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Cyclin D3 Promotes Myogenic Differentiation and Pax7 Transcription

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ABSTRACT

Differentiation of skeletal muscle myoblasts involves activation of muscle-specific markers such as MyoD, Myf5, MRF4, and myogenin, followed by exit from the cell cycle, expression of structural proteins, and fusion into multinucleated myotubes. Cyclin D3 is upregulated during muscle differentiation, and expression of cyclin D3 in proliferating myoblasts causes early activation of myogenesis. In this study, we have identified the genes activated by cyclin D3 expression in C2C12 myoblasts and differentiated cells by real-time PCR analysis. Cyclin D3 expression induced faster differentiation kinetics and increase in levels of myogenic genes such as MyoD, Myf5, and myogenin at an early stage during the differentiation process, although long-term myogenic differentiation was not affected. Transcript levels of the transcription factor Pax7 that is expressed in muscle progenitors were enhanced by cyclin D3 expression in myoblasts. Components of a histone methyltransferase complex recruited by Pax7 to myogenic gene promoters were also regulated by cyclin D3. Further, the Pax7 promoter was upregulated in myoblasts expressing cyclin D3. Myoblasts that expressed cyclin D3 showed moderately higher levels of the cyclin-dependent kinase inhibitor p21 and were stalled in G2/M phase of the cell cycle. Our findings suggest that cyclin D3 primes myoblasts for differentiation by enhancing muscle specific gene expression and cell cycle exit. J. Cell. Biochem. 113: 209–219, 2012. © 2011 Wiley Periodicals, Inc.

KEY WORDS: CYCLIN D3; PAX7; MYOGENIN; HISTONE METHYLTRANSFERASE; MUSCLE DIFFERENTIATION

Skeletal muscle differentiation is a multistep process in which muscle precursor cells initially express early differentiation markers, exit the cell cycle, then express muscle-specific structural genes and fuse to form multinucleated myotubes [Andrés and Walsh, 1996]. The major muscle regulatory factors MyoD, Myf5, MRF4, and myogenin control the commitment and differentiation of myoblasts [Lassar et al., 1994; Rudnicki and Jaenisch, 1995; Arnold and Winter, 1998], and cooperate with the MEF2 family of proteins to synergistically activate expression of muscle-specific genes [Black and Olson, 1998]. The expression of MyoD and Myf5 is in turn regulated by the paired box transcription factors Pax3 and Pax7 that are expressed in muscle cell progenitors [Seale et al., 2000; Bajard et al., 2006; Kuang et al., 2006; Hu et al., 2008; McKinnell et al., 2008]. Pax3 is critical for delamination and migration of muscle precursors from the somites to the limbs [Tajbakhsh et al., 1997]. Pax7 is essential for the production of functional satellite cells, and is expressed in proliferating myoblasts and downregulated upon differentiation [Seale et al., 2000; Olguin and Olwin, 2004; Relaix et al., 2005; Kuang et al., 2006; Zammit et al., 2006; Olguin et al., 2007].

Cell cycle withdrawal during muscle differentiation is characterized by hypophosphorylation of pRb and expression of cyclin-dependent kinase (cdk) inhibitors. Hypophosphorylated pRb plays an essential role in cell cycle exit, inhibition of DNA synthesis, and activation of muscle-specific genes [Schneider et al., 1994; Novitch et al., 1996; Wang and Walsh, 1996]. Cdk activity is blocked by two classes of cdk inhibitors, the p21 and p16 family of proteins [Sherr and Roberts, 1995]. The p21 family contains two other related genes, p27 and p57. Induction of p21 synthesis has been correlated with cell cycle exit during myoblast differentiation [Guo et al., 1995; Halevy et al., 1995; Parker et al., 1995; Skapek et al., 1995]. The expression of most cyclins is downregulated during cell cycle arrest, a notable exception being cyclin D3 which is upregulated and stabilized in differentiating muscle cells [Kiess et al., 1995; Rao and Kohtz, 1995]. MyoD induces the expression of cyclin D3 early in the differentiation process [Cenciarelli et al., 1999]. In differentiated myoblasts, pRb stabilizes cyclin D3 by directly binding to it, which prevents the GSK3β-mediated phosphorylation and consequent degradation of cyclin D3 [De Santa et al., 2007]. Cyclin D3 sequesters pRb into a multi-protein complex that is tethered to the

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nucleoskeleton in differentiated cells [Cenciarelli et al., 1999]. This is accompanied by remodeling of the nuclear lamina [Muralikrishna et al., 2001; Markiewicz et al., 2005], which is specifically induced in muscle cells by cyclin D3 and pRb [Mariappan and Parnaik, 2005; Mariappan et al., 2007; Parnaik, 2008].

Muscle differentiation has been extensively studied using the C2C12 mouse myoblast cell line which has been derived from myoblasts isolated from adult muscle tissues which are capable of further differentiation in response to appropriate environmental cues [Yaffe, 1969]. Expression of myogenin is one of the early markers of myogenesis in this system. We have previously reported that C2C12 myoblasts transiently expressing cyclin D3 expressed higher levels of myogenin compared to normal myoblasts at early times during differentiation [Mariappan and Parnaik, 2005]. However, it is not known whether expression of cyclin D3 plays a role in the induction of myogenic genes in myoblasts.

In the present study, we have expressed cyclin D3 by adenoviral infection in C2C12 mouse myoblasts and determined the effects on muscle-specific markers, cell cycle parameters, and differentiation kinetics. We have carried out a comparative real-time PCR analysis of genes that may be regulated by cyclin D3 expression. Our results indicate that cyclin D3 upregulates Pax7 transcription in myoblasts, and activates expression of myogenic genes and the cdk inhibitor p21 at an early stage during differentiation.

MATERIALS AND METHODS

PLASMID CONSTRUCTS

The 900-bp insert encoding the human cyclin D3 gene from pRC-CMV-cyclin D3HA (provided by P Hinds, Harvard Medical School, MA) was subcloned into the pAdTrack-CMV vector of the pAdEasy-1 system (obtained from B Vogelstein, The Johns Hopkins University School of Medicine, MD) to prepare the adeno-cyclin D3 virus and the empty pAdTrack-CMV vector coding for GFP alone was used for making adeno-control virus. The Pax7-3.8k-Luc construct (provided by S. L. Chen, National Central University, Taiwan) contains the −3,880 to +21 promoter fragment of the mouse Pax7 gene cloned into the pGL3-Basic luciferase reporter gene vector [Lang et al., 2009].

