Molecular and cellular pharmacology

Cholesterol depletion by methyl-β-cyclodextrin enhances cell proliferation and increases the number of desmin-positive cells in myoblast cultures

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ABSTRACT

Skeletal myogenesis comprises myoblast replication and differentiation into striated multinucleated myotubes. Agents that interfere with myoblast replication are important tools for the understanding of myogenesis. Recently, we showed that cholesterol depletion by methyl-β-cyclodextrin (MCD) enhances the differentiation step in chick-cultured myogenic cells, involving the activation of the Wnt/β-catenin signaling pathway. However, the effects of cholesterol depletion on myoblast replication have not been carefully studied. Here we show that MCD treatment increases cell proliferation in primary chick myogenic cell cultures. Treatment of myogenic cells with the anti-mitotic reagent cytosine arabinoside, immediately following cholesterol depletion, blocks the MCD-induced effects on proliferation. Cholesterol depletion induced an increase in the number of desmin-positive mononucleated cells, and an increase in desmin expression. MCD induces an increase in the expression of the cell cycle regulator p53 and the master switch gene MyoD1. Treatment with BIO, a specific inhibitor of GSK3β, induced effects similar to MCD on cell proliferation; while treatment with Dkk1, a specific inhibitor of the Wnt/β-catenin pathway, neutralized the effects of MCD. These findings indicate that rapid changes in the cholesterol content in cell membranes of myoblasts can induce cell proliferation, possibly by the activation of the Wnt/β-catenin signaling pathway.

1. Introduction

Skeletal myogenesis proceeds through the following main sequential stages: muscle cell commitment, myoblast proliferation, cell cycle withdrawal, myoblast alignment and fusion, and their subsequent differentiation into mature multinucleated myotubes.

The control of myoblast proliferation and its subsequent withdrawal from cell cycle are critical steps during myogenesis and are finely regulated by the cell cycle-related proteins p53 and p21. p53 has a role in normal muscle cell differentiation, and p53-defective myoblasts exit from the cell cycle but do not differentiate (Soddu et al., 1996). The cyclin-dependent kinase inhibitor p21 is transcriptionally regulated by p53 and can block replication Expression of p21 in myoblasts correlates with the establishment of the postmitotic state (Andrés and Walsh, 1996). Furthermore, p21 is essential for the coordination of cell cycle exit and differentiation during myogenesis and in the absence of p21, skeletal muscle regeneration is markedly impaired (Hawke et al., 2003).

Other factors control the replication of myoblasts. The Wnt/β-catenin signaling pathway is involved in the regulation of many myoblast functions, including cell fate specification and proliferation. Several Wnt molecules, including Wnt1, Wnt3 and Wnt7, have strong mitogenic activity (Wodarz and Nusse, 1998). Wnt-3a increases cell proliferation in chick somite explants (Galli et al., 2004).

Muscle regulatory factors and muscle-specific proteins are also involved in the regulation of cell cycle. The master switch gene MyoD induces cell cycle withdrawal by activation of cyclins (Wei and Paterson, 2001). MyoD also directly controls the expression of desmin, the first muscle-specific cytoskeletal protein to be expressed during myogenesis. Desmin is one of the key markers of muscle commitment. All muscle cells express desmin and even

in dedifferentiated muscle cells, desmin is not down regulated (Costa et al., 2004). However, the relation between desmin expression and cell cycle withdrawal is not completely understood. The temporal interval between muscle commitment and cell cycle exit varies among species, e.g., desmin expression in mammalian cells differs from that in chicken embryo myoblasts where only a small proportion of replicating chicken embryo myoblasts expresses desmin (Kaufman and Foster, 1988).

Recently, we showed that cholesterol depletion by methyl-β-cyclodextrin (MCD) induces an enhancement in the total amount of DNA (as ascertained by DAPI fluorescence) in chick-cultured myoblast cultures (Mermelstein et al., 2005), indicating a possible increase in cell replication after cholesterol depletion. It has been shown that MCD enhances the proliferation of many cell types, such as vascular smooth muscle cells (Qin et al., 2009). So far, the effects of MCD on myoblast proliferation have not been examined. Here, we studied the relationship between cholesterol depletion, myoblast proliferation, desmin expression and the Wnt signaling pathway. We followed the expression of markers of cell proliferation, cell cycle regulators, muscle-specific proteins and muscle regulatory factors in MCD-treated chick skeletal muscle cells grown in vitro.

Studies on myoblast replication are crucial for the understanding of myogenesis, and can be particularly important for research on muscle degenerative diseases. Methyl-β-cyclodextrin could be a useful tool in muscle cell therapies.

2. Materials and methods

DNA-binding probe DAPI (4,6-diamino-2-phenylindole dihydrochloride) was purchased from Molecular Probes (USA). Rabbit polyclonal anti-desmin, mouse monoclonal anti-α-tubulin (clone DM1A) and mouse monoclonal anti-BrdU antibodies were from Sigma Chemical Co. (USA). Rabbit polyclonal anti-phospho-histone H3 was from Upstate Millipore (USA). Mouse monoclonal anti-MyoD1 (clone 5.8A) was from DAKO (USA). Mouse monoclonal antibodies against p21 (clone SX118) and M-cadherin were from BD Pharmigen (USA). Mouse monoclonal anti-p53 (clone DO-1) antibody was from Santa Cruz (USA). Alexa Fluor 488-goat anti-mouse/rabbit IgG and Alexa Fluor 546-goat anti-mouse/rabbit IgG antibodies were from Molecular Probes (USA). Peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies were obtained from Amersham Biosciences (UK).

2.1. Cell cultures

This study using chick embryos was approved by the Ethics Committee for Animal Care and Use in Scientific Research from the Federal University of Rio de Janeiro and received the approval number: DAHEICB 004. All cell culture reagents were purchased from Invitrogen (São Paulo, Brazil). Primary cultures of myogenic cells were prepared from breast muscles of 11-day-old chick embryos (Mermelstein et al., 2005). Cells were grown in 2 ml of medium (Minimum Essential Medium with the addition of 10% horse serum, 0.5% chick embryo extract, 1% L-glutamine and 1% penicillin-streptomycin) at an initial density of 7.5×10^5 cells/35 mm culture dishes onto 22 mm-aclar plastic coverslips (Pro-Plastics Inc., USA) previously coated with rat tail collagen. Cells were grown under humidified 5% CO_2 atmosphere at 37 °C. After the first 24 h, cultures were fed with fresh cultured medium.

