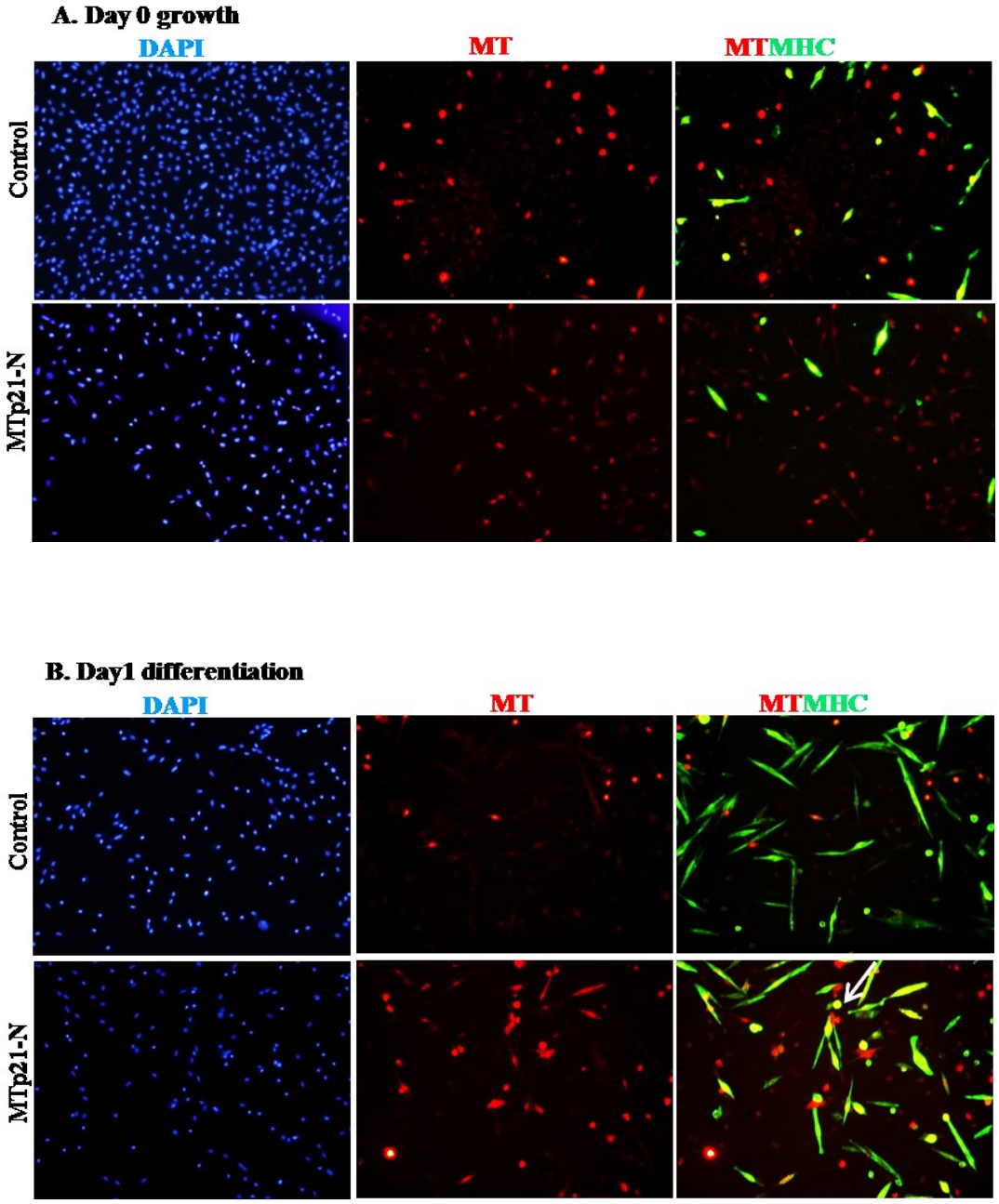
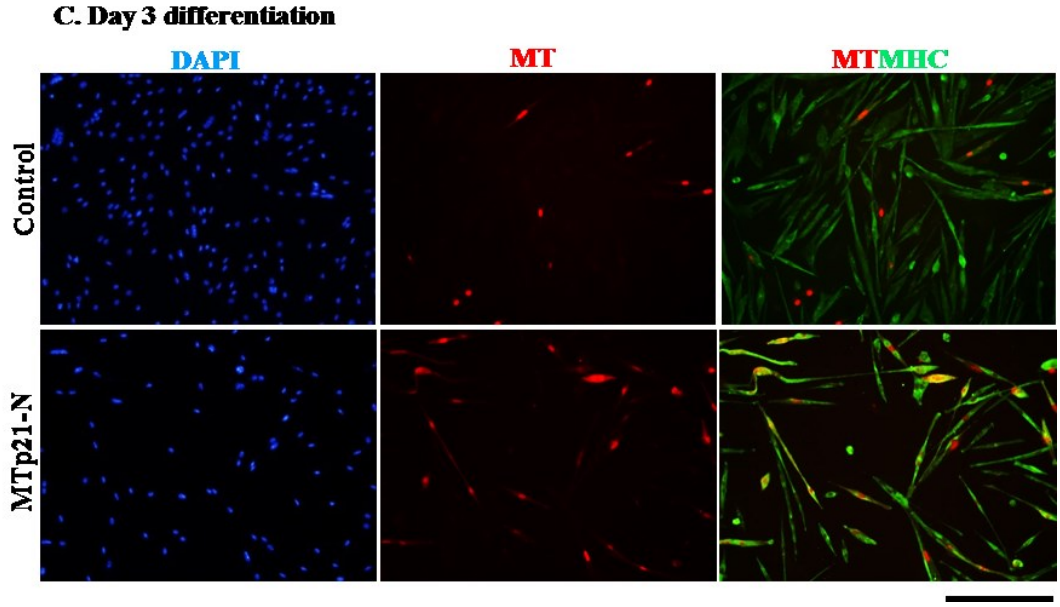
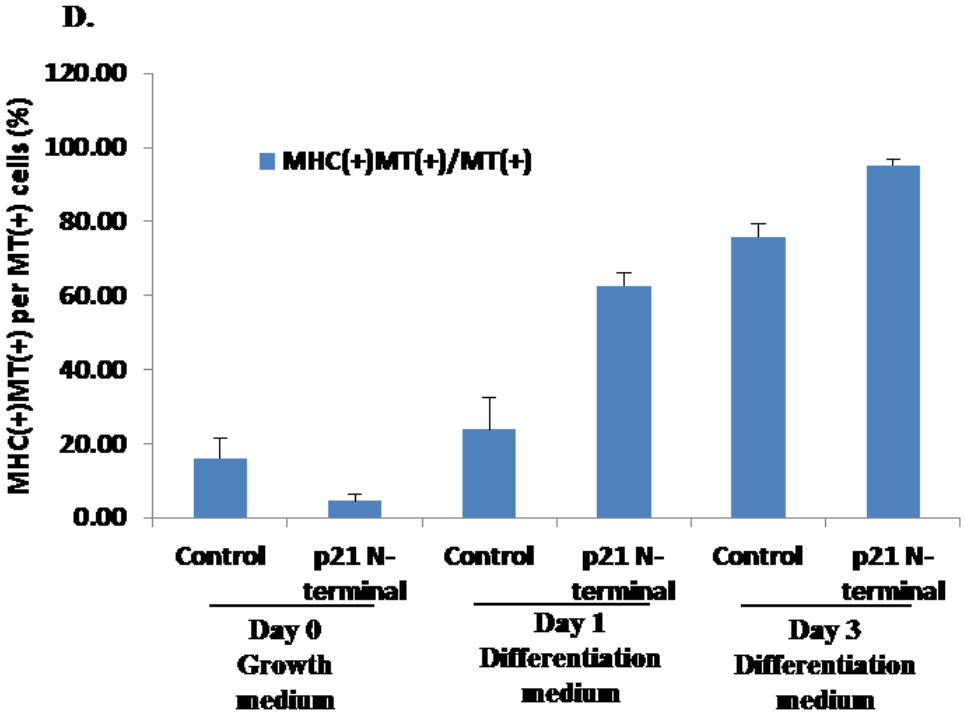


Figure 18. Protein structures of p21, p27 and p57[36]. All three of them have a CDK inhibitory domain in their N-terminal region. Only p21 protein has a PCNA binding domain in its C-terminal region (circled), when compared with p27 and p57.

For this, we synthesised primers to specifically amplify the N-terminal (CDK inhibitory) domain and the C-terminal (PCNA) domain of p21[41]. We cloned the domains into the pMX-MT-puro vector to produce pMX-MT-N-terminal-puro (referred as MTp21-N) and pMX-MT-C-terminal-puro (referred as MTp21-C). We initially observed a decrease in the percentage of MHC(+) cells in both the N-terminal and the C-terminal over-expressed cells (Figs. 15A, 16A). However, at day 1, the N-terminal over-expressing cells showed a distinct increase in the number of MHC(+) cells, whereas the C-terminal over-expressing cells exhibit similar levels to that of the control cells (Figs 15B, 16B). By Day 3, there was no difference in the contribution of either N-terminal or C-terminal towards differentiation (Figs 15C, 16C). A point to note would be the increase in the percentage of MHC(+) cells by the N-terminal over-expressing cells at Day 1 and not by C-terminal, suggests that the N-terminal domain of p21 protein might be the one involved in pushing the myoblasts towards myogenic differentiation.