CELL CULTURE AND DNA TRANSFECTION

C2C12 mouse skeletal myoblasts were maintained at subconfluent densities in DMEM supplemented with 20% FBS (growth medium, GM). Myogenic differentiation was induced by transfer to DMEM containing 2% horse serum (differentiation medium, DM). HEK293 cells were grown in DMEM containing 10% FBS. DNA transfections were carried out with Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were grown for 24 h before transfection. Lipid and DNA complexes were added to cells in serum-free DMEM. After 3 h, the medium was replaced with complete medium and cells were harvested 24–48 h post-transfection.

ADENOVIRAL INFECTION

The adeno-cyclin D3 and adeno-control viruses were prepared according to the protocol described by He et al. [1998]. In vivo recombination was performed by transforming the linearized pAdTrack-cyclin D3HA plasmid or pAdTrack-empty plasmid into BJ5183 bacterial cells that contain the pAdEasy1 plasmid, by electroporation. The recombinant plasmids were linearized and transfected into HEK293 packaging cells in DMEM containing 10% FBS (as described in the previous section) to generate the recombinant adenoviruses. After 3 days of infection, infectious viral lysates were prepared by repeated freeze-thaw of cells in liquid nitrogen, followed by centrifugation. Myoblasts were infected with the viral lysates in GM for 3 h. The viral supernatant was removed and replaced with fresh GM. After 24 h of infection, cells were harvested and used directly or transferred to DM to induce differentiation.

ANTIBODIES

Antibodies to cyclin D3, cyclin D1, cdk2, cdk4, cdk6, and p27 were obtained from BD Transduction Laboratories (Lexington, KY), and anti-pRb-ser807/811 antibody was from Cell Signaling Technology (Beverly, MA). The p21 and lamin A/C antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to lamin B1, pRb, and GFP were from Abcam (Cambridge, MA). An anti-HA antibody was obtained from Roche Applied Sciences Inc. (Indianapolis, IN). An anti-PCNA antibody was from Sigma Chemical Co. (St. Louis, MO). Mouse monoclonal antibodies to myogenin (clone F5D) and Pax7 were from the Developmental Studies Hybridoma Bank (University of Iowa, IA).

IMMUNOFLUORESCENCE MICROSCOPY

Cells were washed with phosphate-buffered saline (PBS) and fixed by treatment with 3.5% formaldehyde for 10 min followed by 0.5% Triton X-100 for 6 min at room temperature. Cells were incubated with 0.5% gelatin or 2% horse serum in PBS for 1 h, followed by incubation with primary antibody for 1 h and then with Cy3-conjugated secondary antibody for 1 h at room temperature. Samples were mounted in Vectashield (Vector Laboratories, Burlingame, CA) containing 1 μg/ml DAPI and viewed under a Zeiss LSM510 META confocal fluorescence microscope (Carl Zeiss, Jena, Germany). Images were analyzed with LSM 510 META software and assembled using Adobe Photoshop. For quantitative estimations of labeled cells, analysis was carried out by inspection of at least n = 100 cells per sample in three independent experiments.

WESTERN BLOTS

Samples of C2C12 myoblasts, myotubes and adenovirus infected myoblasts were harvested, lysed in Laemmli's sample buffer, boiled, and electrophoresed through SDS–polyacrylamide gels. Gels were electroblotted onto nitrocellulose membrane filters and blocked overnight in 5% BLOTTO in Tris-buffered saline (TBS) containing 0.05% Tween-20. Filters were incubated with primary antibody for 2 h, followed by species-specific peroxidase-conjugated secondary antibody in TBS containing 0.05% Tween-20 for 1 h. Bound antibody was visualized using a chemiluminescence kit (Roche Applied Sciences Inc.). Blots probed with antibodies to pRb were processed according to the manufacturer's instructions.

DNA SYNTHESIS

DNA synthesis was measured by a BrdU incorporation assay as described [Muralikrishna et al., 2001]. Myoblasts were pulsed with 10 μM BrdU for 30 min, fixed and permeabilized in chilled 70% ethanol at 4°C for 30 min. DNA was denatured with 2 M HCl containing 0.5% Triton X-100, 0.5% Tween-20, and then neutralized with sodium borohydride. Samples were incubated in 2% horse serum for 1 h, followed by immunostaining with a mouse anti-BrdU antibody (SeroTec, UK).

FLOW CYTOMETRY

Adenovirus infected myoblasts and uninfected myoblasts were harvested and fixed in cold 70% methanol, followed by labeling with 1% Triton X-100 containing 50 mg/ml propidium iodide and 100 mg/ml RNase A for 30 min at 37°C. Samples were analyzed on a FACS-Caliber flow cytometer (BD Biosciences, CA) using Cellquest software. G1, S, and G2/M stages were determined using uninfected myoblasts. Graphical quantitation was carried out using the FlowJo V7.2.4 software package (Tree Star Inc., Ashland, OR).

TRANSCRIPT ANALYSIS

Total RNA was extracted from adenovirus infected myoblasts and 1–5 μg of RNA was reverse transcribed using Superscript III (Invitrogen) according to the manufacturer's protocols. For real-time quantitation, the reverse transcription reaction mixture was mixed with gene-specific primers and SyBR Green dye (Applied Biosystems Inc., Foster City, CA) and fluorescence readings were taken on an ABI PRISM 7900HT machine. The ribosomal protein, large P0 gene (RPLP0) was used as a reference gene as recommended previously, since other commonly used reference genes show variable expression in differentiating myoblasts [Stern-Straeter et al., 2009]. For each gene amplicon, melting curve analysis was done and C_t values calculated using the SDS 2.2.3 software. Fold changes were computed using the $2^{-\Delta\Delta Ct}$ method. The analysis for each gene was done in triplicate and three independent biological replicates were performed. Gene expression in adeno-cyclin D3 infected cells was expressed as fold change in comparison with adeno-control infected cells. The gene-specific primers used for real-time PCR analysis are tabulated in Table I.

REPORTER GENE PROMOTER ASSAY

For Pax7 promoter analysis, myoblasts were transfected with 0.3 μg of Pax7-3.8k-luciferase reporter vector, 0.2 μg of either pRC-CMV-cyclin D3HA or pcDNA3.1 empty vector, and 0.1 μg of pCMV-SPORT-βgal plasmid using Lipofectamine as described earlier. Cells were transfected with pGL3-Basic vector instead of the Pax7 promoter for negative controls. Cells were harvested 24 h post-transfection and lysed. Aliquots were assayed for luciferase activity using a kit from Promega Corporation (Madison, WI) and for β-galactosidase activity by a standard assay. The values for luciferase activity were normalized to the β-galactosidase activity as an internal control and then expressed as fold activation compared to the promoter-less pGL3-Basic vector. Each experiment was repeated three times.

