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Nutlin-3 down-regulates retinoblastoma protein expression and inhibits muscle cell differentiation

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ABSTRACT

The p53 tumor suppressor gene plays a critical role in regulation of proliferation, cell death and differentiation. The MDM2 oncoprotein is a major negative regulator for p53 by binding to and targeting p53 for proteasome-mediated degradation. The small molecule inhibitor, nutlin-3, disrupts MDM2-p53 interaction resulting in stabilization and activation of p53 protein. We have previously shown that nutlin-3 activates p53, leading to MDM2 accumulation as concomitant of reduced retinoblastoma (Rb) protein stability. It is well known that Rb is important in muscle development and myoblast differentiation and that rhabdomyosarcoma (RMS), or cancer of the skeletal muscle, typically harbors MDM2 amplification. In this study, we show that nutlin-3 inhibited myoblast proliferation and effectively prevented myoblast differentiation, as evidenced by lack of expression of muscle differentiation markers including myogenin and myosin heavy chain (MyHC), as well as a failure to form multinucleated myotubes, which were associated with dramatic increases in MDM2 expression and decrease in Rb protein levels. These results indicate that nutlin-3 can effectively inhibit muscle cell differentiation.

1. Introduction

The tumor suppressor p53 plays a crucial role in protection from carcinogenesis by regulating numerous cellular processes, including cell cycle progression, apoptosis, metabolic homeostasis, antioxidant defense, DNA repair and senescence. Under physiological condition, p53 protein levels are maintained low by MDM2-mediated protein degradation. Upon cellular stresses, however, the p53-MDM2 interaction is inhibited, leading to p53 accumulation and activation of downstream responses. Importantly, p53 can potently up-regulate MDM2 gene expression, forming a negative feedback regulatory loop that restrains p53 activity [1].

The specificity of the p53-MDM2 interaction has prompted the development of a family of small molecule inhibitors termed nutlins, which fit in the hydrophobic p53-binding pocket in the MDM2 N-terminal domain and disrupt p53-MDM2 interaction [2]. Treatment of cancer cells with nutlins has been shown to activate the p53 pathway and to promote cell cycle arrest, premature cellular senescence and apoptosis [3,4]. Therefore, nutlins hold good promise for the development of targeted cancer therapies against cancers harboring wild type p53 and MDM2 amplification. One of these types of cancer is rhabdomyosarcoma (RMS), a primarily pediatric malignancy of the skeletal muscle [5]. Indeed, MDM2 amplification and wild type p53 retention has been found in numerous RMS cases exhibiting a non-differentiated phenotype [6–10].

In addition to its role in regulating p53 levels, MDM2 has been shown to possess oncogenic functions that are independent of p53. Notably, MDM2 directly binds to the Retinoblastoma protein (Rb) via its central acidic domain, thus inhibiting Rb growth suppressive function and targeting Rb to proteasomal degradation [11,12]. Rb is an important tumor suppressor that plays pivotal roles in a number of biological processes, including cell cycle control, DNA damage response, senescence and apoptosis [13]. A key function of Rb is to bind and inhibit E2F transcription factors, which would otherwise induce expression of genes that enhance cell cycle progression [14]. In addition, Rb plays a major role in development and muscle cell differentiation, as evidenced from the severe deficiencies in skeletal muscle development exhibited in Rb-null mice [15–17]. Rb promotes muscle differentiation at multiple stages of myogenesis

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by a variety of mechanisms, as shown by studies in which Rb deficiency results in inhibition of myoblast differentiation [18–20]. For instance, negative regulation of E2F-mediated gene expression by Rb induces permanent cell cycle withdrawal, which is required for terminal differentiation [21,22]. Moreover, Rb has been shown to promote activation of myogenic regulatory factors (MRFs), including MyoD, a basic helix-loop-helix (bHLH) transcription factor, and Myocite Enhancer Factor 2 (MEF2), thereby inducing expression of muscle-specific genes, such as Myosin Heavy Chain (MyHC) [23,24].

Since histological analyses of RMS cases usually reveal undifferentiated myoblastic phenotypes [25], we investigated the effects of nutlin treatment on muscle cell differentiation. We found that nutlin treatment led to significant up-regulation of MDM2 and down-regulation of Rb protein levels, and a blockage of myoblast proliferation and differentiation.