A. Day 0 growth
DAPI
MT
MTMHC
Control
MTp21-N
B. Day1 differentiation
DAPI
MT
MTMHC
Control
MTp21-N


C. Day 3 differentiation
DAPI
MT
MTMHC
Control
MTp21-N


D.
MHC(+)MT(+) per MT(+) cells (%)
120.00
100.00
80.00
60.00
40.00
20.00
0.00
MHC(+)MT(+)/MT(+)
Control
p21 N-terminal
Control
p21 N-terminal
Control
p21 N-terminal
Day 0 Growth medium
Day 1 Differentiation medium
Day 3 Differentiation medium

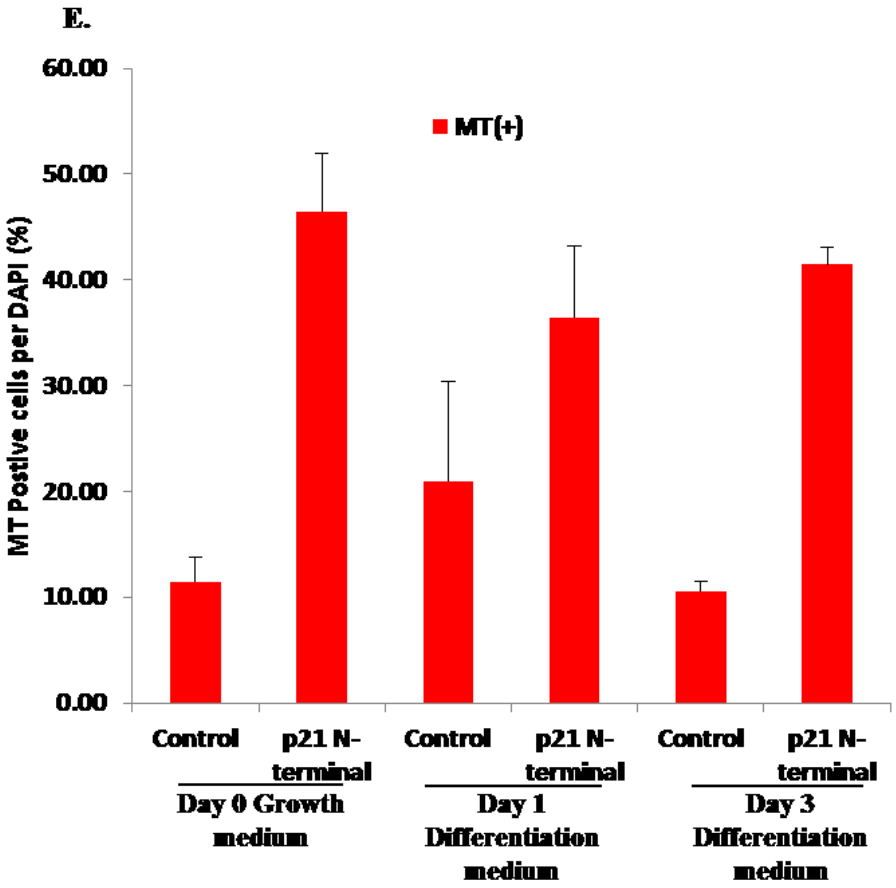
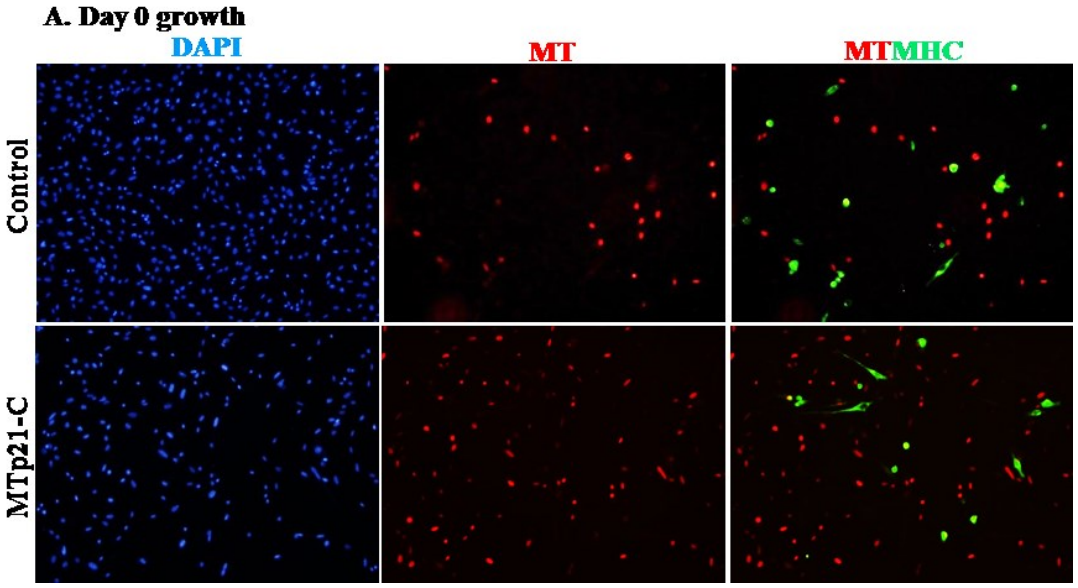
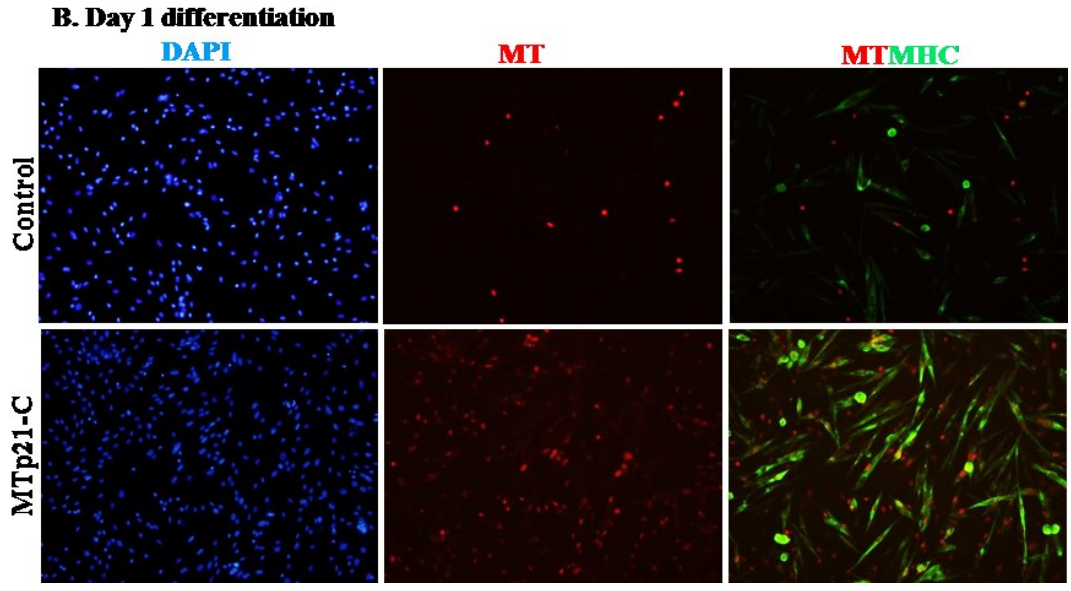
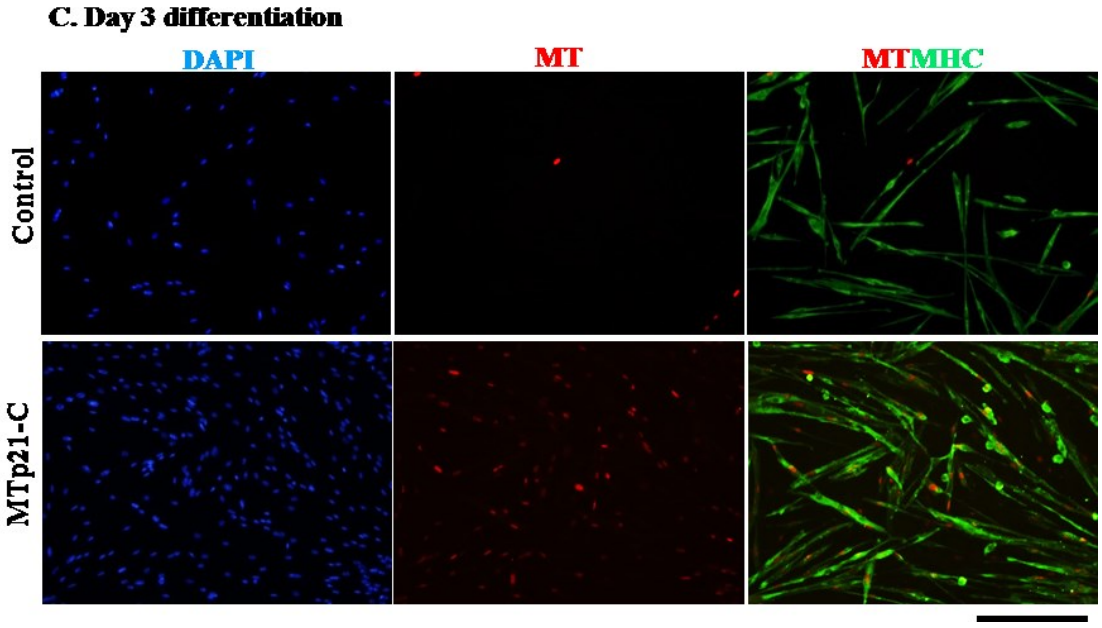
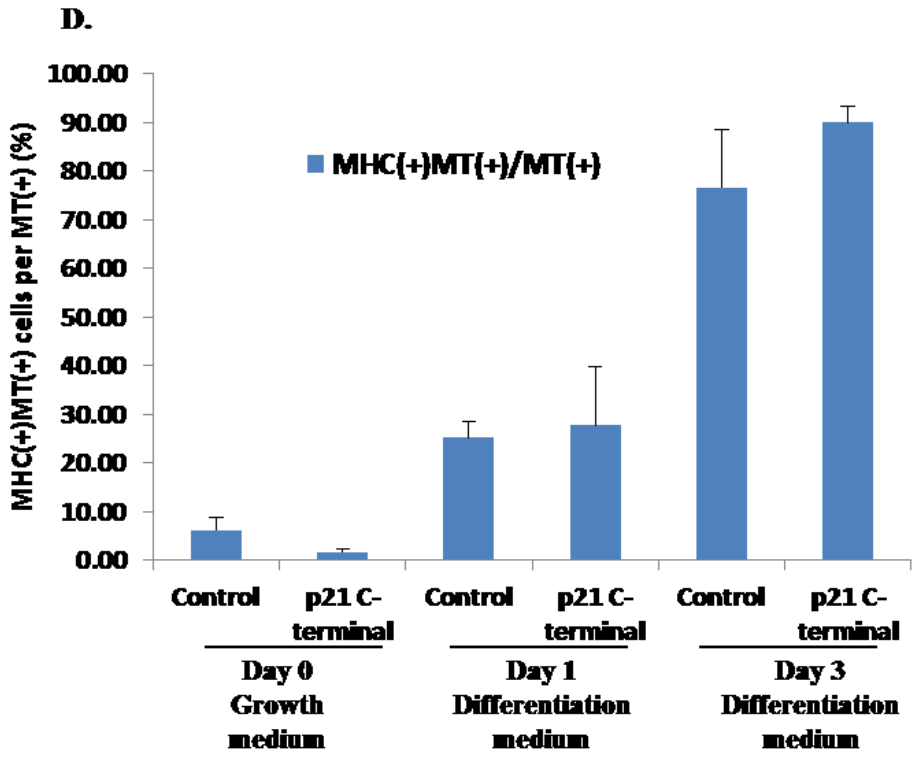