The percentage of myoblasts in these cell cultures was calculated by the double-labeling of 24 h cultures with both DAPI (nuclear staining) and anti-desmin antibody (applied herein to define a muscle-specific marker) and subsequently counting the number of desmin-positive cells out of the total number of cells in the field. On average, myoblasts made up 80% of each culture and non-myogenic cells comprised 20%.

Myogenic cultures were treated for 30 min with methyl-β-cyclodextrin (MCD; Sigma) at a final concentration of 2 mM after the first 24 h of culture. After MCD exposure, cultures were washed with culture medium and grown for the next 1, 3, 6 or 24 h. Some myogenic cultures were treated with the anti-mitotic reagent cytosine arabinoside (ara-c; Sigma) at a final concentration of 10^{-5} M in two different experiments: immediately following MCD treatment (in 25 h cultures) or 1 h before MCD treatment (in 23 h cultures). Ara-c was left in the cultures for 24 h.

2.2. Quantification of DNA

Chick myogenic cells were plated at the following densities: 0.8×10^5, 1.5×10^5 and 3.0×10^5 cells/24-well plates previously coated with rat tail collagen. Cells were grown in 1 ml of medium (Minimum Essential Medium with the addition of 10% horse serum, 0.5% chick embryo extract, 1% L-glutamine and 1% penicillin-streptomycin), under humidified 5% CO_2 atmosphere at 37 °C. After the first 24 h, some cultures were treated for 30 min with methyl-β-cyclodextrin (MCD; Sigma) at a final concentration of 2 mM. After MCD exposure, cultures were washed once and fed with fresh culture medium and grown for the next 1, 3 or 6 h. Cultures were then quickly washed in ice-cold PBS, scraped off the dish with a plastic cell scraper and transferred to a microcentrifuge tube. Cell extracts were centrifuged and supernatants were discarded. PBS containing 0.5% Triton X-100 was added to the pellets and suspensions were vortexed at top setting for 5 min and kept at -20 °C for 15 min. Samples were thawed and centrifuged again. Pellets were discarded and TE buffer (100 mM Tris–HCl, 10 mM EDTA, pH 8.0) were added to supernatants. The total amount of DNA present in samples was analyzed in a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA).

2.3. BrdU incorporation assay

5-bromo-2′-deoxyuridine (BrdU, Sigma, 3 ug/ml) was added to MCD-treated and control myogenic cells (with 24 h) for 1 h. Cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and subsequently washed twice with PBS at 37 °C for 15 min and once with deionized water at 37 °C for 10 min. Cells were incubated with 2 N HCl at 50 °C for 30 min and washed with 0.1 M Borate buffer (pH 8.5) at 37 °C for 10 min and once with PBS. Cells were then double-stained with anti-desmin and anti-BrdU antibodies and analyzed in immunofluorescence microscopy. For the quantification of the percentage of desmin-positive/BrdU-positive cells, at least 50 cells for each culture condition were scored in three independent experiments.

2.4. Cell viability

Cell viability was tested with the Live/Dead assay (Invitrogen, USA). The Live/Dead assay allows the simultaneous determination of viable and nonviable cells. Fluorescein diacetate (FDA) indicates intracellular esterase activity, and the propidium iodide (PI) indicates membrane integrity. Myogenic cultures were treated for 30 min with methyl-β-cyclodextrin (MCD) at a final concentration of 2 mM after the first 24 h of culture. 3 or 24 h after MCD exposure, cultures were washed with fresh culture medium and stained with the LIVE/DEAD assay reagents (FDA and PI) following the protocols supplied by Invitrogen. Finally, the number of live (green) and dead (red) cells was determined by

using an Axiovert 100 fluorescence microscope (Carl Zeiss, Germany). This experiment was carried out in triplicate.

2.5. BIO, Dkk and simvastatin experiments

Myogenic cultures were treated for 30 min with methyl-β-cyclodextrin (MCD) at a final concentration of 2 mM after the first 24 h of culture. After MCD exposure, cultures were washed with fresh culture medium and incubated with either 5 µM 6-bromoindirubin-3′-oxime (BIO, Sigma), or 0.1 µg/ml recombinant mouse Dkk1 protein (R&D Systems, USA) or 0.5 µM Simvastatin for the next 24 h at 37 °C. Cells were then fixed with 4% paraformaldehyde in PBS for 10 min at room temperature and permeabilized with 0.5% Triton X-100 in PBS (three times for 10 min each). Cells were labeled with the nuclear dye DAPI (0.1 µg/ml in 0.9% NaCl), and examined with an Axiovert 100 microscope (Carl Zeiss, Germany). Comparisons have shown that flow cytometry and image analysis provide similar efficacy of DNA quantification for diagnostic purposes (Bertino et al., 1994). Images were acquired with a DP71 camera (Olympus, Japan). For each experiment, the illumination and acquisition conditions were kept constant. Since the background was completely dark and corresponded to zero in the captured (8 bits) images, we assumed that all gray levels above zero correspond to DAPI emitted fluorescence, and therefore they were directly proportional to DNA amount. By measuring with the public domain software ImageJ (http://rsb.info.nih.gov/ij/) the amount of fluorescence (total gray level, or RawIntDen) of regions of images with defined numbers of nuclei, we built for each experiment a formula (with correlation higher than 0.99) that relates gray levels to cell numbers. Data were obtained from three independent experiments.

2.6. Immunofluorescence and digital image acquisition

Cultures were rinsed with PBS and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. They were then permeabilized with 0.5% Triton X-100 in PBS 3 times for 10 min. The same solution was used for all subsequent washing steps. Cells were incubated with primary antibodies for 1 h at 37 °C. After incubation, cells were washed for 30 min and incubated with Alexa Fluor-conjugated secondary antibodies for 1 h at 37 °C, and nuclei were labeled with DAPI (0.1 µg/ml in 0.9% NaCl). Cells were examined with an Axiovert 100 microscope (Carl Zeiss, Germany) and images were acquired with a DP71 camera (Olympus, Japan).