2. Materials and methods

2.1. Cell culture and drug treatment

C2C12 mouse myoblasts were maintained in growth media Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin G and streptomycin sulfate. Rat L6 myoblasts were cultured in DMEM supplemented with 10% FBS and 1% penicillin G and streptomycin sulfate. Cells were maintained at 37 °C under 5% CO_2 in a humidified incubator. For induction of differentiation, 4.2×10^5 C2C12 cells or 10.8×10^5 L6 cells were plated in 100 mm dishes and grown in normal growth media for 24 h. Then, the media were replaced with differentiation media, which were supplemented with 2% horse serum (C2C12) or 2% calf serum (L6) instead of FBS. Cells were treated with 10 μM nutlin-3 or vehicle (DMSO), as indicated. Media were replaced with fresh media with or without nutlin-3 every 48 h.

2.2. Western blot analysis

Whole cell lysates were prepared using EBC_{250} lysis buffer (50 mM Tris–HCl pH 8.0, 250 mM NaCl, 0.5% Nonidet P-40, 1 mM PMSF, 2 μg/mL aprotinin, 40 mM NaF, and 0.5 mM $NaVO_4$). Equal amounts of total protein were separated by SDS-PAGE and then transferred onto PVDF membranes and hybridized to a specific primary antibody and HRP-conjugated secondary antibody for subsequent detection by ECL (Pierce). Primary antibodies and dilutions used: Rb 1:500 (G3-245, BD Bioscience); myogenin 1:400 (F5D, Santa Cruz); actin 1:1000 (C-11, Santa Cruz); murine MDM2 (mixture of 2A10, 4B2, and 4B11; 1.0 mL each in 20 mL), and MyHC 1:400 (F-20, DSHB). Secondary antibodies were purchased from Santa Cruz Biotechnology and used at 1:3000 dilutions, including goat anti-mouse IgG-HRP (sc-2005), goat anti-rabbit IgG-HRP (sc-2004), and donkey anti-goat IgG-HRP (sc-2020).

2.3. Immunofluorescence

For immunofluoresence, 7.0×10^4 C2C12 cells or 1.8×10^5 L6 cells were grown on glass microscope coverslips in 35 mm plates, each containing 3 coverslips. Twenty-four hours after plating, cells were fixed with cold methanol and acetone (1:1) for ten minutes and air-dried overnight. The samples were blocked for one hour at room temperature with PBS containing 5% bovine serum albumin (BSA, Fisher), incubated for one hour at 37 °C with antibody specific for Myosin Heavy Chain (1:200 dilution), washed three times with PBS, and then incubated with a secondary antibody (anti-mouse IgG-Alexa488, Invitrogen) at 1:200 dilution for one hour at room temperature. The cells were washed with PBS, counterstained with propidium iodide (500 nM in water) for one minute and then washed three more times with PBS. Glass coverslips were mounted onto microscope slides with Prolong Antifade (Invitrogen). Fluorescent images were captured using an inverted Axiovert 200M microscope (Carl Zeiss) with a CCD ORCA-ER camera (Hamamatsu).

3. Results

3.1. Nutlin-3 blocks myoblast cell differentiation

The small molecule nutlin-3 is a potent inhibitor of the MDM2-p53 interaction. Since p53 promotes MDM2 expression as part of a negative-feedback regulatory loop, and MDM2 has been shown to induce Rb degradation, which is known to play an important role in muscle differentiation, we investigated the effects of nutlin-3 on muscle cell differentiation. To this end, we used rat myoblast L6 cells and induced differentiation into myotubes using differentiation medium (DM) in the presence or absence of nutlin-3. Myoblast differentiation into myotubes is characterized by strong expression of myogenin, an early differentiation marker, and myosin heavy chain (MyHC), a late differentiation marker, and it culminates with cell fusion to form multi-nucleated myotubes [26]. As shown in Fig. 1A, control L6 cells remained proliferative within the first 48 h in differentiation medium, as evidenced from the cell density gradually reaching confluency. Cell differentiation first became evident after 24 h, at which point a number of single cells stained positive for MyHC. After 48 h, a large portion of cells exhibited phenotypic characteristics of myotubes, including MyHC expression and multiple nuclei. By 72 h, the majority of the cells had terminally differentiated into myotubes, which were usually large with a dramatic increase in the number of nuclei. On the other hand, nutlin-3 treatment notoriously down-regulated cell proliferation, and dramatically inhibited myoblast differentiation. Indeed, only a minimal number of cells displayed MyHC staining after 72 h of exposure to DM, and there was virtually no morphological evidence of myotube formation. Moreover, while control cells exhibited marked up-regulation of Myogenin expression as early as 24 h post exposure to DM that was maintained through 72 h, cells treated with nutlin-3 exhibited only modest Myogenin up-regulation that declined back to basal levels through the course of the treatment. Similarly, MyHC expression was markedly down-regulated in cells treated with nutlin-3, compared to control cells. Notably, MDM2 protein levels in control cells were not affected by differentiation, and remained low throughout the course of the experiment. By sharp contrast, nutlin-3 treatment led to a dramatic MDM2 up-regulation within 24 h that was maintained through the course of the experiment, likely due to increased transcription of the MDM2 gene by up-regulated p53 (Fig. 1B).