Figure 15.Over-expression of MTp21-N in myoblast culture causes increase in the number of MHC(+) under differentiation conditions, compared to control. Myoblasts were infected with retroviral supernatant of pMX-MTp21-N -puro and pMX-MT-puro (control). Cells were fixed and stained using antibodies for MHC (green) and MT (red) under A) growth condition at day 0, and B) and C) differentiation conditions at day 1 and day 2 (n=1). DAPI was used as a nuclear stain (blue). Scale bar = 100 µm. D) Histogram representing the percentage of MHC(+)MT(+)/MT(+)on all three days. E) Percentage of MT(+) cells per DAPI(+) nuclei. Error bars = standard error mean.


A. Day 0 growth
DAPI
MT
MTMHC
Control
MTp21-C


B. Day 1 differentiation
DAPI
MT
MTMHC
Control
MTp21-C


C. Day 3 differentiation
DAPI
MT
MTMHC
Control
MTp21-C


D.
MHC(+)MT(+) cells per MT(+) (%)
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
MHC(+)MT(+)/MT(+)
Control
p21 C-terminal
Control
p21 C-terminal
Control
p21 C-terminal
Day 0 Growth medium
Day 1 Differentiation medium
Day 3 Differentiation medium

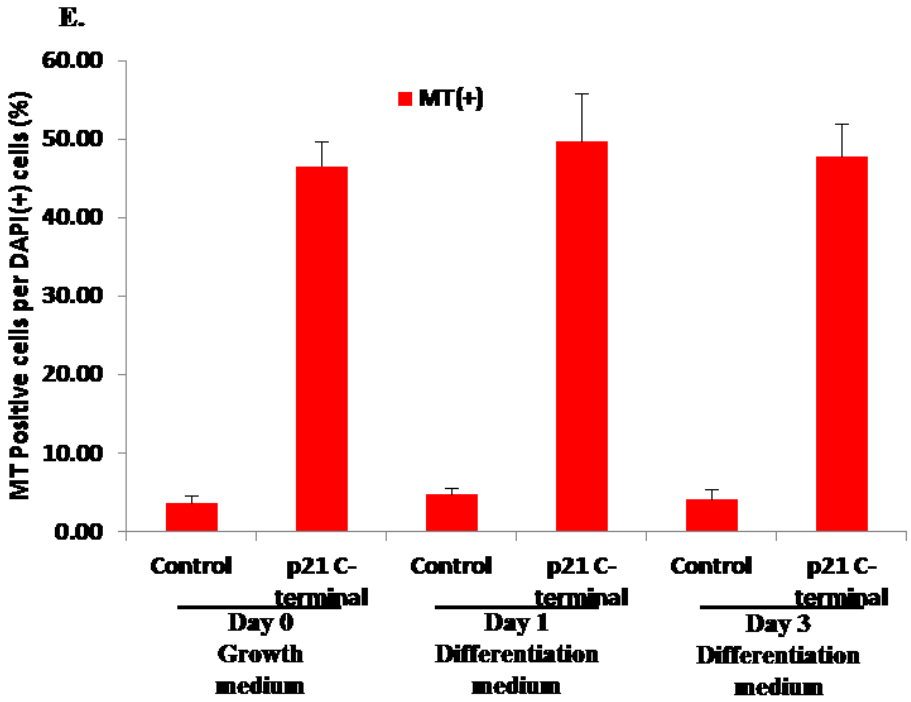


Figure 16.Over-expression of MTp21-C in myoblast culture showed similar number of MHC(+) cells under differentiation conditions compared to control. Myoblasts were infected with retroviral supernatant of pMX-MTp21-C-puro and pMX-MT-puro (control). Cells were fixed and stained using antibodies for MHC (green) and MT (red) under A) growth condition at day 0, and B) and C) differentiation conditions at day 1 and day 2 (n=1). DAPI was used as a nuclear stain (blue). Scale bar = 100 µm. D) Histogram representing the percentage of MHC(+)MT(+)/MT(+) on all three days. E) Percentage of MT(+) cells per DAPI(+) nuclei. Error bars = standard error mean.

3.4 Construction of retroviral vectors for non-Myc tagged CDKIs

The eleven amino acid sequence of Myc epitope (EQKLISEEDL) derived from leucine zipper of Myc protein, which is involved in heterodimerization, has been widely used as a tag for monitoring expression of protein[42]. Myc belongs to helix-loop-helix leucine zipper family of transcription factors and regulates various biological effects through DNA binding as well as interaction with other proteins. In addition, Myc has also been shown

to inhibit function of the cell cycle inhibitors. For instance, Myc has been shown to inhibit the activity of p27 protein by blocking its binding with the newly formed cyclin E/Cdk2 conplex[43]. Myc was also shown to reverse the growth arrest induced by over-expression of p27 protein, which was found to be independent of p27-ubiquitination[44]. Myc has also been shown to repress p21 protein by sequestration of Sp1/Sp3 transcription factors, which are required for production of p21 protein[45]. Furthermore, there is also a speculation that Myc inhibits p57, possibly in the same way as it inhibits p27[46].

Above, we demonstrated that Myc-tagged CDKIs (MTp21, MTp21-N, MTp21-C, MTp27 and MTp57) showed inhibitory effects on myogenic differentiation when over-expressed in myoblast cultures, which contradicts the previous work. Therefore, we could speculate that the presence of Myc tag might be inhibiting the activities of p21, p21-N, p21-C, p27 and p57. To test our hypothesis, we constructed retroviral vectors for CDKIs without Myc tag by excising the Myc tag fragment using appropriate restriction enzymes (Fig. 17).