RESULTS

ECTOPIC EXPRESSION OF CYCLIN D3 IN MYOBLASTS DOES NOT INDUCE MYOGENIC DIFFERENTIATION

In order to analyze the effects of cyclin D3 expression in myoblasts on cell cycle and myogenic markers, we have employed an efficient system of cyclin D3 expression using adenoviral expression. C2C12 myoblasts were infected with adenovirus expressing HA-tagged cyclin D3 (adeno-cyclin D3) or with a control adenovirus (adeno-control) and were analyzed 24 h after viral infection. At this time point, ~90% of cells expressed cyclin D3 and expression levels were more uniform than those obtained by transient transfection. We tested the levels of a number of cell cycle markers, as well as muscle-specific markers such as myogenin, in adeno-cyclin D3, and adeno-control cells in comparison with normal myoblasts and myotubes (after 48 h in DM) to validate the system (Fig. 1). The level of HA-tagged cyclin D3 in adeno-cyclin D3 myoblasts was approximately twice that of endogenous cyclin D3 expression in myotubes, and it migrated slower, probably due to the presence of the HA tag.

The profile of cell cycle markers such as cyclin D1, cdk2, cdk4, cdk6, and PCNA in adeno-cyclin D3 over-expressed myoblasts was similar to that in control myoblasts. Cdk6, cyclin D1, and PCNA

TABLE I. List of Primers for Real-Time PCR Analysis

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
Ash2L	AGTCACTGCCGGACACCTACAAAG	TGTACAGTGAGATGGCTGGGAAG
cyclin D1	AAGTGCGTGCAGAAGGAGATTGTG	TCGGGCCGGATAGAGTTGTCAGT
MEF2C	TCCATCAGCCATTTCAACAA	AGTTACAGAGCCGAGGTGGA
MLL2	AAGCCGACATGCTGAGACTCTTCC	TGATCATGAGCGGTAACTCCATCAG
Foxk1	TGGTTACCACCTCTGCCAACTC	TGGCAAATGCTATGGTGGGCTTC
Myf5	CCCCACCTCCAACTGCTCTG	CCAAGCTGGACACGGAGCTT
MyoD1	CGCTCGTGAGGATGAGCAT	AGCGTCTCGAAGGCCTCAT
Myogenin	TGCCGTGGGCATGTAAGGT	TGCGCAGGATCTCCACTTTAG
p21	CCACAGCGATATCCAGACATTCAGAG	ATGAGCGCATCGCAATCACG
p27	TGGTGGACCAAATGCCTGACTC	AGCTGTTTACGTCTGGCGTCGAAG
Pax3	TGGCCTTTCACCTCAGGTAATGG	TAGCCTGCGGTGCTATAGGTG
Pax7	AAGCAGGCAGGAGCTAACCAG	ACCCTGATGCATGGTTGATGG
Pax7′	CATGGTGGGCCATTTCCACT	GGCCCGGGGCAGAACTAC
RbBP5	TGGACAGAACTACCCAGAGGA	CCATCGTTACAGCCAACAGC
RPLP0	ATGCAGCAGATCCGCATGTCG	TGGCACCTTATTGGCCAACAGC
Wdr5	TCCGTGACAGGCGGGAAGTG	TAACGCTGCTGAGGCAATGATG

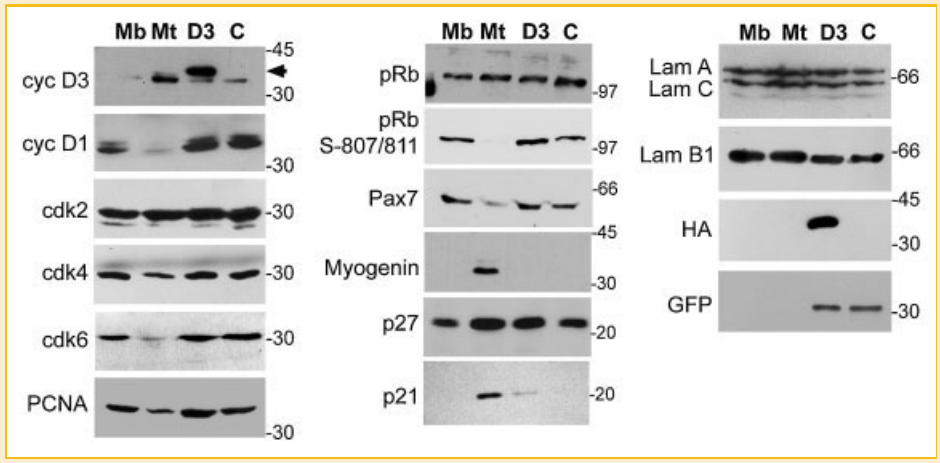

Fig. 1. Effects of cyclin D3 expression on cell cycle and muscle markers. Whole cell lysates of normal myoblasts (Mb), myotubes (Mt), and myoblasts expressing adeno-cyclin D3 (D3) or adeno-control (C) were analyzed by western blotting with the indicated antibodies. Lamins A/C and B1 were used as loading controls. HA-tagged cyclin D3 has a slower mobility and is indicated by an arrow. Molecular mass markers (in kDa) are indicated to the right of each panel.

were downregulated in growth-arrested myotubes, as expected. Cdk2 and cdk4 levels were relatively constant in both myoblasts and myotubes, consistent with earlier studies [Guo et al., 1995; Skapek et al., 1995]. The hypophosphorylation of pRb at ser-807/811 is a marker for quiescent cells [Nitta et al., 2006] and this phosphoform of pRb was depleted in myotube samples but not in adeno-cyclin D3 myoblasts. The levels of whole pRb protein remained comparable in all samples. Adeno-cyclin D3 cells did not upregulate myogenin under growth conditions, and low basal levels of myogenin expression were observed in all myoblast samples. We tested the levels of the muscle stem cell marker Pax7, which is downregulated during differentiation of C2C12 cells. Both proliferating control myoblasts and cyclin D3 over-expressed myoblasts expressed Pax7, whereas myotube samples showed low levels of Pax7, consistent with earlier reports [Olguin and Olwin, 2004]. Interestingly, the level of p21 was higher in cyclin D3 over-expressed myoblasts than in control myoblasts. However, there were no significant changes in the levels of the cdk inhibitor p27. Both adeno-cyclin D3 and adeno-control myoblasts expressed the GFP marker encoded by the adenoviral vector. Further control experiments indicated that there were no changes in the p38 MAP kinase or Akt kinase signaling pathways in adeno-cyclin D3 myoblasts (data not shown). These results suggest that the expression of key cell cycle and myogenic genes is similar in adeno-cyclin D3 and adeno-control myoblasts, except for a moderate increase in p21 levels.