2.7. SDS-PAGE and immunoblotting

Myogenic cultures were treated for 30 min with MCD at a final concentration of 2 mM after the first 24 h of culture. Cultures were then washed twice with culture medium and led to grow for the following 3 or 24 h. Cultures were quickly washed in ice-cold PBS. 50 µL of ice-cold sample buffer (4% SDS, 20% glycerol, 0.2 M dithioethreitol, 125 mM Tris–HCl pH 6.8) were added to the cultures and then cells were scraped off the dish with a plastic cell scraper. Cell extracts were then recovered in a tube, centrifuged, and boiled for 5 min. The amount of protein in each sample was determined according to Bradford method (Bradford, 1976), using bovine serum albumin as a standard. Equal amounts of protein were loaded on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to PVDF membranes. The proteins immobilized on the membranes were immediately blocked for 1 h at room temperature with 5% non-fat dry milk in Tris buffered saline-Tween 20 solution (0.001%) (TBS-T). Then the membranes were incubated for 12 h at 37 °C

with one of the following antibodies: anti-desmin, anti-MyoD1, anti-p21, anti-p53 or anti-M-cadherin. After 5 washes in TBS-T (3 min each), the membranes were incubated for 1 h at 37 °C with either an anti-rabbit or an anti-mouse peroxidase conjugated antibodies (Amersham, dilution 1:10,000 in TBS-T), washed again as described above and the bands were visualized using the ECL plus Western Blotting Detection System (Amersham). To check sample loading, other membranes with the same samples were incubated with a mouse monoclonal anti-α-tubulin antibody (Sigma, dilution 1:3000 in TBS-T-milk). After 5 washes in TBS-T (3 min each), membranes were incubated with anti-mouse peroxidase conjugated antibody (Amersham, dilution 1:10,000 in TBS-T) and developed as described above. Quantification of protein bands was performed using the public domain software ImageJ (http://rsb.info.nih.gov/ij/) with data obtained from three independent experiments.

2.8. Statistical analysis

All the values were represented as the means $\pm$ standard deviation. Statistical comparisons were performed by Student's t-test. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Cholesterol depletion enhances cell proliferation in myogenic cell cultures

Our previous studies showed that cholesterol depletion by methyl-β-cyclodextrin (MCD) enhances chick myogenic proliferation and differentiation after 72 h of treatment (Mermelstein et al., 2005). To further characterize the effects of MCD on the proliferation of myogenic cells, we quantified the total amount of DNA present in chick myogenic cells using a Nanodrop spectrophotometer (Fig. 1). Chick myogenic cells were plated at different densities (0.8×10^5, 1.5×10^5 and 3.0×10^5 cells/well) and after the first 24 h they were treated with MCD for 30 min. After MCD exposure, cultures were grown for the next 1, 3 or 6 h and their DNA content was analyzed in a Nanodrop spectrophotometer. Quantification of DNA shows that MCD treatment enhances the total amount of DNA in all cell density conditions analyzed
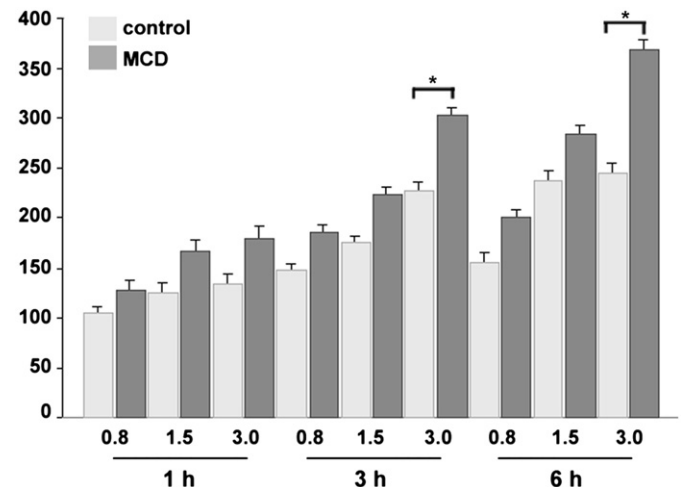
Fig. 1. Cholesterol depletion enhances the total amount of DNA in myogenic cell cultures. Primary cultures of chicken myogenic cells were plated at different densities (0.8×10^5, 1.5×10^5 and 3.0×10^5 cells/dish) and treated with MCD for 30 min. After MCD exposure, cultures were grown for the next 1, 3 or 6 h and their DNA content was analyzed in a Nanodrop spectrophotometer. (*$P < 0.05$; Student's t-test; compared with control; $n=3$).

(Fig. 1), but a higher increase in the total amount of DNA was observed after 3 and 6 h of MCD treatment in the higher cell density condition. So, after these initial results we decided to concentrate our analysis in two time points: a short period of time after MCD treatment (3 h after MCD) and a long period of time after MCD (24 h after MCD).

We then decided to evaluate the expression of phospho-histone H3, which is a cellular marker for cell proliferation, since histone H3 is specifically phosphorylated during mitosis. Primary chick myogenic cells were grown for 24 h, treated with MCD for 30 min and grown for the next 3 or 24 h. Cells were fixed with 4% paraformaldehyde and stained with an antibody against the phospho-histone H3 protein (Fig. 2). By the quantification of the percentage of phospho-histone H3-positive cells in each culture condition, we estimated a 1.5-fold increase in phospho-histone H3-positive cells after 3 h of MCD treatment. These results, in conjunction with the data shown in Fig. 1, show that cholesterol depletion by MCD enhances the proliferation of myogenic cells.

In order to confirm MCD-induced effects on cell replication, we probed the effects of the anti-mitotic agent cytosine arabinoside (ara-c) in primary chick myogenic cultures (Fig. 3). Two different experiments were performed: (1) cells were grown for 24 h, treated with MCD for 30 min and exposed to ara-c for 24 h; (2) cells were exposed to ara-c 1 h before MCD treatment. As previously reported by our group (Mermelstein et al., 2005), MCD treatment (without ara-c) induces a 50% enhancement in the total number of nuclei present in chick myogenic cell cultures

(Fig. 3). Treatment with ara-c alone induces a 60% decrease in the total number of nuclei present in these cultures (Fig. 3). Treatment with ara-c immediately after MCD resulted in a 90% decrease in the number of nuclei, while treatment with ara-c 1 h before MCD treatment resulted in a 20% decrease in the number of nuclei (Fig. 3). These experiments tell us that ara-c can inhibit the myogenic cell proliferation induced by MCD only when used immediately after MCD treatment.