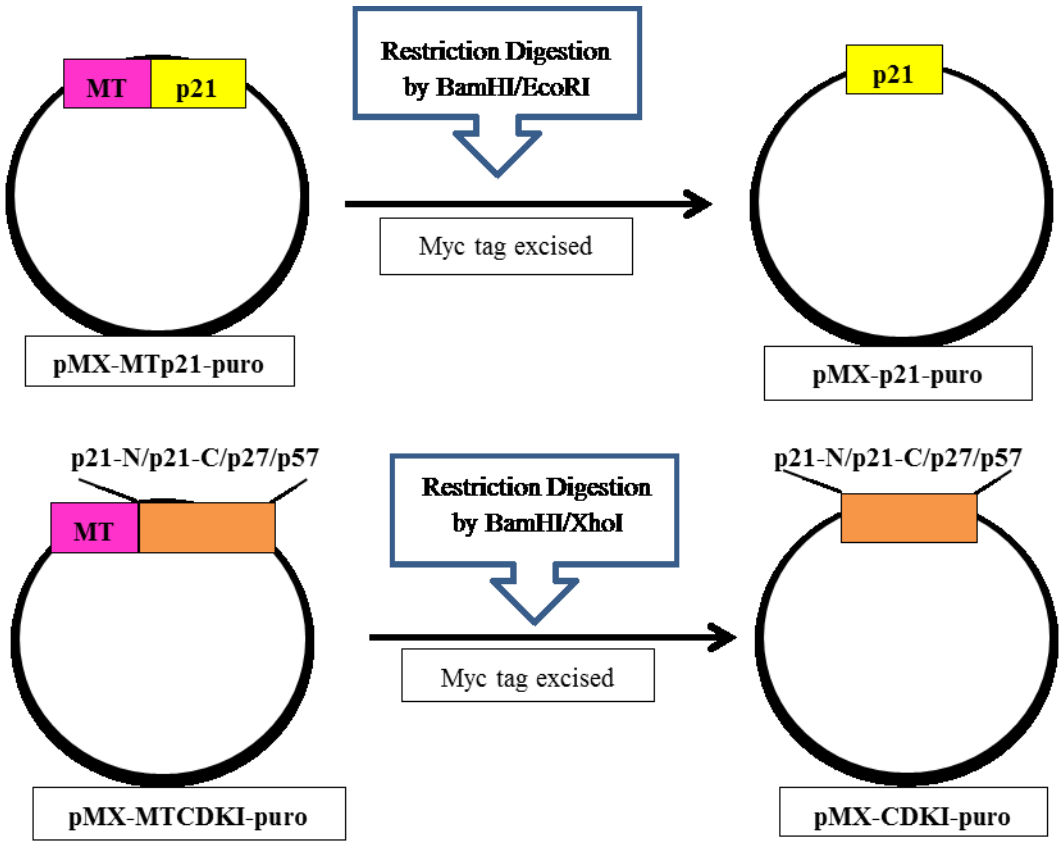
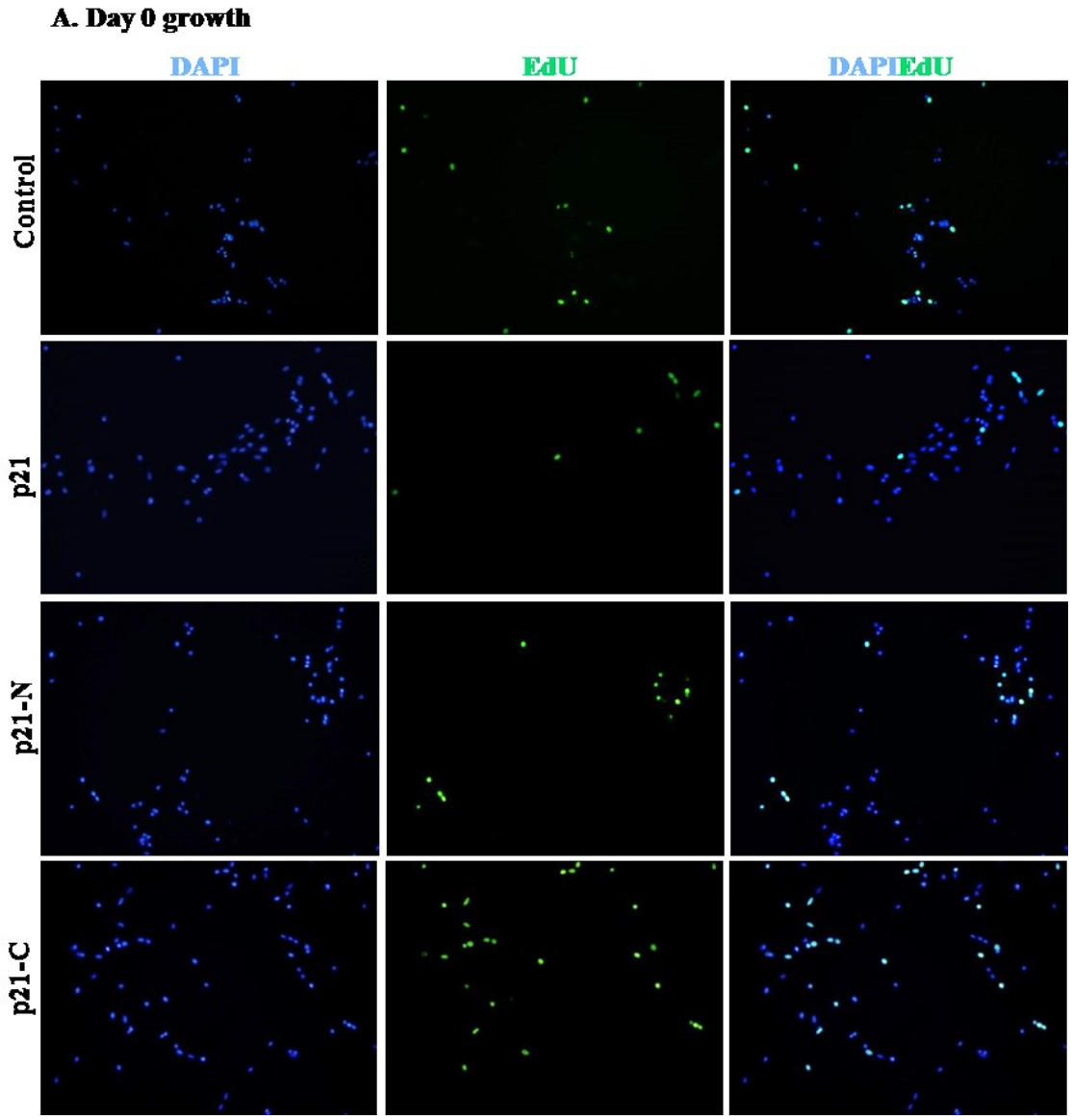


Figure 17. Schematic showing the construction of non-Myc tagged CDKI retroviral vectors. Myc tag was excised out using appropriate restriction enzymes. Since p21 was cloned using the EcoRI sites, BamHI and partial EcoRI sites were used to excise Myc tag out. All the other vectors were cloned using XhoI and Not I sites, and thus, Myc tag was removed using BamHI and XhoI sites. After restriction digestion, these vectors were allowed for self-religation.

3.5 Proliferation was decreased upon overexpression of non-MT CDKI compared to control

First we investigated the percentage of proliferating cells amongst all the cells that were subjected to CDKI over-expression by EdU assay, on the day of infection. EdU (5'-ethynyl-2'deoxyuridine) assay is a commonly used method for detection of proliferation, using EdU incorporation into the DNA. The EdU molecule incorporates during active

DNA synthesis, thus indicative of the proliferative cells. We utilised this method to look for percentage of EdU(+) cells upon over-expression of all the CDKI. We found that the percentage of EdU(+) cells was decreased amongst all the samples i.e over-expression of p21, p21-N-terminal, p21 C-terminal, p27 and p57 when compared to the control cells infected with pMX-MT-puro (Fig. 18A, 18B). These data suggests that all of the CDKI suppress proliferation effectively when compared with the control. Interestingly, we found that the percentage of EdU(+) cells in the p21 C-terminal over-expressing cells was almost equal to that of the control cells. In the case of p21 N-terminal also, the percentage of proliferating cells was found to be higher than that of full length p21. This was similar to the results obtained previously[41], which showed that the truncated form of p21 N-terminal and C-terminal may not be as effective as full length p21 in suppressing DNA synthesis.