Next, we explored whether the effect of nutlin-3 on myoblast differentiation is cell type specific. Thus, we induced differentiation in the presence or absence of nutlin-3 in C2C12 mouse myoblasts and observed for an extended period of seven days. Consistent with our previous results, cells treated with DM continued to proliferate, exhibited MyHC expression, and fused to form elongated, multi-nucleated myotubes. Conversely, in the presence of nutlin-3, cell proliferation was greatly inhibited and MyHC was not detectable by immunostaining even after seven days (Fig. 1C). As shown in Fig. 1D, western blot analyses revealed that induction of Myogenin and MyHC expression was greatly down-regulated in nutlin-3-treated cells, compared to control cells, while MDM2 expression was strongly up-regulated. Moreover, Rb protein levels in control cells were maintained at a near constant level. On the other hand, nutlin-3 treatment resulted in dramatically reduced Rb protein levels. These data indicate that nutlin-3 not only inhibits cell proliferation, but also effectively blocks myoblast cell differentiation.

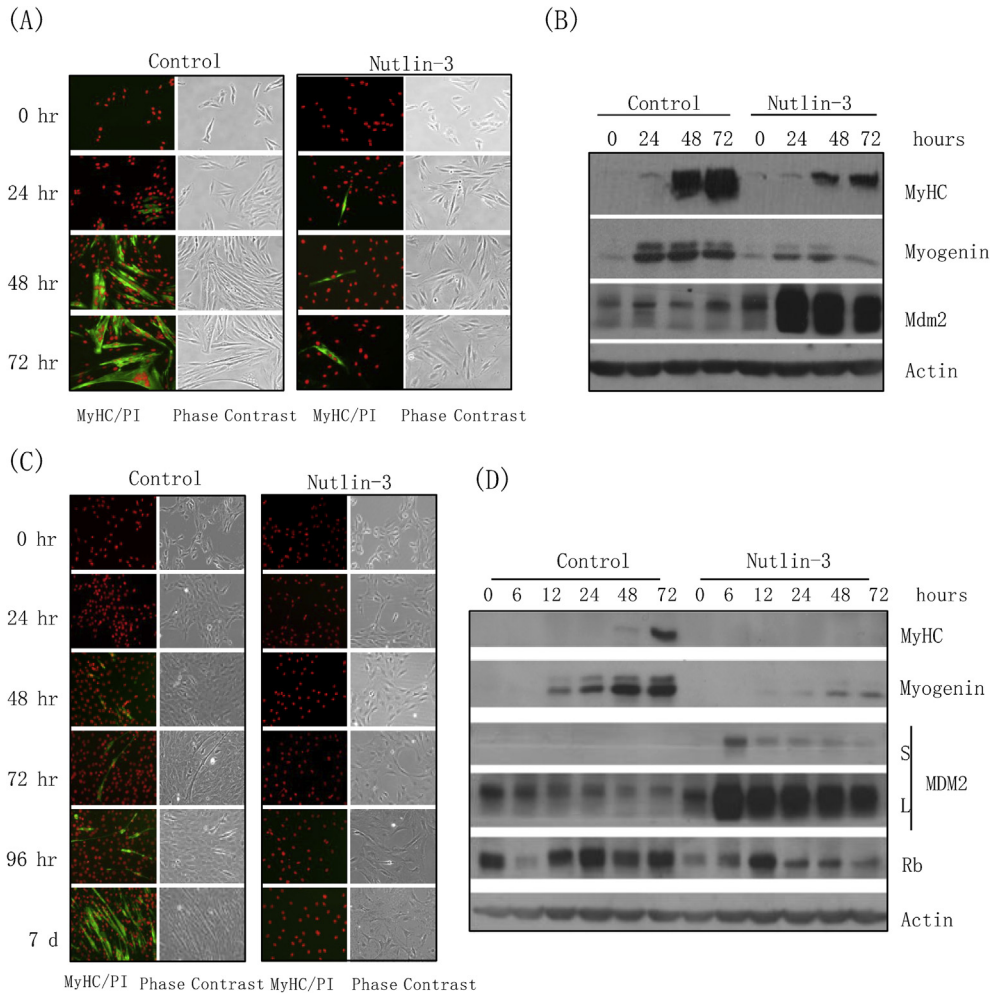

Fig. 1. Nutlin-3 inhibits L6 and C2C12 cells proliferation and differentiation, correlated with down-regulation of Rb. Rat myoblast L6 and mouse myoblast C2C12 cells were grown in DMEM containing 10% FBS. L6 cells were induced to differentiation in DMEM containing 2% calf serum in the presence or absence of 10 μM nutlin-3 for 24, 48 or 72 h. C2C12 cells were induced to differentiate in DMEM containing 2% horse serum in the presence or absence of 10 μM nutlin-3 for 24, 48, 72, 96 h or 7 days. DMSO was used as a vehicle control. (**A**, **C**) L6 (A) and C2C12 (C) cells were immunostained for myosin heavy chain (MyHC) and counterstained with propidium iodide (PI). Images were captured by fluorescence or phase contrast microscopy. (**B**, **D**) Whole-cell lysates were subjected to western blot analyses, as indicated. S = Short exposure; L = Long exposure.