3.2. Cholesterol depletion has no effect on cell viability

To determine whether cholesterol depletion by MCD has any effect on cell viability, experiments using the Live/Dead assay (Invitrogen) were performed in untreated and MCD-treated chick primary myogenic cultures grown for 3 and 24 h after MCD. Live/Dead viability assays showed that all untreated and MCD-treated cells were fluorescein diacetate (FDA) positive and propidium iodide (PI) negative (data not shown). These results show that MCD treatment do not induce cell death.

3.3. Cholesterol depletion enhances the number of desmin-positive cells in myogenic cultures

Since the results of Figs. 1 and 2 show that MCD enhances cell proliferation, we asked whether cholesterol depletion was affecting both myogenic and non-myogenic (fibroblastic) cells. Desmin is one of the first structural proteins that are expressed in myoblasts and it
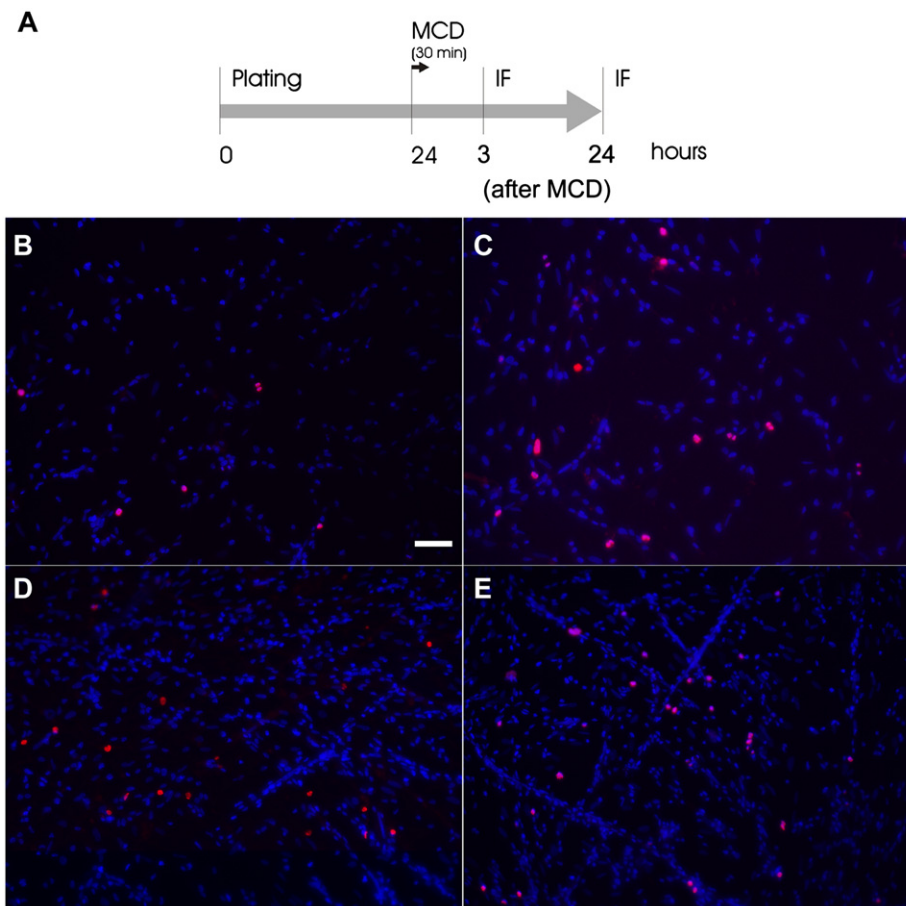

Fig. 2. Cholesterol depletion enhances cell proliferation in myogenic cell cultures. A schematic representation of the experiments is shown in (**A**). Primary cultures of chicken myogenic cells were grown for 24 h, treated with MCD (2 mM) for 30 min and grown for the next 3 or 24 h. Cells were fixed and immunostained (**IF**) with an anti-phospho-histone H3 antibody (red) and with the nuclear dye DAPI (blue). (B) 3 h control cells, (C) 3 h MCD-treated cells, (D) 24 h control cells, and (E) 24 h MCD-treated cells. Scale bar (**B**) represents 50 μm. Quantification of the percentage of phospho-histone H3-positive cells/field in each culture condition revealed a 1.5-fold increase in phospho-histone H3-positive cells after 3 h of MCD treatment. At least 50 microscopic fields for each culture condition were scored in at least three independent experiments.
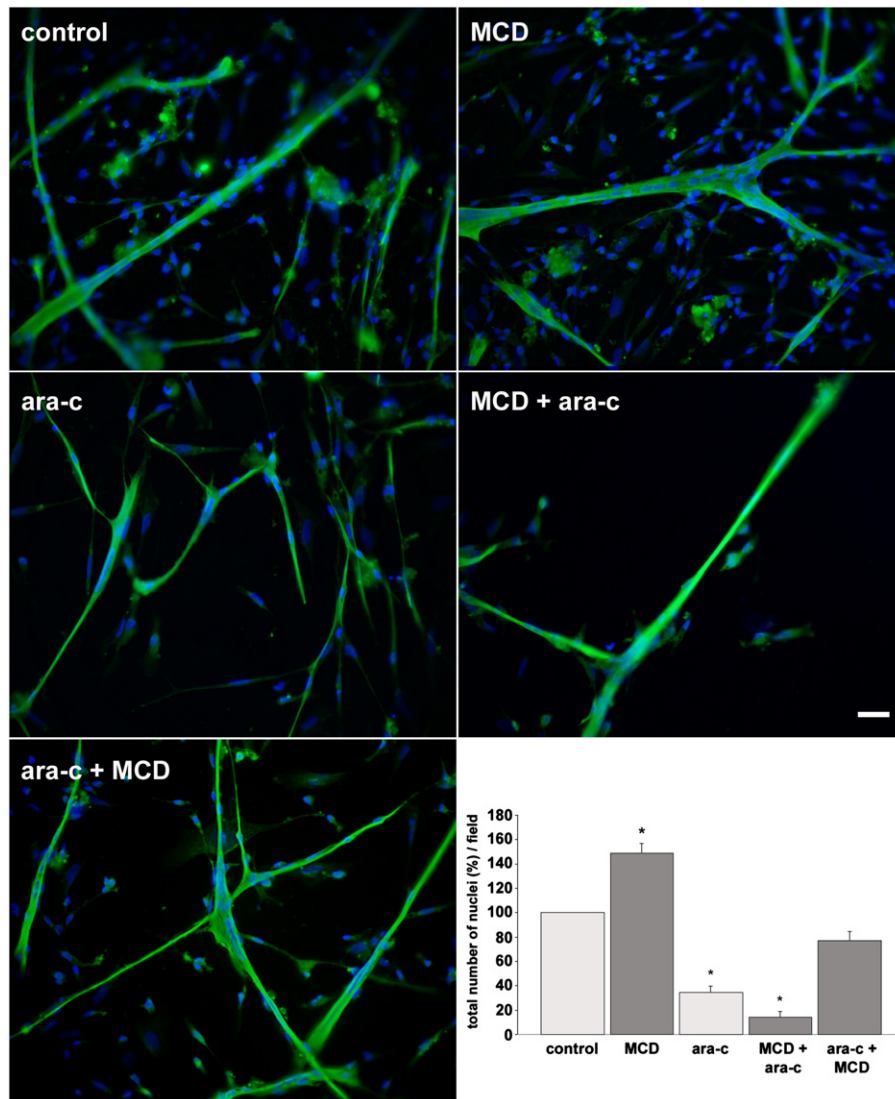
Fig. 3. Inhibition of cell proliferation blocks MCD-induced effects on myogenic differentiation. Primary cultures of chicken myogenic cells were grown for 24 h and treated as described: treated with MCD (2 mM) for 30 min and grown in normal medium for 24 h; treated with ara-c for 24 h; treated with MCD (2 mM for 30 min) and treated with ara-c for 24 h; treated with ara-c 1 h before MCD treatment (2 mM for 30 min) and then grown in normal medium for 24 h. All culture conditions were fixed with 48 h and double-stained with anti-desmin antibody (green) and the nuclear probe DAPI (blue). Scale bar represents 50 μm. The total number of nuclei per field (percentage) was quantified in each culture condition. (*$P < 0.05$; Student's t-test; compared with control; $n=3$). At least 50 microscopic fields for each culture condition were scored in at least three independent experiments.