A. Day 0 growth
DAPI
EdU
DAPIEdU
Control
p21
p21-N
p21-C

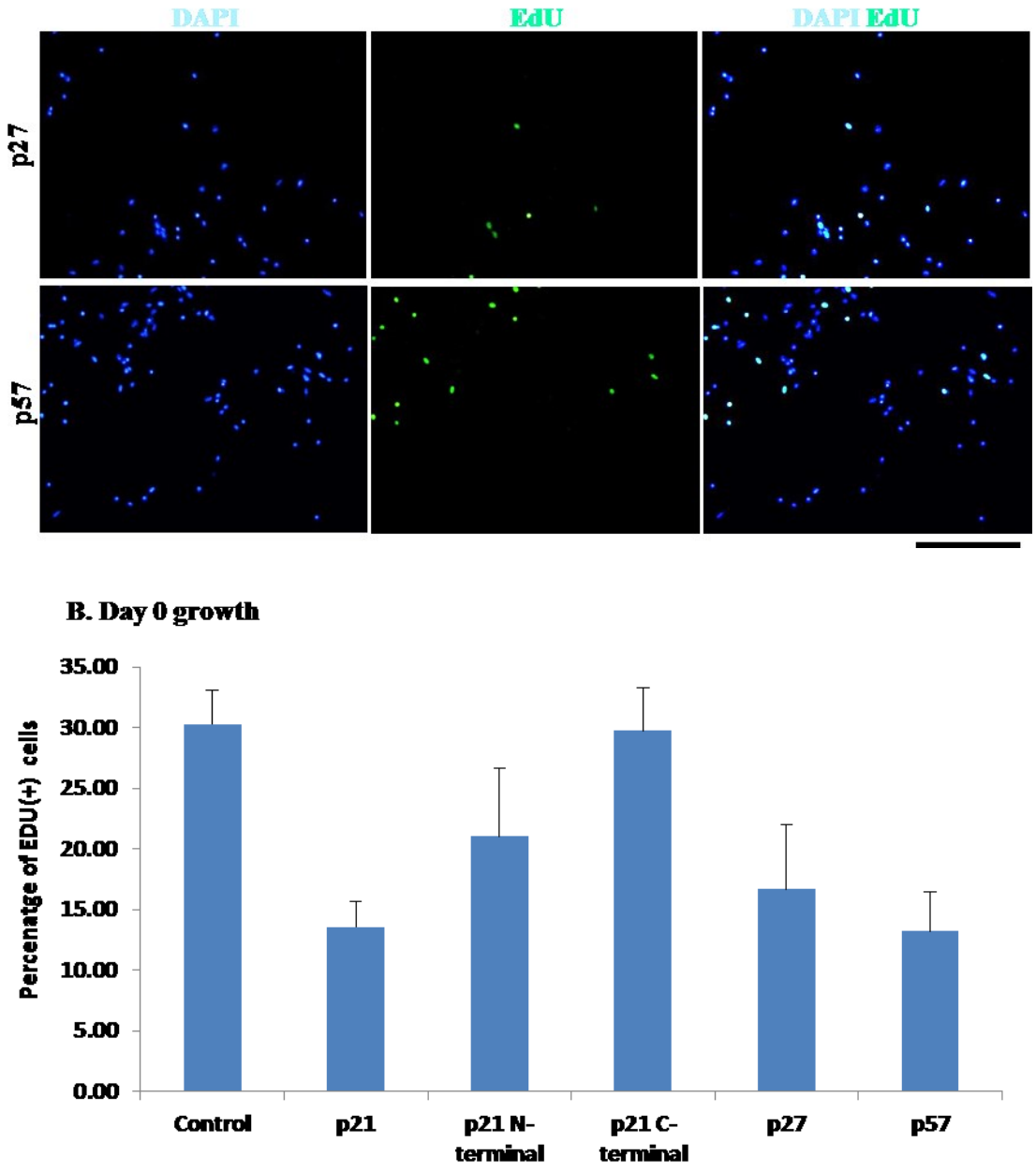
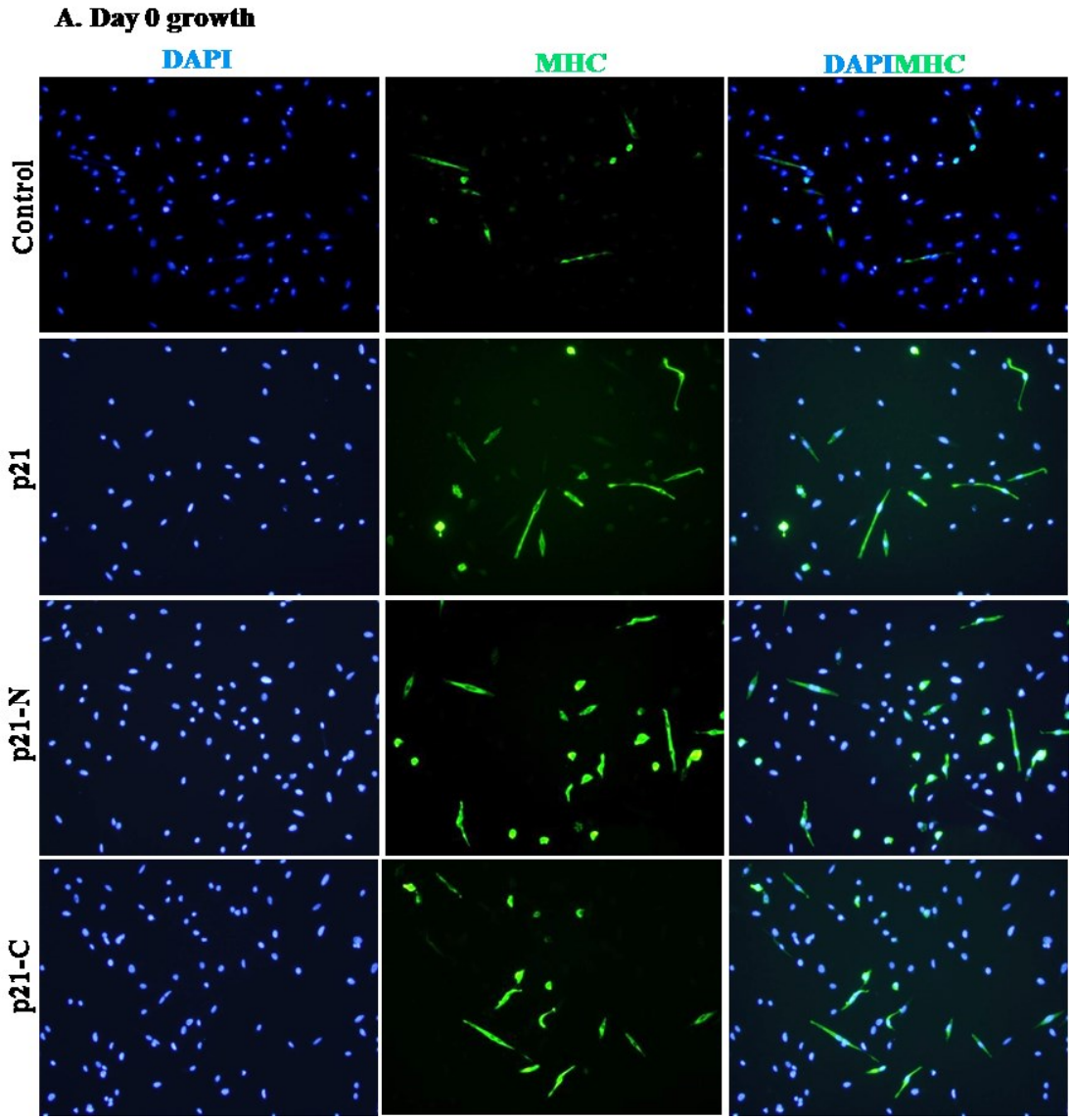


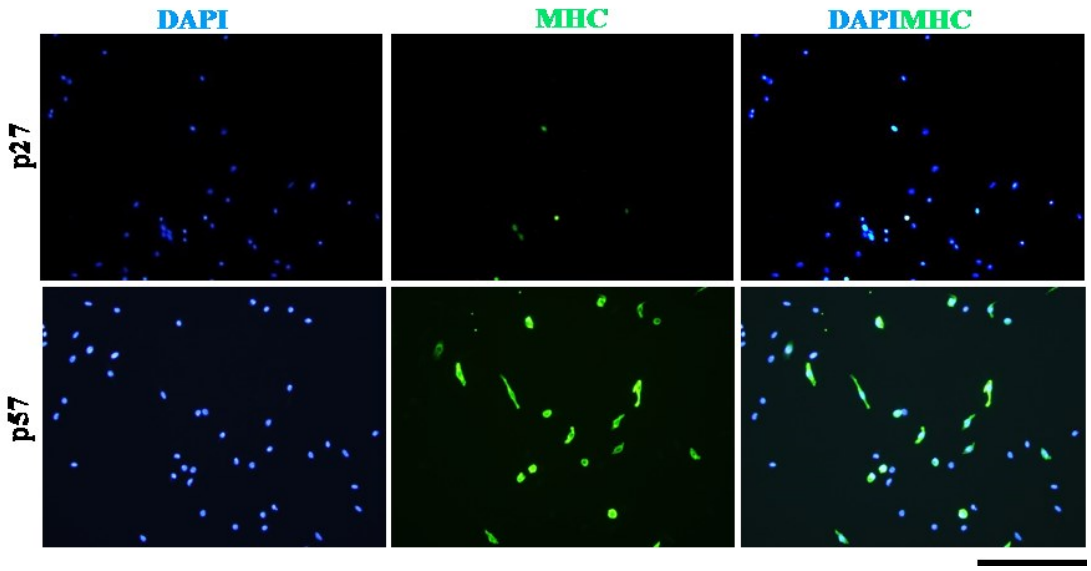
Figure 18. Proliferation was decreased upon over-expression of CDKIs using non-Myc tag vectors. After retroviral infection, myoblasts cultured on growth medium, were incubated with EdU for 3 hours and then fixed for immunostaining. A) Cells were stained for EdU (green) and nuclei were counterstained with DAPI (blue) (n=1). Scale bar = 100µm. B) Graphical representation of the number of EdU(+) cells per nuclei among all samples compared with control (pMX-MT-puro). Error bars = standard error mean.