3.2. Short-term nutlin-3 treatment blocks proliferation and differentiation

Since prolonged nutlin-3 treatment dramatically blocks muscle cell differentiation, we were interested in determining the effects of short-term treatment with nutlin-3. To this effect, we examined the ability of myoblasts to recover from acute exposure to nutlin-3 treatment. We subjected C2C12 cells to differentiation conditions with or without nutlin-3 for 24 h, after which time we removed nutlin-3 and added fresh DM back to the cells. As shown in Fig. 2, acute nutlin-3 treatment significantly delayed proliferation and differentiation, compared to control cells. However, removal of nutlin-3 from the DM allowed differentiation to proceed. These results suggest that nutlin-3 inhibits myoblast commitment to differentiation, and indicate that this inhibition is not irreversible, since continued exposure is required to prevent differentiation into myotubes.

3.3. Nutlin-3 blocks the progression of muscle differentiation

Thus far, we have shown that nutlin-3 inhibits the initiation of differentiation by treating myoblasts simultaneously with DM and nutlin-3. Next, we examined the effect of nutlin-3 on the progression of myoblasts through differentiation by adding nutlin-3 after the cells had already been subjected to differentiation conditions. To this end, we induced differentiation in C2C12 cells, and added nutlin-3 24 h after addition of DM. As shown in Fig. 3A, addition of nutlin-3 at 24 h post-differentiation resulted in markedly reduced differentiation, as evidenced by decreased MyHC staining, compared to control cells. Likewise, MyHC expression was markedly down-regulated in nutlin-3-treated cells, as assessed by western blotting (Fig. 3B). In addition, nutlin-3 strongly up-regulated MDM2 and down-regulated Rb. Moreover, DM induced the early differentiation marker Myogenin within 24 h. Notably, in control cells Myogenin levels continued to increase throughout the course of the experiment. On the other hand, addition of nutlin-3 after 24 h halted Myogenin up-regulation (Fig. 3B).