is expressed after the commitment of cells to the myogenic program by master switch genes, such as MyoD (Costa et al., 2004; Holtzer et al., 1991). Therefore, desmin can be used as an early marker of muscle differentiation. Primary chick myogenic cells were grown for 24 h, treated with MCD for 30 min and grow for the next 3 or 24 h. Cells were fixed with 4% paraformaldehyde and double-stained with an anti-desmin antibody and with the nuclear probe DAPI (Fig. 4). By the quantification of the average number of desmin-positive and desmin-negative mononucleated cells/field, we estimated a 40% increase in desmin-positive cells after 3 and 24 h of MCD treatment (Fig. 4C), compared to untreated counterparts. A decrease in desmin-positive cells was found both in 48-h untreated cultures as well as in 48-h MCD-treated cultures, as compared to 24 h-cultures (Fig.4C). This decrease in desmin-positive mononucleated cell is concomitant with the increase in multinucleated cells (myotubes), which resulted from cell fusion. In contrast, no significant differences were found in the average number of desmin-negative cells/field after 3 or 24 h of MCD treatment, compared to untreated cultures. Overall these results clearly show that cholesterol depletion by MCD enhances the cell proliferation of desmin-positive myogenic cells.

3.4. Cholesterol depletion enhances the number of desmin-positive/BrdU-positive cells

During myogenesis, myoblasts cease replication after the expression of desmin. Kaufman and Foster (1988) have shown that only a small proportion of replicating chicken embryo myoblasts express desmin in contrast to mammalian myoblasts. To analyze the relationship between cell cycle and desmin expression, we decided to study the type of cells (myogenic and/or fibroblastic) that was induced to proliferate after MCD treatment by analyzing BrdU incorporation in these myogenic cultures. BrdU, a thymidine analog, becomes stably incorporated into DNA during the S-phase of mitosis, and thus allows the quantification of dividing cells. Untreated and MCD-treated 24 h myogenic cells were labeled (right after MCD treatment) with BrdU (3 ug/ml) for 1 h, fixed with 4% paraformaldehyde and double-stained with anti-BrdU and anti-desmin antibodies (Fig. 5A). BrdU-positive nuclei were found in desmin positive-myoblasts and desmin negative-fibroblastic cells, but we estimated a 2-fold increase in the percentage of desmin-positive cells incorporating BrdU after MCD treatment (Fig. 5B).
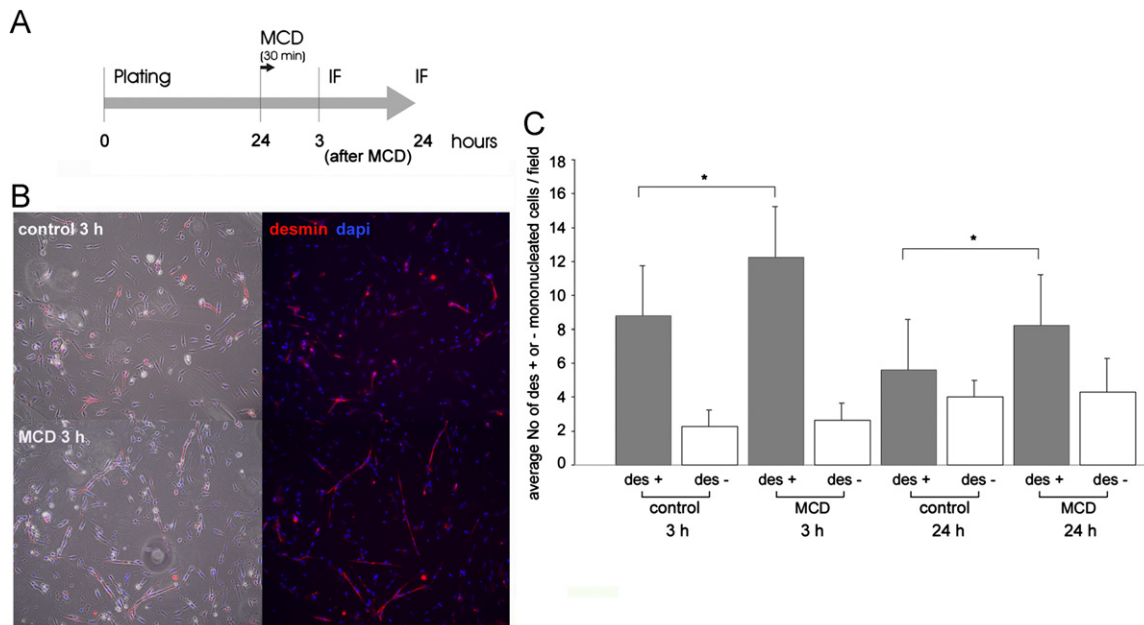
Fig. 4. Cholesterol depletion enhances the number of desmin-positive cells in myogenic cultures. A schematic representation of the experiments is shown in (**A**). Primary cultures of chicken myogenic cells were grown for 24 h, treated with MCD (2 mM) for 30 min and grown for the next 3 or 24 h. Cells were fixed and double-stained (**IF**) with an anti-desmin antibody (red) and with the nuclear probe DAPI (blue). Quantification of the average number of desmin-positive and desmin-negative mononucleated cells/field revealed a 40% increase in desmin-positive cells after 3 and 24 h of MCD treatment, whereas no differences were found in the average number of desmin-negative cells/field compared to untreated cultures (**C**). (*$P < 0.05$; Student's t-test; compared with control; $n = 3$). At least 50 microscopic fields for each culture condition were scored in at least three independent experiments.
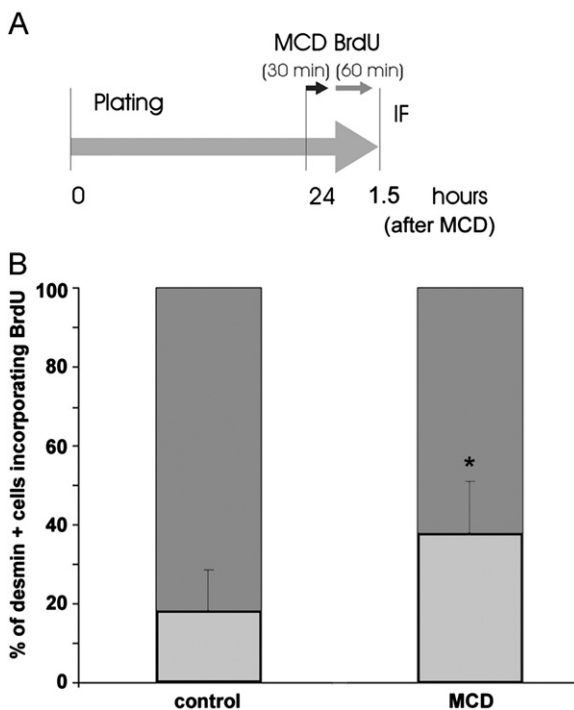