CYCLIN D3 EXPRESSION LEADS TO EARLY ENTRY INTO MYOGENESIS

In order to confirm that cyclin D3 over-expressed myoblasts were competent to differentiate and to study early myogenic events during differentiation, adeno-cyclin D3, and adeno-control myoblasts were induced to differentiate by serum depletion. Cells were analyzed for expression of the early myogenic marker myogenin from 0 to 30 h of differentiation by immunostaining with antibody to myogenin followed by quantitation of myogenin positive cells. Cyclin D3 and GFP-expressing cells were also quantitated during the time course. As shown in Figure 2A and B, the percentage of adeno-cyclin D3 myoblasts expressing myogenin was approximately threefold higher than control cells at 3 h, twofold higher upto 12 h, and continued to be higher than control cells upto 30 h. This indicates that myoblasts expressing cyclin D3 enter the differentiation pathway earlier than control myoblasts. Cyclin D3 was expressed in ~90% of adeno-cyclin D3 cells upto 12 h and this was decreased to ~60% thereafter. Endogenous cyclin D3 increased steadily during the time course. There was a reduction of GFP positive cells in both adeno-cyclin D3 and adeno-control samples from ~90% to ~70% after 24 h, which is probably due to the deleterious effects of prolonged adenovirus expression. The morphology of myotubes expressing adeno-cyclin D3 was similar to that of control myotubes (Fig. 2C), indicating that cyclin D3 over-expression did not have a detrimental effect on myotube formation. By 30 h, which is the earliest time at which myotubes with more than two nuclei are formed under our conditions, the fusion index was three to four nuclei per myotube in both adeno-cyclin D3 and adeno-control samples. It may be noted that myogenin levels are similar by this time point in both types of cells. Hence, cyclin D3 over-expression does not affect long-term myogenic differentiation in this system.

The expression of a few myogenic and cell cycle markers was analyzed in cells induced to differentiate over 24 h by immunoblotting extracts of adeno-cyclin D3 and adeno-control cells (Fig. 3). Myogenin levels were higher in cyclin D3 over-expressed cells as observed in the immunofluorescence analysis. Myf5 levels were also higher early during the differentiation time course (0–8 h), but at later time points Myf5 was low in both adeno-cyclin D3 and adeno-control cells. Myf5 has a key role in regulating the onset of differentiation but Myf5 protein is proteolytically degraded once differentiation ensues [Lindon et al., 1998]. There was also an upregulation of MyoD levels in adeno-cyclin D3 cells from 16 to 24 h of differentiation. An increase in MyoD was not observed at early time points and this could be attributed to the rapid turnover of the protein in dividing cells [Song et al., 1998]. HA-tagged cyclin D3

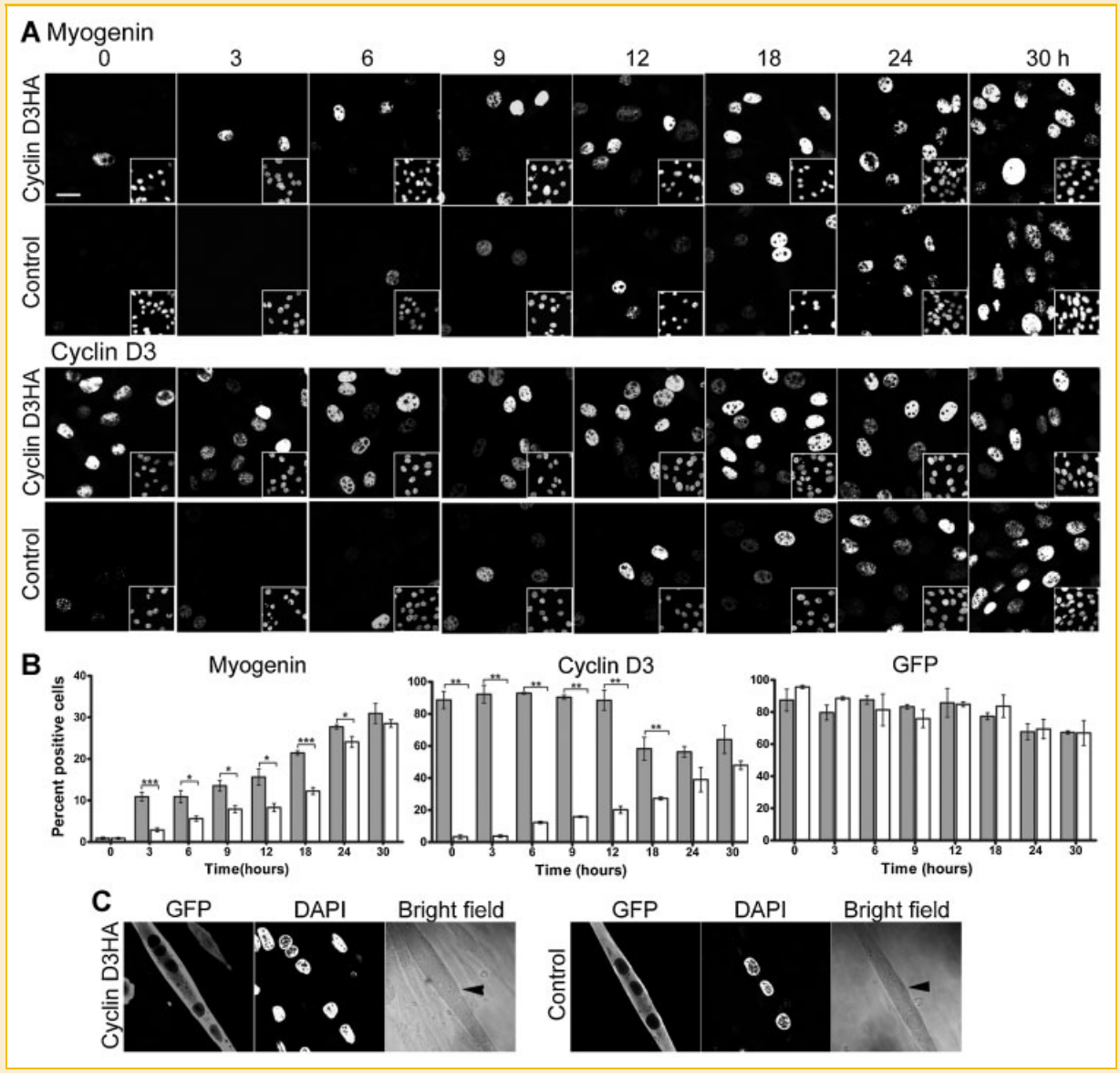

Fig. 2. Expression of myogenin and cyclin D3 during differentiation. Adeno-cyclin D3 and adeno-control myoblasts were grown in GM and then induced to differentiate by transfer to DM. (A) Cells were fixed at 0, 3, 6, 9, 12, 18, 24, and 30 h of differentiation and immunostained with antibodies to myogenin or cyclin D3 and counterstained with DAPI (insets, DAPI stained cells). (B) Graphical analysis of cells positive for myogenin, cyclin D3, or GFP at 0–30 h of differentiation (mean ± SD of three independent experiments; solid bars, adeno-cyclin D3; open bars, adeno-control); $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. (C) Adeno-cyclin D3 and adeno-control myotubes were stained with anti-GFP antibody, counterstained with DAPI and also visualized in bright field (arrowheads). Bar, 20 μM.