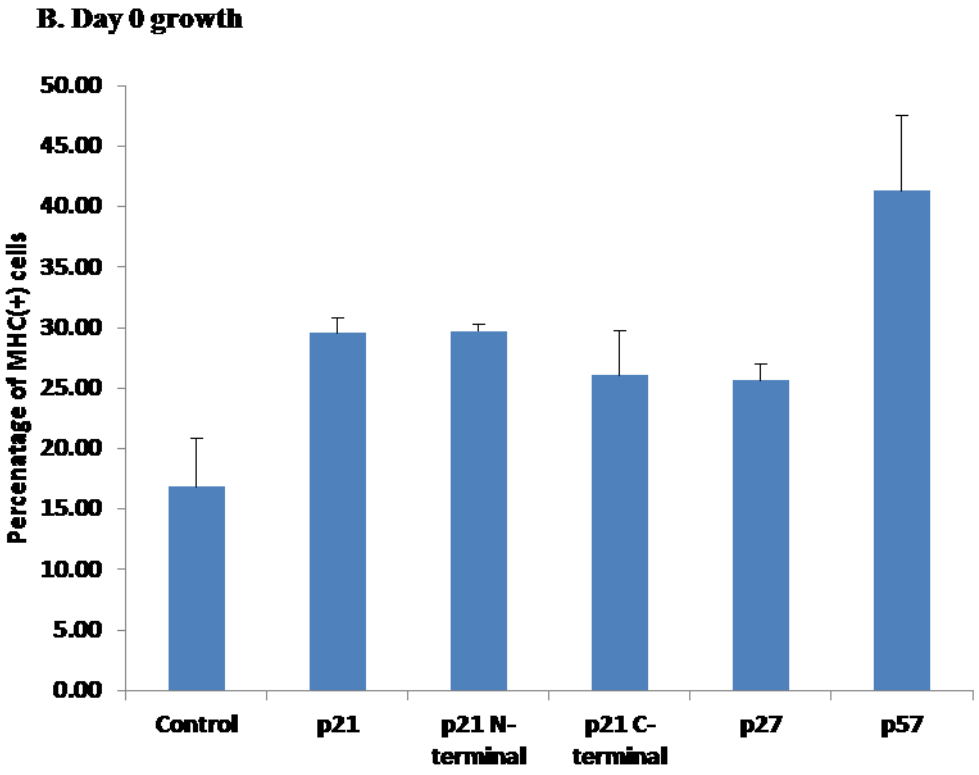
3.6 Differentiation efficiency was increased by the overexpression of non-MT CDKI

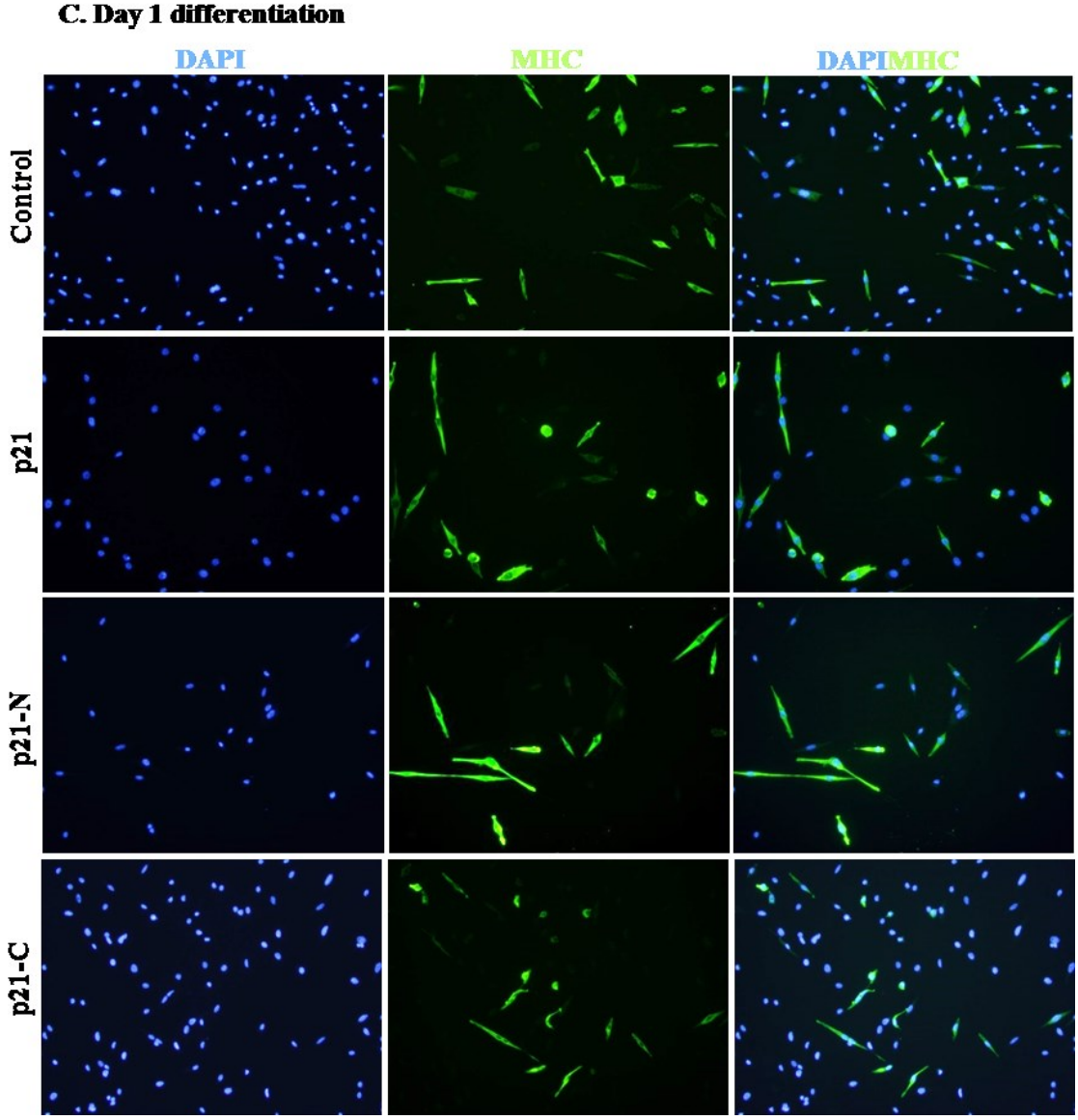
We next investigated the myogenic differentiation efficiency of myoblast culture, by looking at the number of MHC(+) cells, upon overexpression of non-MT CDKIs. For this experiment, we synthesised retroviral supernatants of the non-Myc tag vectors for CDKIs (pMX-p21-puro, pMX-p21-N-puro, pMX-p21-C-puro, pMX-p27-puro and pMX-p57-puro) and infected into myoblasts to look for MHC-positive differentiated muscle cells. We found an increase in the percentage of MHC(+) cells, on all the three culture conditions, upon over-expression of each of the non-MT CDKI, in comparison with the control cells (Fig. 19). Cells over-expressing full length p21 show a steady increase in the percentage of MHC(+) from 29% at day 0 to 54% at day 1 and finally to 65% at day 3, indicating that overexpression of p21 might promote myogenic differentiation, which was consistent with the previously reported works. Interestingly, we found that the cells over-expressing the p21-N demonstrated a number of MHC(+) cells similar to the level of full length p21 amongst all the three time points, compared to the p21-C. Surprisingly, by Day 3, the p21-N over-expressing cells showed a higher percentage of MHC(+) cells (85%), when compared to the cells over-expressing full length p21 (66%) (Fig. 19C, 19F). These data suggest that p21-Nmight be involved in the p21-mediated induction of myogenic differentiation in myoblast culture, in comparison with p21-C. Similarly, cells over-expressing p27 also exhibited a steady increase in MHC(+) cells ranging from 25% at day 0 to 47% at day 1 to 63% at day 3. The percentage of MHC(+) cells in cells over-expressing p57 also coincided with the pattern observed in p21 and p27 over-expressing

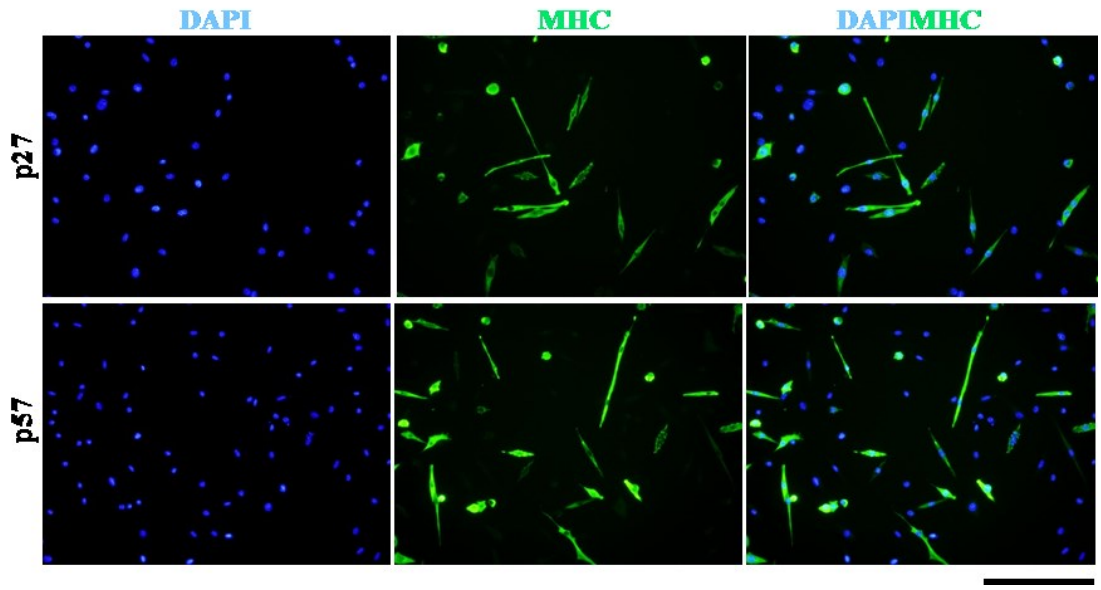
cells, with percentages ranging from 41% at day 0 to 53% at day 1 and 70% at day 3. Overall, removal of Myc tag enhanced the differentiation potential of CDKI when over-expressed in myoblast cultures.