Fig. 5. Cholesterol depletion induces the incorporation of BrdU into desmin positive-mononucleated cells. A schematic representation of the experiments is shown in (**A**). MCD-treated and control myogenic cells (with 24 h) were labeled with BrdU (3 µg/ml) for 1 h. Cells were then fixed with 4% paraformaldehyde and double-stained with anti-BrdU and anti-desmin antibodies. The ratio of the number of desmin-positive/BrdU-positive cells relative to the total number of BrdU-positive cells was recorded in control (untreated) cultures and in MCD-treated cells (**B**). (*$P < 0.05$; Student's t-test; compared with control; $n = 3$). At least 50 BrdU-positive cells for each culture condition were scored in three independent experiments.

Since our group has previously shown that myotubes formed after MCD treatment display more nuclei than untreated cells (Mermelstein et al., 2005, 2007), we can conclude that cholesterol depletion by MCD enhances the proliferation of fusion competent desmin-positive cells.

3.5. Cholesterol depletion enhances the expression of desmin in myogenic cultures

To quantify the amount of desmin protein expression after cholesterol depletion, we performed Western blotting (Fig. 6A). Cells were grown for 24 h, treated with MCD for 30 min and grown for the next 3 or 24 h. Cell extracts were analyzed by 10% SDS-PAGE followed by immunoblotting. Quantification of immunoblots revealed a 4-fold increase in the expression of desmin when comparing control 48 h-cultures with control 24 h-cultures that can be explained by the expected increase in the number of myogenic cells in 48 h cultures. 3 h treatment of MCD induced a 60% increase in the levels of desmin expression when compared to control cells (Fig. 6B), compared to a 14% increase in desmin expression in cells after 24 h of MCD treatment when compared to control cells (Fig. 6B).