Next, we examined whether nutlin-3 affects myoblast differentiation when added 48 post-induction of differentiation, when most cells are already committed to differentiation. As show in Fig. 4A, MyHC up-regulation and differentiation, which were underway at 48 h, was halted by addition of nutlin-3, as evidenced by no changes in the levels of MyHC staining. Again, nutlin-3 led to increased MDM2 levels and Rb down-regulation. Importantly,

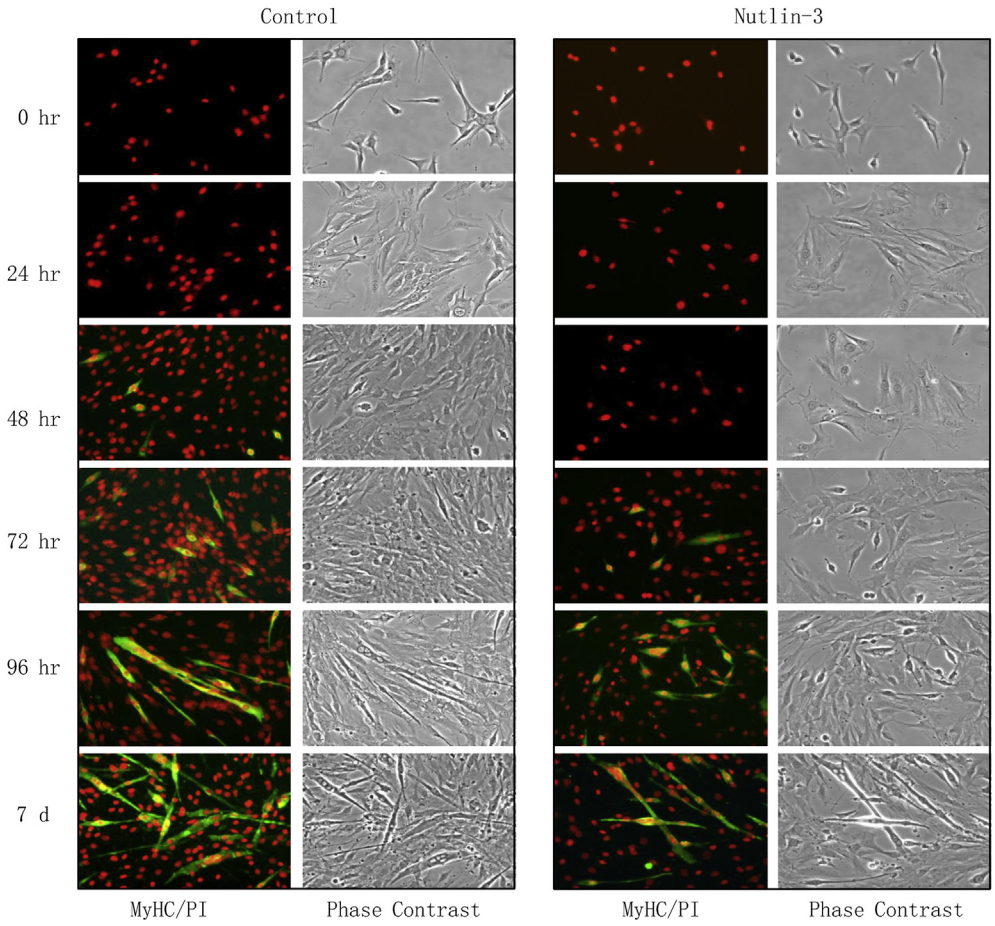

Fig. 2. Short-term treatment with nutlin-3 blocks C2C12 proliferation and differentiation. C2C12 cells were induced to differentiate in DMEM containing 2% horse serum in the presence or absence of 10 μM nutlin-3 for 24 h. DMSO was used as a vehicle control. Cells were washed twice with PBS and were then maintained in differentiation media for the indicated times. Cells were immunostained for myosin heavy chain (MyHC) and counterstained with propidium iodide (PI). Images were captured by fluorescence or phase contrast microscopy.

Myogenin levels, which were reaching strong up-regulation by DM, were down-regulated after addition of nutlin-3 (Fig. 4B). Together, these data indicate that nutlin-3 strongly inhibits progression through the differentiation process, even after initial commitment.