levels peaked at 8 h while endogenous cyclin D3 increased in both adeno-cyclin D3 cells and control cells during the time course. Expression of cell cycle markers was determined by blotting with antibodies to the cdk inhibitors p21 and p27 and the differentially expressed pRb phosphoform ser-807/811. In adeno-cyclin D3 cells, p21 was expressed at moderate levels at early time points and upregulated by 8–16 h of differentiation, ahead of control myoblasts in which p21 levels increased by 16 h of differentiation. Alterations in the levels of p27 were not detectable in adeno-cyclin D3 or adeno-control cells during this differentiation time-course; p27 has been reported to increase to a small extent by 96 h of differentiation in C2C12 cells [Halevy et al., 1995]. The hypophosphorylation of pRb at ser-807/811 occurred by 16 h in both adeno-cyclin D3 and adeno-control cells.

CYCLIN D3 EXPRESSING MYOBLASTS ARE STALLED IN G2/M PHASE

As adeno-cyclin D3 myoblasts expressed higher levels of p21, we determined the effects of cyclin D3 on the cell cycle by flow cytometric analysis of adeno-cyclin D3 myoblasts (in GM). Compared to control myoblasts, a higher percentage of adeno-cyclin D3 cells were present in the G2/M phase of the cell cycle (Fig. 4). Analysis of the S phase cells by BrdU incorporation studies indicated that there was a moderate reduction in the percentage of adeno-cyclin D3 myoblasts in S phase (56.2%, n = 390) compared to

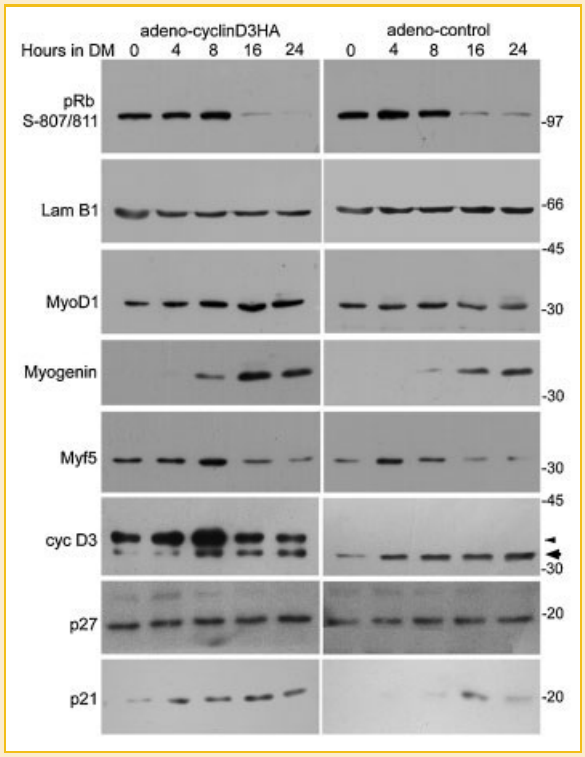

Fig. 3. Induction of cell cycle markers during differentiation. Adeno-cyclin D3 and adeno-control myoblasts were transferred to DM to induce differentiation, and lysates of samples were analyzed at 0, 4, 8, 16, and 24 h of differentiation by western blotting with the indicated antibodies. Lamin B1 was used as the loading control. HA-tagged cyclin D3 has a slower mobility and is indicated by an arrowhead; endogenous cyclin D3 is indicated by an arrow. Molecular mass markers (in kDa) are indicated to the right of the panel.

adeno-control myoblasts (61.0%, n = 250). These results suggest that cyclin D3 over-expressed cells have slower cell cycle kinetics and are stalled in G2/M phase of cell cycle, probably due to expression of the cell cycle inhibitor p21.

CYCLIN D3 UPREGULATES TRANSCRIPTION OF MYOGENIC MARKERS IN DIFFERENTIATED CELLS

As cyclin D3 induced higher levels of expression of myogenin and other muscle markers early during differentiation, we assessed the effects of cyclin D3 overexpression on the transcription of a number of myogenic and cell cycle genes by real-time PCR analysis of adeno-cyclin D3 myoblasts normalized to adeno-control myoblasts at 0 h (in GM) and 16 h (in DM) of differentiation (Fig. 5). As compared to control cells, there was a marked upregulation of myogenic regulators in 16 h-differentiated myocytes expressing exogenous cyclin D3. Myocytes expressing cyclin D3 showed twofold higher levels of myogenin, and Myf5 and MyoD were upregulated by 2.5-fold. This corroborated well with the increase in myogenin and MyoD protein expression observed in previous experiments (Figs. 2 and 3), though Myf5 protein levels were lower, most probably due to its known instability in differentiated cells. Transcript levels of MEF2C were not affected by cyclin D3 expression at 16 h, probably because MEF2C is expressed later in the differentiation program [Martin et al., 1993; Mariappan and Parnaik, 2005]. An interesting finding was that Pax7 was upregulated fourfold in cyclin D3 over-expressed myoblasts but downregulated upon differentiation. Pax7 is essential for the survival and differentiation potential of myogenic progenitors and is tightly regulated during differentiation [Olguin and Olwin, 2004; Relaix et al., 2005; Kuang et al., 2006; Olguin et al., 2007].

An analysis of cell cycle markers indicated a small increase in p27 transcripts at 16 h of differentiation, though this could not be clearly detected at the protein level. As there was no increase in p21 transcripts in differentiated cells, the upregulation of p21 that was detectable in western blots of adeno-cyclin D3 samples at 8–16 h of differentiation, may be due to increased protein stability. We also observed that a myogenic forkhead/winged helix transcription factor, Foxk1, which has been implicated in suppressing p21 [Hawke et al., 2003], was upregulated in differentiated cells. The marked reduction of cyclin D1 along with Pax7 in differentiated cells suggests that growth promoting signals are downregulated upon differentiation of myoblasts expressing cyclin D3 and, together with an increase in myogenic transcripts, this leads to rapid activation of myogenesis. The transcript levels of other cell cycle regulatory genes such as cdkn2A and cdk4 were not altered to a significant extent. Cyclin D3 transcripts were higher in adeno-cyclin D3 cells at both 0 and 16 h time points.