Next we asked whether MCD effects on myogenic cell proliferation was independent of cell differentiation. Low initial myoblast cell densities lead to a reduction in cell fusion and a decrease in the formation of fully differentiated multinucleated myotubes. High serum content in the media also inhibits differentiation. Therefore, we used a culture media with fetal bovine serum (instead of horse serum) and different initial cell densities to study the effects of cholesterol depletion independent of differentiation. Myogenic cells were plated at the following densities: 1.8×10^5 (low density), 3.6×10^5 (medium density) and 7.5×10^5 (high density). 24 h-cultures were treated with MCD and after 3 h cells were double labeled with an anti-desmin antibody and with the nuclear dye DAPI (Fig. 7). We observed an 2.5, 1.5 and 2-fold increase in the total number of
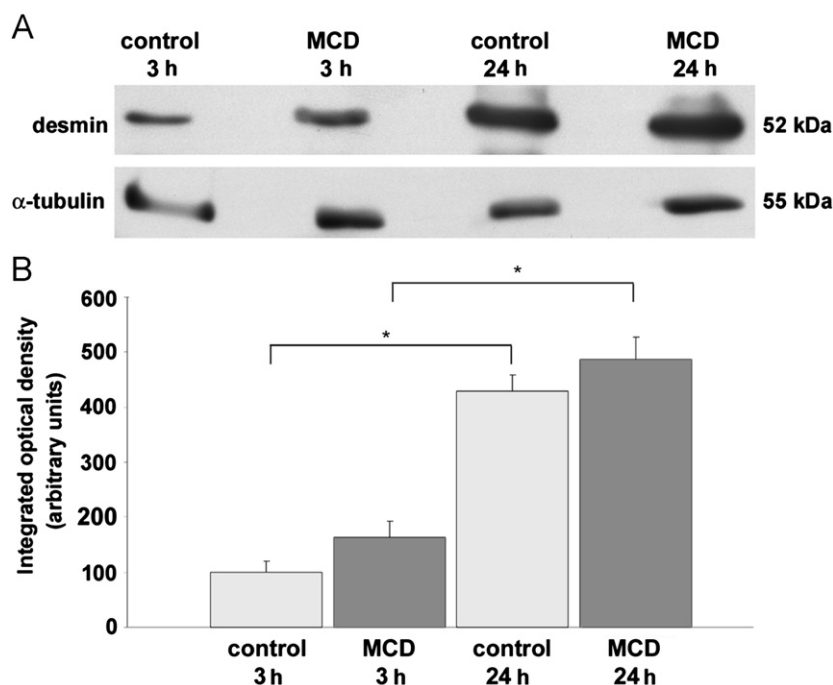
Fig. 6. Cholesterol depletion enhances the expression of desmin in myogenic cultures. Total cell extracts from primary cultures of chicken myogenic cells were analyzed by Western blotting using anti-desmin antibody (**A**). Cells were grown for 24 h, treated with MCD (2 mM) for 30 min and grown for the next 3 or 24 h. Quantification of immunoblots revealed a 60% increase in the levels of desmin expression (52 kDa bands) in cells after 3 h of MCD treatment when compared to control cells, and a 14% increase in desmin expression in cells after 24 h of MCD treatment when compared to control cells (**B**). An 4-fold increase in the expression of desmin was observed when comparing control 48 h-cultures with control 24 h-cultures, and can be explained by an increase in the number of fully differentiated myotubes in 48 h cultures (**B**). (*P < 0.05; Student's t-test; compared with control; n = 3). One representative image of this experiment is shown on the top and the semi-quantitative analysis is shown on the bottom, representing the average of three independent experiments.

nuclei/field in MCD-treated cultures plated at low, medium and high densities, respectively, as compared to control cultures plated at the same densities (Fig. 7). These results show that the effects of MCD on replication are independent of differentiation and of cell density.

3.6. Influence of cholesterol depletion on MyoD, p53 and p21 expression

The results described above suggest that MCD interferes with myoblast proliferation. To compare the expression of muscle regulatory factors with cell cycle regulators, we analyzed MyoD, p53 and p21 expression by immunoblotting

The expression of MyoD1 was examined after 3 h of MCD treatment in extracts of cultured chick myogenic cells by immunoblotting (Fig. 8A). Quantification of immunoblots revealed a 2.5-fold increase in the levels of MyoD1 expression in MCD treated-cells when compared to control cells (Fig. 8B). These results are in accordance with the observed increase in myogenic cell proliferation induced by MCD.

Progression through cell cycle is regulated by cyclin-Cdk complexes and cyclin-Cdk inhibitors. MyoD blocks replication by directly interfering with cell cycle regulators. To understand the influence of cholesterol depletion in the regulation of cell proliferation, we analyzed the expression of the cell cycle regulators p53 and p21 after 3 h of MCD treatment in extracts of cultured chick myogenic cells by immunoblotting (Fig. 8A). Cell extracts were analyzed by 10% SDS-PAGE followed by immunoblotting. Quantification of immunoblots revealed a 2.5-fold increase in the levels of p53 expression and no significant change in the levels of p21 expression in MCD treated-cells when compared to control cells (Fig. 8B). Expression of the cyclin-dependent kinase inhibitor p21 in myoblasts correlates with the establishment of the postmitotic state (Andrés and Walsh, 1996). p21 has been shown to be up-regulated by the p53 tumor

suppressor gene in vitro in response to DNA-damaging agents. However, p21 expression can be regulated independently of p53 in various tissues during normal development (Macleod et al., 1995). We can hypothesize that the expression of p21 is independent of p53 in chick muscle cells. Further studies are necessary to understand the relationship between the expression of p21 and p53 in chick myoblasts.