4. Discussion

Targeted cancer therapies are designed to be highly effective at reducing tumor size, while minimizing side effects on normal tissues. As a potential chemotherapy drug, it is important to examine the effects of nutlin-3 on different cell types. In this study, we showed that both long-term and short-term nutlin-3 treatment block myoblast differentiation, which is concomitant with up-regulated MDM2 and markedly lower Rb protein levels. Importantly, we had previously shown that MDM2 induces Rb degradation in an ubiquitin-independent, but proteasome-dependent mechanism [11,27]. Since nutlin-3-bound MDM2 retains its ability to bind Rb via its central acidic domain and to promote Rb degradation, these results suggest that nutlin-3-mediated p53 up-regulation induces MDM2 expression, which leads to increased Rb degradation and impaired myoblast differentiation.

Nutlin-3 has been suggested as a treatment for rhabdomyosarcomas (RMS) with MDM2 amplification. Of note, it has been shown in xenograft studies using human RMS cell lines that standard chemotherapy treatment leads to a reduction of tumor size, but not to a complete elimination of the tumor, thus highlighting the need for developing more efficacious therapies against RMS [28]. For example, treatment of RMS cell lines with nutlin-3 was shown to induce cell cycle arrest and p53-dependent apoptosis [29]. Similarly, MI-63, a different small molecule inhibitor of the p53-MDM2 interaction that also fits in the p53-binding pocket in MDM2, was also shown to effectively impair cell proliferation and viability of RMS cells [30]. More recently, a newer generation nutlin family member termed RG7112 was shown to be a potent inhibitor of tumor growth in pre-clinical mouse xenograft models, especially in osteosarcoma cells with amplified MDM2 expression [31]. In addition, RG7112 was independently shown to inhibit RMS tumor growth and enhance survival of xenograft-bearing mice [32]. Nevertheless, our results indicate that nutlin-3 treatment disrupts myogenesis, therefore suggesting that there may be potential complications for clinical application.

In this study, we found that nutlin-3 treatment inhibits both initiation of differentiation, as well as progression through the differentiation program. Notably, these effects were accompanied by up-regulated MDM2 expression and a dramatic down-regulation of Rb protein levels. These observations are consistent with our previous study showing that nutlin-3 treatment of cancer cells leads to marked reductions in Rb protein levels [33]. Since it has been shown that Rb is required for myoblasts to exit the cell cycle and initiate differentiation [19], it is likely that nutlin-3 inhibits myoblast differentiation via down-regulation of Rb protein by MDM2. Surprisingly, nutlin-3 still blocked differentiation when