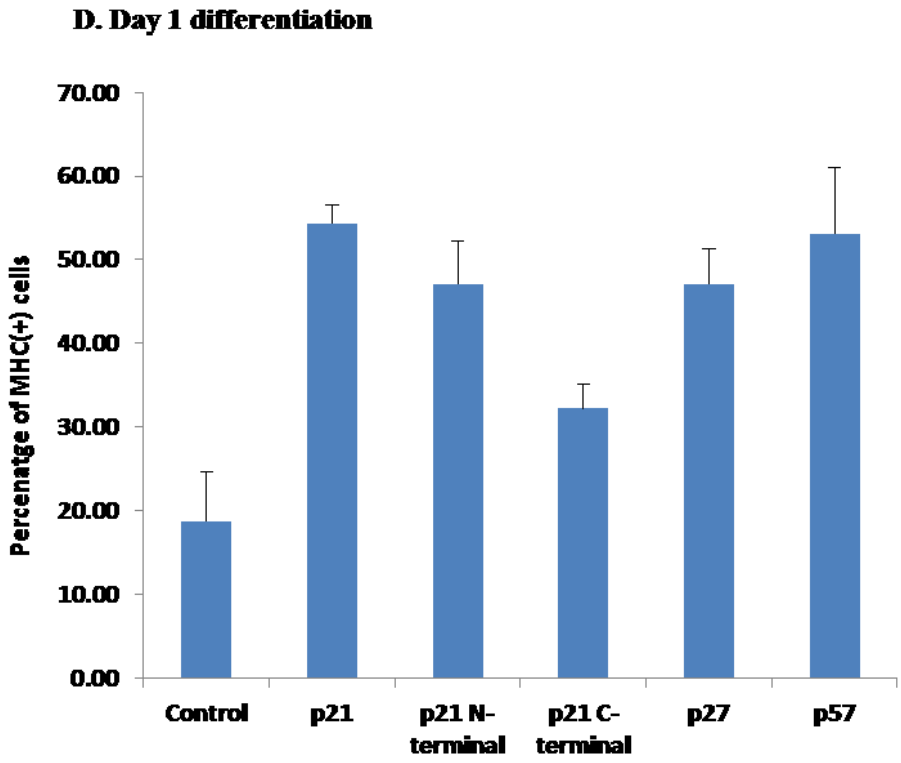


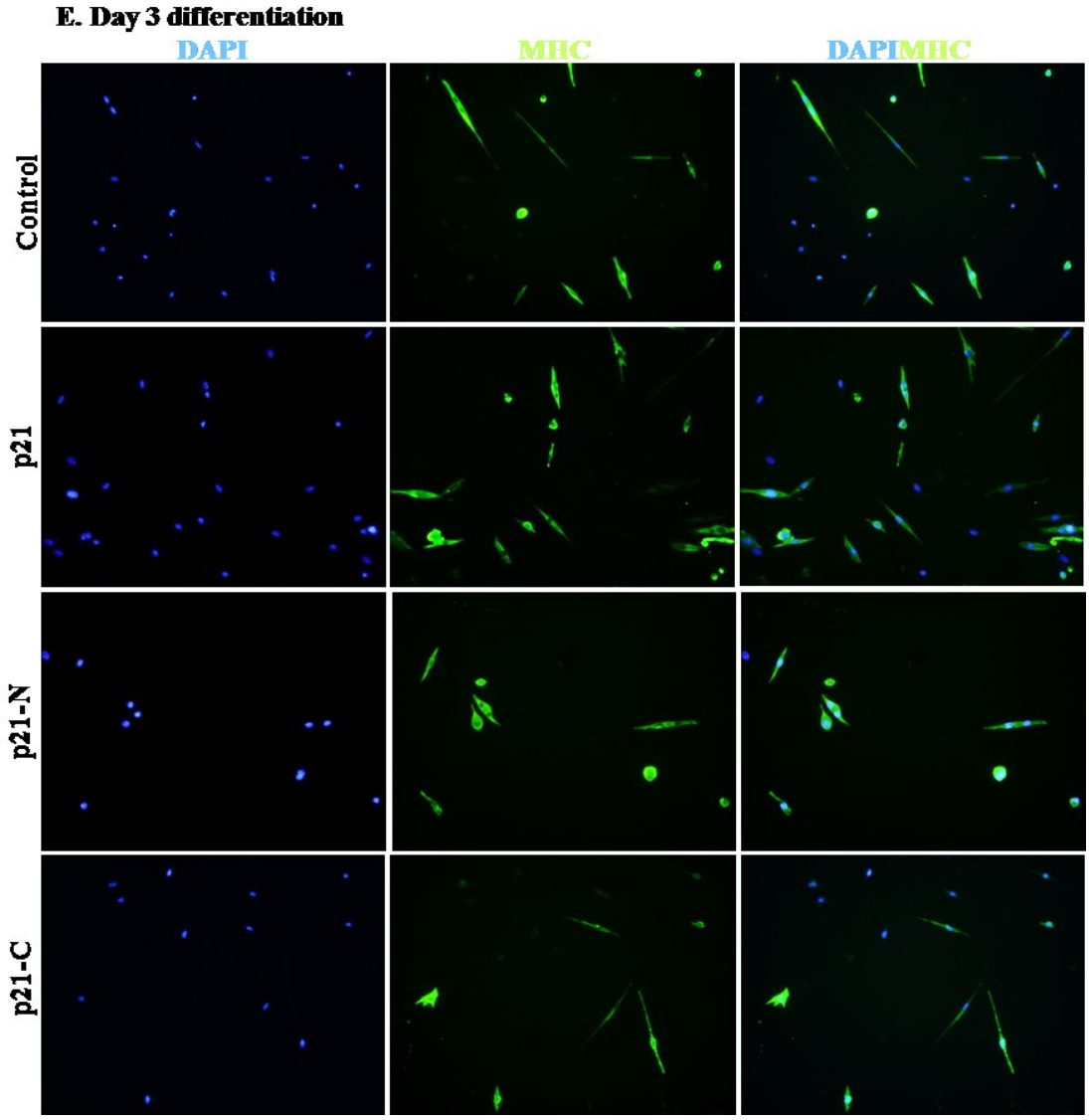

DAPI
MHC
DAPIMHC
p27
p57

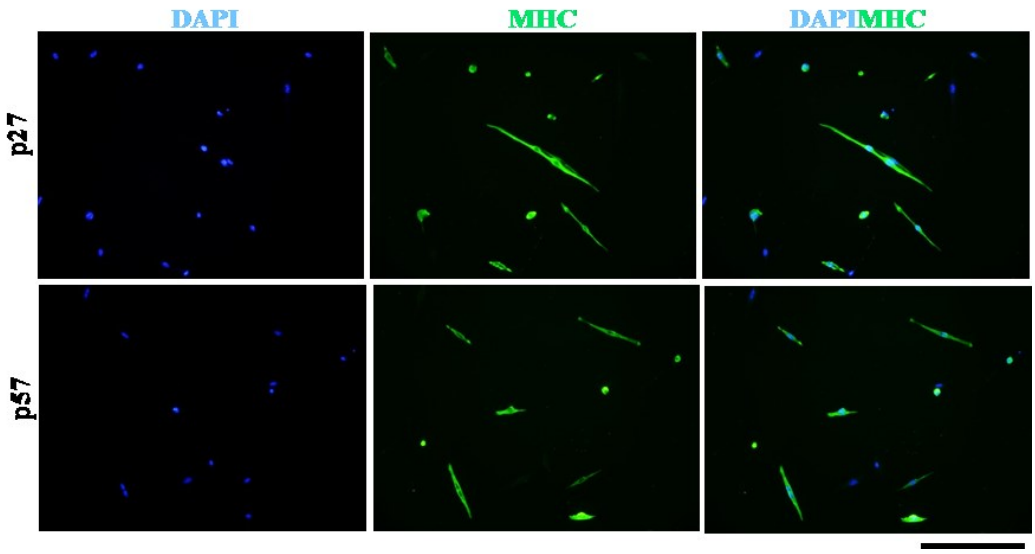

B. Day 0 growth
Percenatage of MHC(+) cells
50.00
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Control
p21
p21 N-terminal
p21 C-terminal
p27
p57


C. Day 1 differentiation
DAPI
MHC
DAPIMHC
Control
p21
p21-N
p21-C


DAPI
MHC
DAPIMHC
p27
p57


D. Day 1 differentiation
Percenatge of MHC(+) cells
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Control
p21
p21 N-terminal
p21 C-terminal
p27
p57