Pax7 ASSOCIATED GENES ARE UPREGULATED IN CYCLIN D3-EXPRESSING CELLS

Since Pax7 is essential for the functions of muscle cell progenitors, we analyzed its role in more detail. Pax7 has been shown to upregulate the Myf5 promoter by recruitment of components of a histone methyltransferase (HMT) that methylates histone 3 at lysine 4 (H3K4) [McKinnell et al., 2008]. These components include the trithorax-group protein Ash2L, the HMT MLL2 (myeloid/lymphoid or mixed-lineage leukemia 2), the WD40 repeat domain 5 protein Wdr5 that binds to histone 3, and the retinoblastoma binding protein-5 RbBP5. We determined the relative levels of these transcripts by real-time PCR analysis of adeno-cyclin D3 myoblasts normalized to adeno-control myoblasts at 0 and 16 h of differentiation (Fig. 6). The levels of Ash2L, MLL2, and RbBP5 transcripts were upregulated and reached approximately twofold at 16 h. The regulation of Pax7 transcripts by cyclin D3 was confirmed using a different set of primers for real-time PCR analysis from those described in Figure 5. The effect on expression of Pax3 and Wdr5 genes was below 1.5-fold at either time point. These data indicate that Pax7 together with certain components of its regulatory complex are upregulated upon cyclin D3 expression in myoblasts. However, Pax7 transcript levels are substantially reduced upon differentiation, probably due to the increased expression of muscle regulatory factors, while those of the HMT components remain high.

CYCLIN D3 EXPRESSION ACTIVATES Pax7 PROMOTER

Since the above real-time PCR data indicated an increase in Pax7 transcripts upon cyclin D3 expression, although higher Pax7 protein levels were not evident in the western blot, we confirmed the ability of cyclin D3 to activate Pax7 transcription by a promoter assay. We studied the ability of cyclin D3 to activate a 3.8 kb Pax7 promoter in

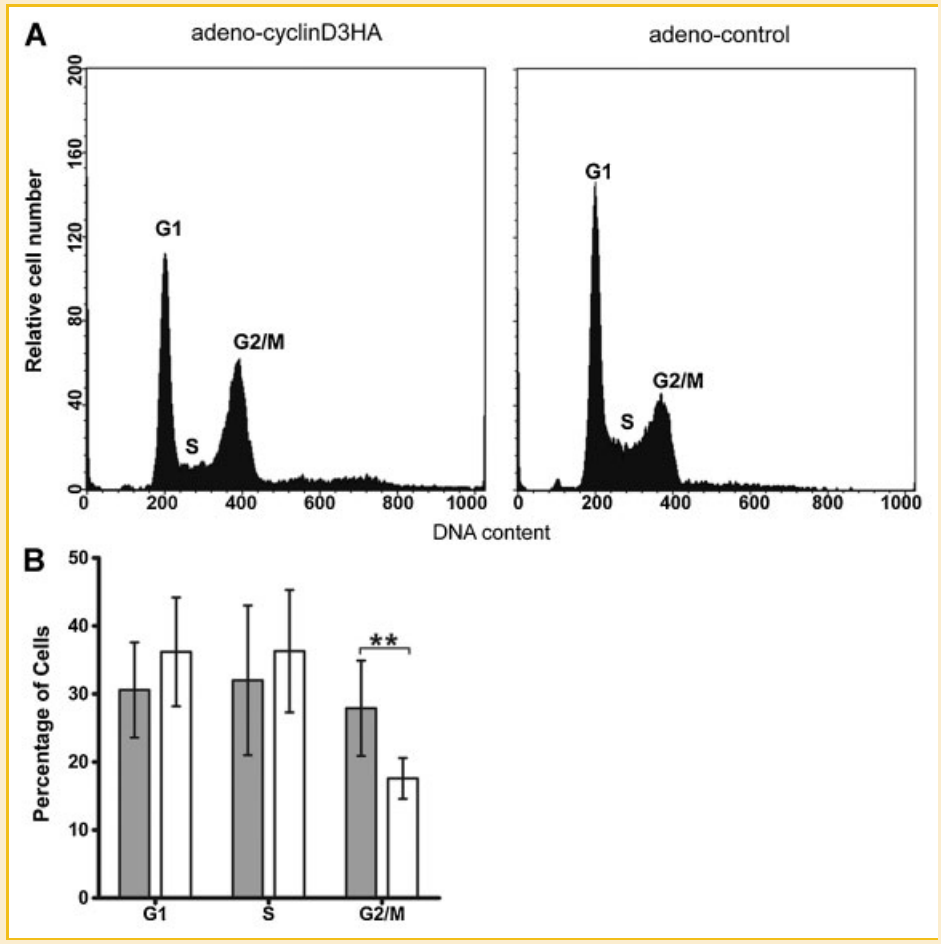

Fig. 4. Cell cycle analysis by flow cytometry. (A) Adeno-cyclin D3 and adeno-control C2C12 cells grown in GM were fixed in chilled 70% methanol after 24 h of infection and fractionated by FACS; representative flow cytometric data is shown. Three independent experimental samples were analyzed with at least 10,000 cells per fraction. Gates for G1, S, and G2/M phases were set using normal myoblasts. The data was analyzed using the FlowJO software. (B) Graphical analysis of percentages of adeno-cyclin D3 (solid bars) and adeno-control (open bars) cells in different phases of the cell cycle at 24 h after infection (mean ± SD from three independent experiments); $^{**}P \leq 0.01$.

myoblasts by a luciferase reporter assay (Fig. 7). This construct harbors essential binding motifs for Oct4 and Nanog that regulate Pax7 activity in muscle progenitors [Lang et al., 2009]. The Pax7 promoter displayed a moderate level of luciferase reporter activity in myoblasts cultured in GM. Myoblasts co-transfected with the Pax7-luc construct and an expression vector for cyclin D3 showed a threefold higher promoter activity. On the other hand, myoblasts co-transfected with the promoter-less pGL3-Basic vector and the cyclin D3 expression vector showed only basal levels of luciferase activity. Taken together with the real-time PCR data, this result suggests that cyclin D3 expression can upregulate Pax7 transcription in myoblasts.