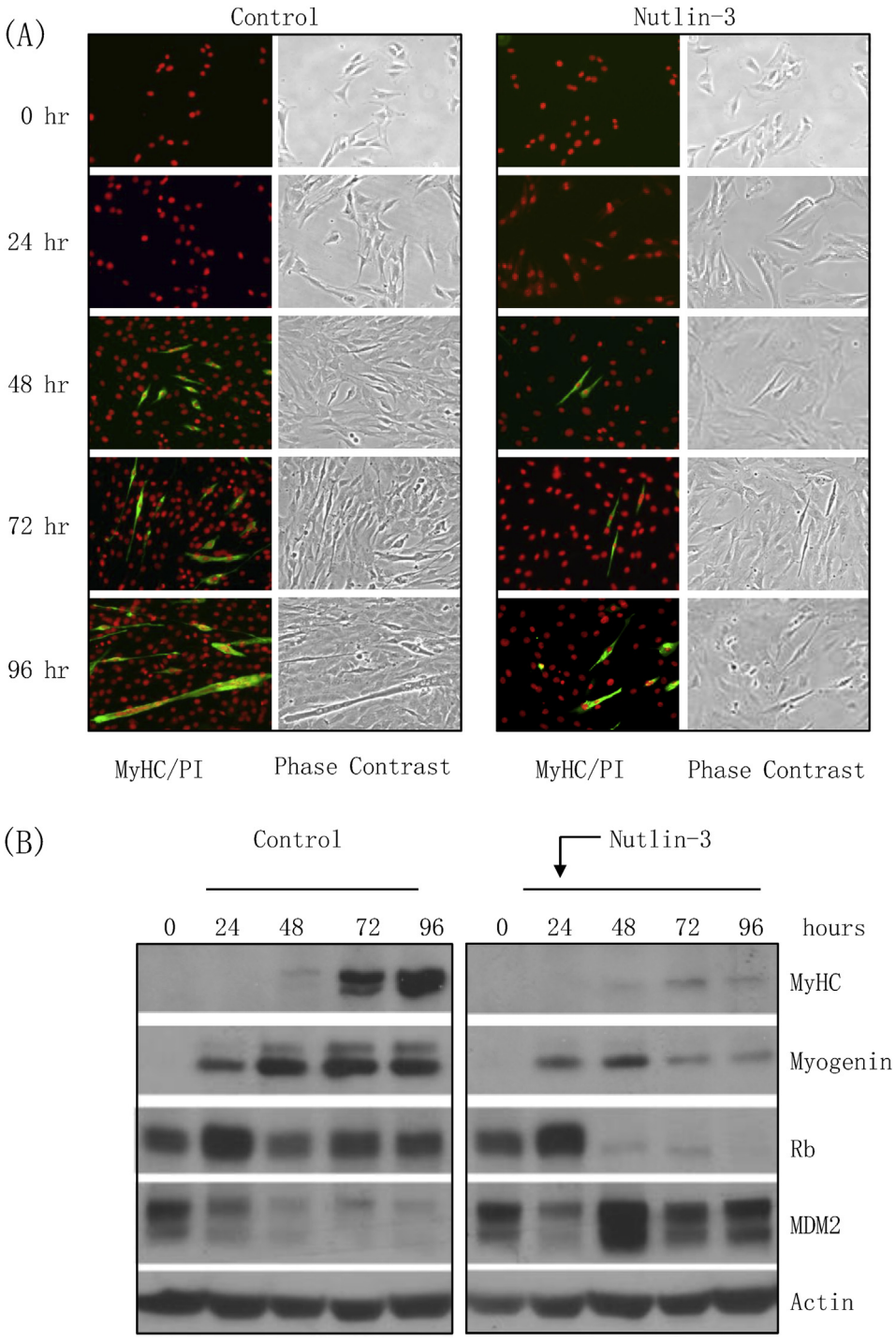

Fig. 3. Nutlin-3 treatment 24 h post-differentiation dramatically inhibits C2C12 differentiation, correlated with down-regulation of Rb. C2C12 cells were subjected to differentiation conditions in DMEM containing 2% horse serum for 24 h prior to the addition of 10 μM nutlin-3. DMSO was used as a vehicle control. (**A**) At the indicated times, cells were immunostained for myosin heavy chain (MyHC) and counterstained with propidium iodide (PI). Images were captured by fluorescence or phase contrast microscopy. (**B**) Whole-cell lysates were subjected to western blot analyses, as indicated.

added 48 h after differentiation, thus indicating that Rb is required not only for exit from the cell cycle, but also for induction of the muscle differentiation program, as has been previously shown [23,24]. Analogously, low MDM2 levels have been shown to be important for maintaining myoblast differentiation potential, as MDM2 over-expression inhibits myoblast differentiation [34]. On the other hand, the role of p53 in muscle differentiation is not yet clear. While $p53^{-/-}$ mice have normal skeletal muscle development [35], it has been shown that activation of p53 promotes the differentiation of myogenic lineages [36]. In addition, it was shown that p53 directly regulates Rb gene transcription and thereby MyoD activity during initiation of differentiation in C2C12 cells, and that p53 deficiency prevents differentiation, which can be rescued by reintroducing Rb expression [37].