E. Day 3 differentiation
DAPI
MHC
DAPIMHC
Control
p21
p21-N
p21-C



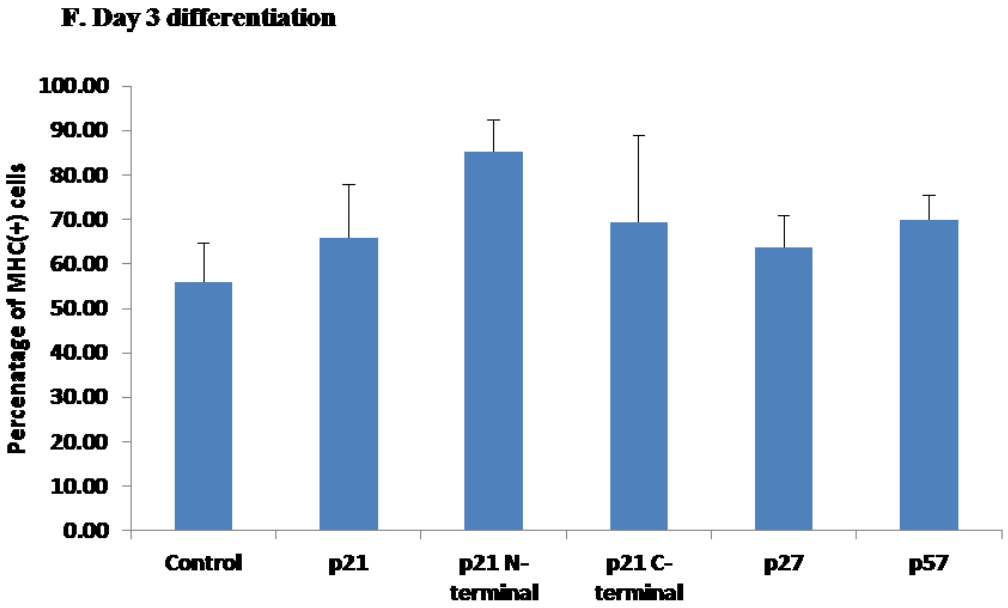


Figure 19.Myogenic differentiation was increased upon over-expression of CDKIs lacking Myc tag. Myoblasts were infected with viral supernatants of non-Myc tag vectors for CDKIs (pMX-p21-puro, pMX-p21-N-puro, pMX-p21-C-puro, pMX-p27-puro and pMX-p57-puro) under growth (day 0) and differentiation (day 1 and day 2) conditions. Cells were stained with MHC (green) and nuclei were counterstained with DAPI (blue) and compared with control cells (pMX-puro) on A) day 0, C) day 1 and E) day 2 (n=1). Graphical representation of the number of MHC(+) cells per DAPI(+) nuclei on B) day 0. D) day 1 and F) day 2. Scale bar = 100µm. Error bars = standard error mean.

4. DISCUSSION

In this study, we have attempted to determine the molecular mechanisms involved in the myogenic differentiation of myoblasts derived from satellite cells isolated from adult skeletal muscle. Cell cycle exit is a necessary event for the induction of terminal differentiation of muscle cells, which is mediated by CDKI. With that knowledge in hand, we started our study by looking into the endogenous levels of the CDKIs, p21, p27 and p57 in the myoblast culture during myogenic differentiation. We started by looking at p21 expression and observed a steady increase in the expression of p21, during myogenic differentiation of myoblasts. Previous work indicated a similar up-regulation of p21 in the C2C12 myoblast differentiation[38]. Similarly, we found an increase in the expression of p27 protein during myogenic differentiation of myoblasts. However, there was not an increase in the number of MHC(+) cells among day 0 (growth medium) and day 1 (Differentiation) in both the experiments, which could be explained by a lower cell density among the two plates. It is known that expression of MHC and myogenic differentiation is influenced by cell density, and the lower cell density could be the possible reason for lower expression of MHC. In a similar way, we found that there was a significant increase in the levels of p57 through the process of myogenic differentiation from day 0 (growth medium) to day 1 and day 2 (differentiation medium), which correlated with the increase in the levels of MHC amongst all the three time points. This observation was similar to previous findings which demonstrated that p57 is up-regulated during myogenic differentiation in the C2C12 cells[39]. We also confirmed this pattern of

expression of the CDKIs with Western Blotting. We checked the expression levels of p27 and p57 proteins, under growth (day 0) and differentiation (day 1, day 3 and day 5) conditions, and found that the levels of p27 and p57 steadily increased, from growth to differentiation conditions. These data suggested that p21, p27 and p57 might play a role in myogenic differentiation.

Asakura lab had already found that upon over-expression of p21 in the myoblasts, there seems to be a reduction in the percentage of Pax7(+)MyoD(-) reserve cells, which is a quiescent satellite cell population observed *in vitro* (unpublished data). On the other hand, muscle from mice lacking p21 gene ($p21^{-/-}$) exhibited an increase in the number of Pax7(+) satellite cells *in vivo* (unpublished data). These observations prompted us to look further in the direction of over-expression of CDKIs and their effect on the satellite cell population. To continue with the previous work, we cloned p21, p27 and p57 into retroviral plasmid vectors containing Myc tag for monitoring the expression of the specific CDKI and its effect on myoblast differentiation. We showed that upon over-expression of MTp21, MTp27 and MTp57 in the myoblasts, the differentiation efficiency decreased, which can be correlated with the decrease in the percentage of MHC(+) cells, amongst all the three time points (day 0, day 1 and day 2). This result was a little surprising as normal myoblasts show an up-regulation in the levels of endogenous CDKIs and MHC, during the process of myogenic differentiation.

To better interpret our results, we also looked at the functions of the Myc tag more closely. Through literature search, we found that Myc gene has the ability to inhibit

the functions of CDKIs. Myc was shown to inhibit the function of p27 by blocking its binding with the CyclinE/Cdk2 complex[43]. In addition, Myc was also involved in the reversal of the growth arrest induced by over-expression of p27 protein[44]. Furthermore, Myc was shown to collaborate with Ras to inhibit the function of p27 by accumulating active CyclinE/Cdk2 complex[47]. Finally, Myc was shown to directly interact with the p21 promoter, controlled by the DNA binding protein Miz-1. Miz-1 interaction blocks p21 induction by p53 and other activators of p21[40]. By looking at these previous works, we had a speculation that the decrease in the differentiation efficiency upon over-expression of Myc-tagged CDKIs, might be due to the Myc-inhibition of the CDKIs. As a confirmation of our speculation, removal of Myc tag enabled an increase in the number of MHC(+) cells, upon over-expressing each of the CDKI, when compared with the control cells. This was consistent at all the three time points (day 0, day 1 and day 3). We also demonstrated that there was a decrease in the proliferating cells upon over-expression of non-MT CDKIs, further supporting that the Myc tag might have been involved in inhibiting the function of the CDKIs.

By looking into the structures of all the three CDKI, we found that the mouse p21 protein has a unique C-terminal domain, containing a *Proliferating cell nuclear antigen* (PCNA) binding domain, which was absent in p27 and p57. PCNA is involved in the activation and processing of DNA polymerase δ and ε[48,49]. PCNA has also been shown be involved in nucleotide excision repair (NER)[50].p21 protein inhibits PCNA-dependent DNA replication *in vitro* by binding to PCNA using its C-terminal domain[51,52,53]. The N-

terminal of all the three CDKI were similar and contained a CDK-inhibitory domain, which binds to cyclin-CDK complexes and inhibits their activity[54]. With this knowledge, we speculated that probably the p21 C-terminal might be the reason for the decrease in the reserve cell population, which was observed *in vitro*. We then constructed retroviral vectors containing either the N-terminal region of p21 or the C-terminal region. We showed that the p21 N-terminal increased the percentage of MHC(+) cells under differentiation conditions and not the C-terminal region, when compared with the control cells. These data suggest that the p21 N-terminal might have higher contribution in the induction of myogenic differentiation, when compared with the C-terminal region. On the whole, this study throws light on two speculations: 1) Myc tag might have an inhibitory function on the CDKIs when fused, leading to a decrease in the number of MHC(+) cells during myogenic differentiation, possibly due to a dominant-negative effect. 2) The N-terminal region (CDK inhibitory domain) of p21 might have a higher contribution towards the induction of differentiation in the myoblasts, when compared with C-terminal of p21. All the observations were derived by performing each of the experiments once (n=1). These experiments have to be repeated to obtain a significant difference.

Further studies would be directed towards looking at the Pax7(+)MyoD(-) reserve cells in the myoblast culture upon over-expression of the CDKI. Previous study had shown that MyoD can transcriptionally up-regulate the expression of p21 through binding to p21 upstream regulatory regions[38]. Thus, down-regulation of MyoD in the reserve cells may be essential for down-regulation of p21 in the cells. Asakura lab showed that over-

expression of p21 suppresses reserve cells in the myoblast culture (unpublished data). For the future experiments, we would examine whether over-expression of p21-N, p21-C, p27 and p57 suppresses reserve cells in the myoblast cultures. We also plan to construct mutant forms of p21 N-terminal and C-terminal domain, to look at the effect of those constructs in the myoblasts. Since this study indicates the involvement of domain-specific interaction of p21, in the promotion of differentiation, looking at the binding partners of p21 N-terminal would throw more light on the exact genes involved in the differentiation process. Previous study has shown that MyoD can bind to p21 upstream regulatory regions and transcriptionally upregulate its expression[38]. Since the decision to undergo self-renewal or differentiation involves cyclin-dependent kinase inhibitors, this study helps in understanding the molecular mechanisms involved in that decision in myoblasts. These molecular mechanisms would also contribute to better understand the satellite cell differentiation and self-renewal, thus helping us to manipulate them for therapeutic use in muscle diseases such as Duchenne Muscular Dystrophy (DMD). Studies have also indicated that introducing antisense oligonucleotides for p21 and p57 enhances the cell proliferation in primary human skeletal muscle culture[55]. This could be used as a possible therapy for DMD patients to enhance their regeneration potential.

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