DISCUSSION

Cyclin D3 is expressed at very low levels in myoblasts due to GSK3β-mediated phosphorylation and consequent degradation which is prevented in differentiating cells, thus allowing upregulation of cyclin D3 during myogenesis [De Santa et al., 2007]. We have previously shown that cyclin D3 expression in myoblasts induces earlier activation of muscle-specific genes like myogenin in differentiating cells [Mariappan and Parnaik, 2005]. The results described in this study suggest that the transcription of Pax7 and associated members of the HMT complex is regulated by cyclin D3 expression. Cyclin D3 expression leads to faster differentiation kinetics and increase in levels of myogenic genes such as MyoD, Myf5, and myogenin. Cyclin D3 over-expressed myoblasts also show higher levels of the cdk inhibitor p21 compared to control myoblasts and are stalled in G2/M phase.

REGULATION OF Pax7 AND ASSOCIATED GENES BY CYCLIN D3

Pax7 is required for the biogenesis of muscle progenitor cells but is tightly regulated during differentiation due to inhibition by muscle regulatory factors [Seale et al., 2000; Olguin and Olwin, 2004; Relaix et al., 2005; Kuang et al., 2006; Zammit et al., 2006; Olguin et al., 2007]. McKinnell et al. [2008] have demonstrated that Pax7 recruits the HMT complex comprised of Wdr5, Ash2L, MLL2, and RbBP5 to methylate H3K4 at the Myf5 promoter in C2C12 myoblasts. The methylation of H3K4 has been shown to be indicative of active

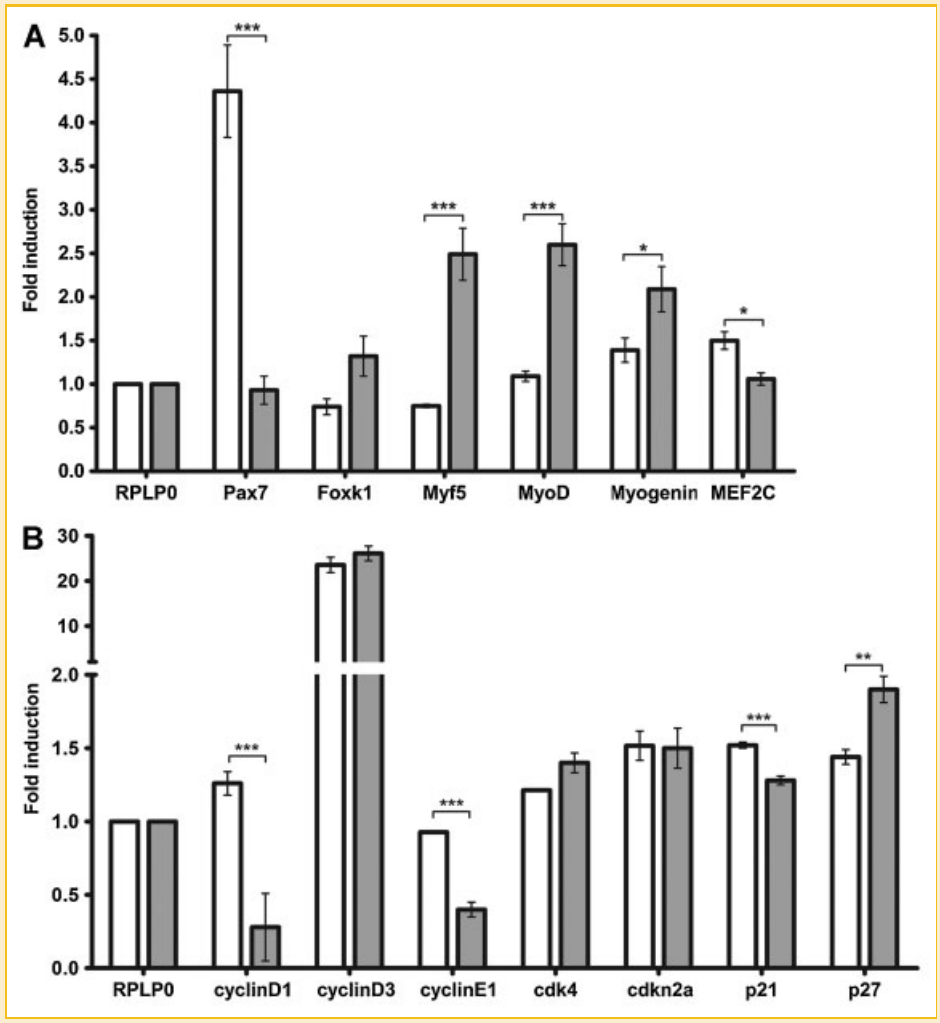

Fig. 5. Real-time PCR analysis of cell cycle and myogenic genes. Adeno-cyclin D3 and adeno-control myoblasts were grown in GM and then transferred to DM to induce differentiation for 16 h. Transcripts were analyzed by real-time PCR and relative gene expression was calculated by normalizing to RPLP0 gene of control at 0 h (open bars) and 16 h (solid bars). (A) Myogenic and (B) cell cycle genes were analyzed. The fold-induction represents the ratio of adeno-cyclin D3 cells to adeno-control cells at each time point and was plotted as mean ± SE from three independent experiments; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

or transcriptionally permissive chromatin [Bernstein et al., 2002]. Although their chromatin is poised for activation, myogenic genes remain repressed until appropriate differentiation conditions prevail. We have observed that low-mitogen conditions that promote differentiation result in increased transcript levels of active chromatin determinants like Ash2L, MLL2, and RbBP5 in adeno-cyclin D3 cells. Furthermore, myogenic genes like MyoD, Myf5, and myogenin are upregulated upon differentiation of these cells. However, in the absence of increased Pax7 protein in undifferentiated myoblasts, there might not be a direct correlation between activation of Pax7 and enhanced myogenesis upon cyclin D3 expression, and other factors might also play a role in the promotion of myogenesis by cyclin D3. The expression of critical factors like Pax3/7 during the biogenesis of muscle precursors is controlled by diverse signaling pathways including Wnt/β-catenin and Notch [Kuang and Rudnicki, 2008]. Of particular interest is the potential for regulation of Pax7 by β-catenin/GSK3β signaling [Perez-Ruiz et al., 2008]. Since cyclin D3 is also regulated by GSK3β activity, this brings up the interesting possibility of cross-talk between events that modulate the turnover of both Pax7 and cyclin D3.