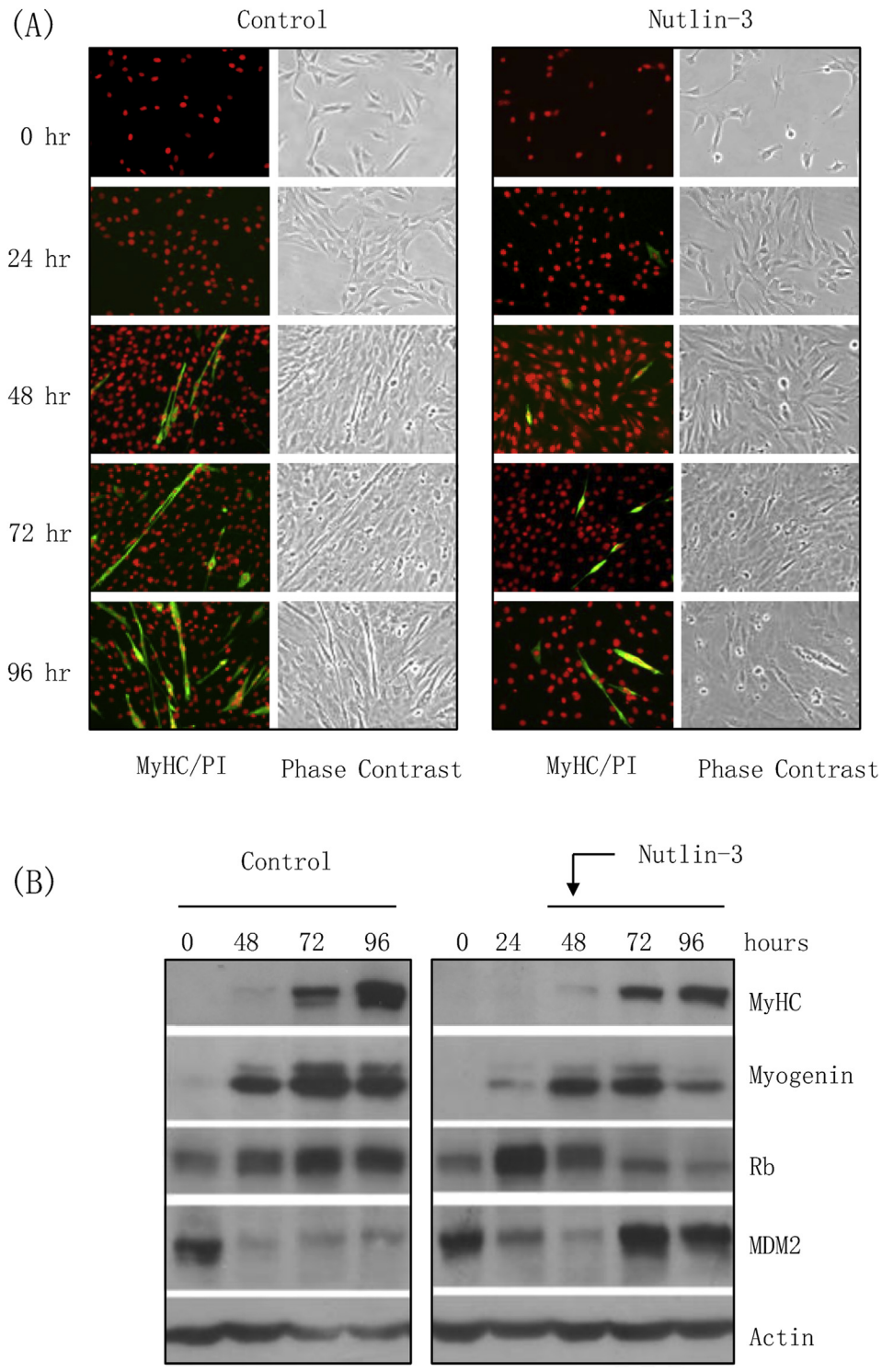

Fig. 4. Nutlin-3 treatment 48 post-differentiation partially inhibits C2C12 differentiation at later stage of differentiation. C2C12 cells were subjected to differentiation conditions in DMEM containing 2% horse serum for 48 h and then the media was changed to differentiation media in the presence or absence of 10 μM nutlin-3 for an additional 48 h. DMSO was used as a vehicle control. (**A**) The cells were analyzed by immunostaining for MyHC and counterstained with PI. Images were captured by fluorescence or phase contrast microscopy. (**B**) Whole-cell lysates were subjected to western blot analyses, as indicated.

Nutlin-3 has been found to affect differentiation of other cell types. For example, nutlin-3 was shown to inhibit pre-osteoclast proliferation and differentiation in a p53-dependent manner [38]. On the other hand, nutlin-3 induced apoptosis in primary acute myeloid leukemia and promoted maturation of the surviving cells [39]. In addition, nutlin-3 has been shown to induce rapid differentiation of embryonic stem cells [40]. Interestingly, it was shown that short-term nutlin-3 treatment of p53 wild-type osteosarcoma U2-OS and colon adenocarcinoma HCT116 cancer cells can lead to polyploidy and drug resistance [41], thus suggesting that

polyploidy in myoblast satellite cells could potentially lead to sporadic RMS.

Conflict of interest

None.

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