INDUCTION OF THE CDK INHIBITOR p21 BY CYCLIN D3

The upregulation of the cdk inhibitor p21 is an essential feature of myocyte terminal differentiation [Guo et al., 1995; Halevy et al., 1995; Parker et al., 1995]. Moreover, ectopic expression of p21 is sufficient for cell cycle arrest in GM [Guo et al., 1995; Skapek et al., 1995]. The expression of p21 has been shown to lead to arrest at both the G1/S and G2/M phase transitions of the cell cycle in a number of cell types [Niculescu et al., 1998]. We have observed that myoblasts that express cyclin D3 have moderately higher levels of p21 than control myoblasts and express high levels of p21 by 8–16 h of differentiation, ahead of control myoblasts. Cyclin D3-expressing myoblasts are stalled in G2/M phase but not completely arrested, probably because p21 levels are not sufficiently high. Levels of p21

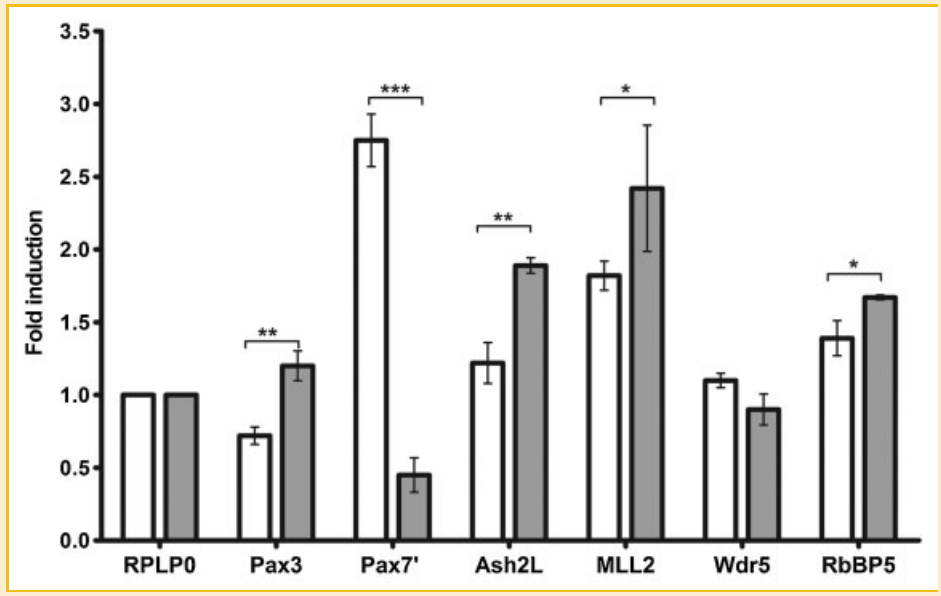

Fig. 6. Real-time PCR analysis of Pax3/7 and HMT complex genes. Adeno-cyclin D3 and adeno-control myoblasts were grown in GM and then transferred to DM to induce differentiation for 16 h. Transcripts were analyzed by real-time PCR and relative gene expression was calculated by normalizing to RPLP0 gene of control at 0 h (open bars) and 16 h (solid bars). Pax3, Pax7, Ash2L, MLL2, Wdr5, and RbBP5 genes were analyzed. The fold-induction represents the ratio of adeno-cyclin D3 cells to adeno-control cells at each time point and was plotted as mean ± SE from three independent experiments; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

are also elevated in myoblasts treated with the GSK3β inhibitor LiCl, which stabilizes cyclin D3 [De Santa et al., 2007]. Cyclin D3 has been reported to interact with and increase nuclear localization of a G2/M kinase p58-PITSLRE that is required for normal cytokinesis; elevated levels of this kinase lead to delay in M phase [Zhang et al., 2002]. Hence, increased levels of both cyclin D3 and p21 in myoblasts are likely to contribute to stalling in G2/M phase.

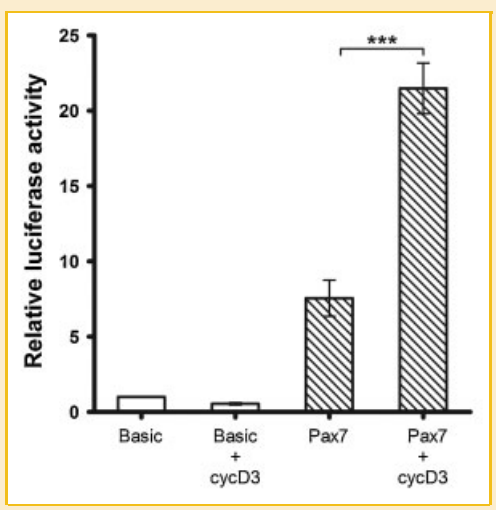

Fig. 7. Activation of Pax7 promoter by cyclin D3. Myoblasts were transfected with pGL3-Basic vector (Basic), pGL3-Basic with pRC-CMV-cyclin D3HA (Basic + cycD3), Pax7-3.8k-luciferase reporter (Pax7), or Pax7-3.8k-luciferase reporter with pRC-CMV-cyclin D3HA (Pax7 + cycD3). Luciferase activity was normalized to β-galactosidase activity and plotted as fold induction relative to pGL3-Basic vector. Values represent mean ± SD from three independent experiments; $^{***}P \leq 0.001$.

EFFECTS OF CYCLIN D3 OVER-EXPRESSION ON MYOGENESIS

We observed that cyclin D3 over-expression in myoblasts led to upregulation of myogenic markers upto 24 h of differentiation. However, cyclin D3 did not appear to have an effect on long-term myogenic differentiation, as the myotube fusion index in cyclin D3 over-expressed cells was similar to control cells at 30 h. The lack of effect on long-term differentiation might be caused by the adenovirus expression system, which is relatively inhibitory to myogenic differentiation after 24 h in serum-depleted medium.

Although endogenous cyclin D3 is upregulated in differentiated cells, we noted that cyclin D3 over-expression was not sufficient to induce the myogenic marker myogenin in myoblasts. LiCl-treated myoblasts also upregulate cyclin D3 but do not express differentiation markers [De Santa et al., 2007]. A critical requirement for differentiation is the hypophosphorylation of pRb which occurs in response to cdk inactivation due to serum withdrawal [Schneider et al., 1994; Novitch et al., 1996; Wang and Walsh, 1996]. Our data indicates that pRb is dephosphorylated at ser-807/811 in myotubes but not in cyclin D3 over-expressed myoblasts. Other factors that are likely to contribute to absence of myogenin in these myoblasts are the presence of growth promoting signals like cyclin D1.

In summary, cyclin D3 expression in myoblasts promotes early activation of myogenic genes during differentiation and upregulates transcription of the premyogenic factor Pax7 as well as components of the HMT complex. Cyclin D3-expressing myoblasts also exhibit higher levels of p21 and show stalling of cell cycle progression, which leads to faster cell cycle exit during differentiation.

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