3.7. The Wnt/β-catenin pathway is involved in the proliferation of myogenic cells after cholesterol depletion

Wnt proteins have been shown to have a proliferative role in a variety of cells and tissues, including chick somites (Galli et al., 2004). In the absence of a Wnt signal, GSK3β phosphorylates β-catenin, leading to its proteolytic degradation. Upon binding of Wnt to its receptor, GSK3β kinase activity is inhibited. The inactivation of GSK3β allows β-catenin to enter the nucleus and regulate transcription of Wnt target genes. In a previous work we have shown that cholesterol depletion by MCD activates the Wnt/β-catenin signaling pathway (Mermelstein et al., 2007). MCD promotes the nuclear translocation of β-catenin and a significant increase in its expression. Moreover, the frizzled-related protein Frzb-1 can inhibit the activation of the Wnt pathway induced by MCD. A soluble and active form of Wnt-3 is significantly enhanced in MCD conditioned medium (Portilho et al., 2007). In order to confirm the hypothesis that Wnt/β-catenin signaling pathway is involved in the proliferation of myogenic cells observed after cholesterol depletion, we tested the effects of 6-bromoindirubin-3′-oxime (BIO), a specific inhibitor of GSK3β. Chick primary myogenic cells were treated for 30 min with either MCD (2 mM) or BIO (5 μM). After 24 h, cells were fixed with 4% paraformaldehyde, stained with the nuclear dye DAPI and the total number of nuclei was quantified (Fig. 9). We found a 65% increase in the total number of nuclei after MCD treatment and a
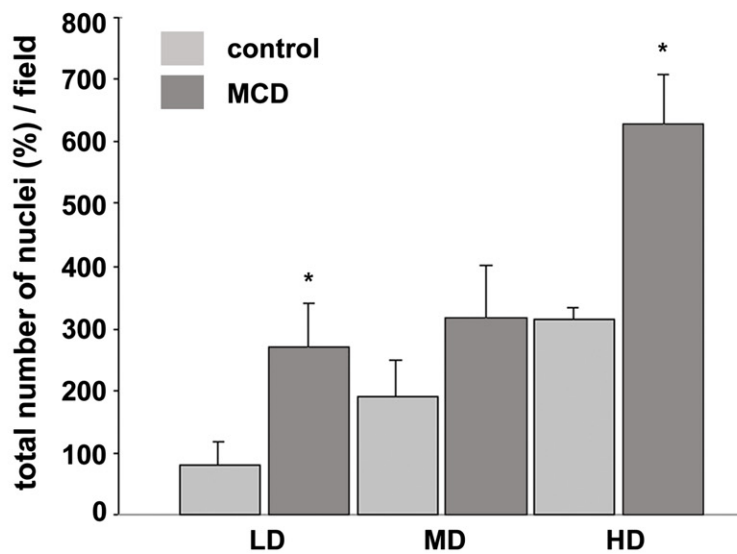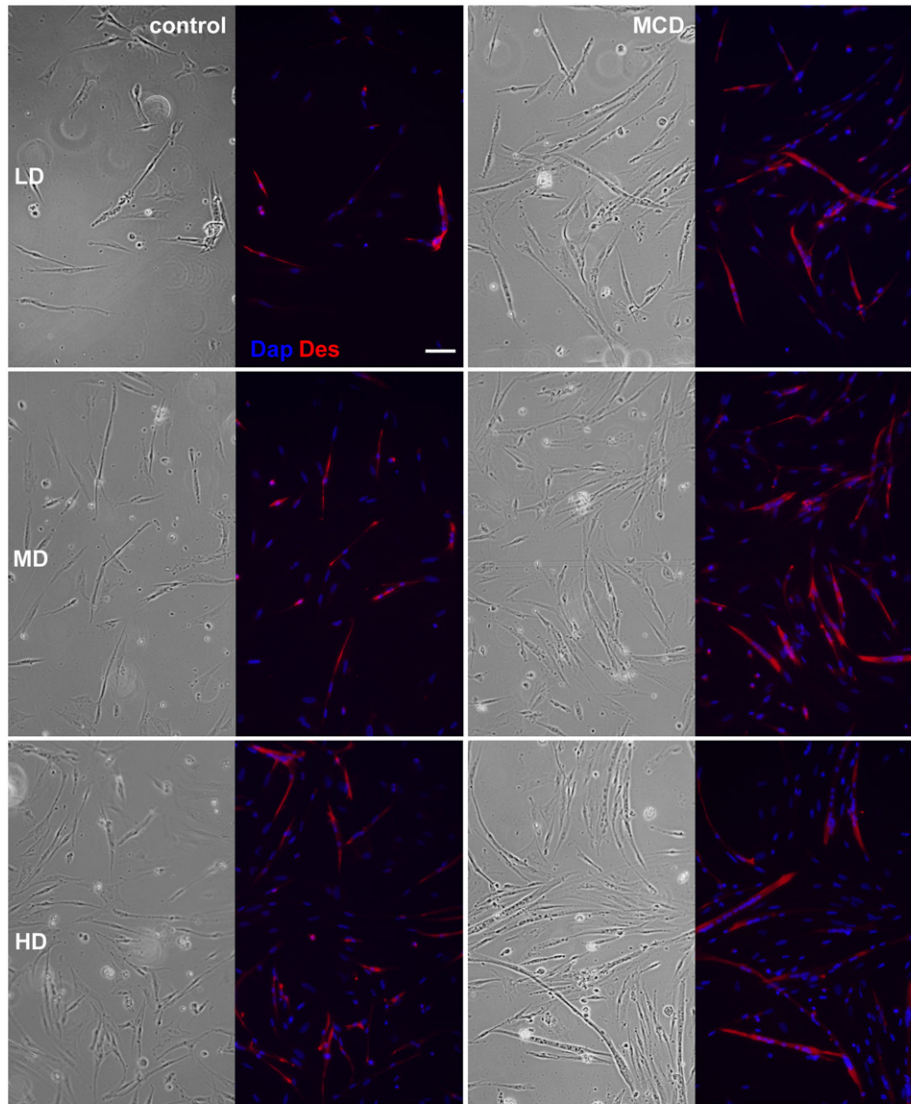
Fig. 7. MCD effects on replication are independent of differentiation. Primary cultures of chicken myogenic cells were plated at the following densities: 1.8×10^5 (low density, LD), 3.6×10^5 (medium density, MD) and 7.5×10^5 (high density, HD). 24 h-cultures were treated with MCD (2 mM) for 30 min and after 3 h cells were double labeled with an anti-desmin antibody (red) and with the nuclear probe DAPI (blue). Scale bar represents 50 μm. The total number of nuclei per field was quantified. Control conditions are represented in light gray and MCD conditions in dark gray. (*$P < 0.05$; Student's t-test; compared with control; $n=3$). At least 50 microscopic fields for each culture condition were scored in at least three independent experiments.
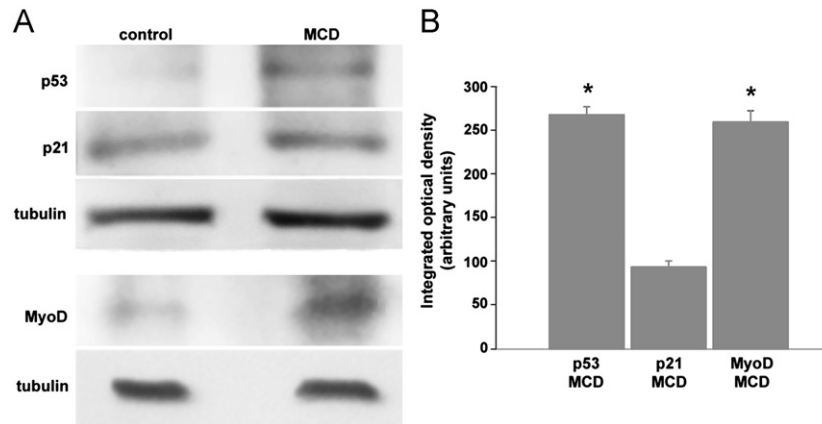
Fig. 8. Influence of cholesterol depletion on MyoD, p53 and p21 expression. Primary cultures of chicken myogenic cells were grown for 24 h, treated with MCD (2 mM) for 30 min and after 3 h cell culture extracts were analyzed by Western blot using antibodies against MyoD, p53 and p21. Quantification of immunoblots revealed a 2.5-fold increase in both MyoD1 and p53, and no significant change in the levels of p21 in MCD treated-cells when compared to control cells. (*$P < 0.05$; Student's t-test; compared with control; $n=3$). One representative image of the experiment is shown (**A**) and the semi-quantitative analysis (**B**) represents the mean of three independent experiments.
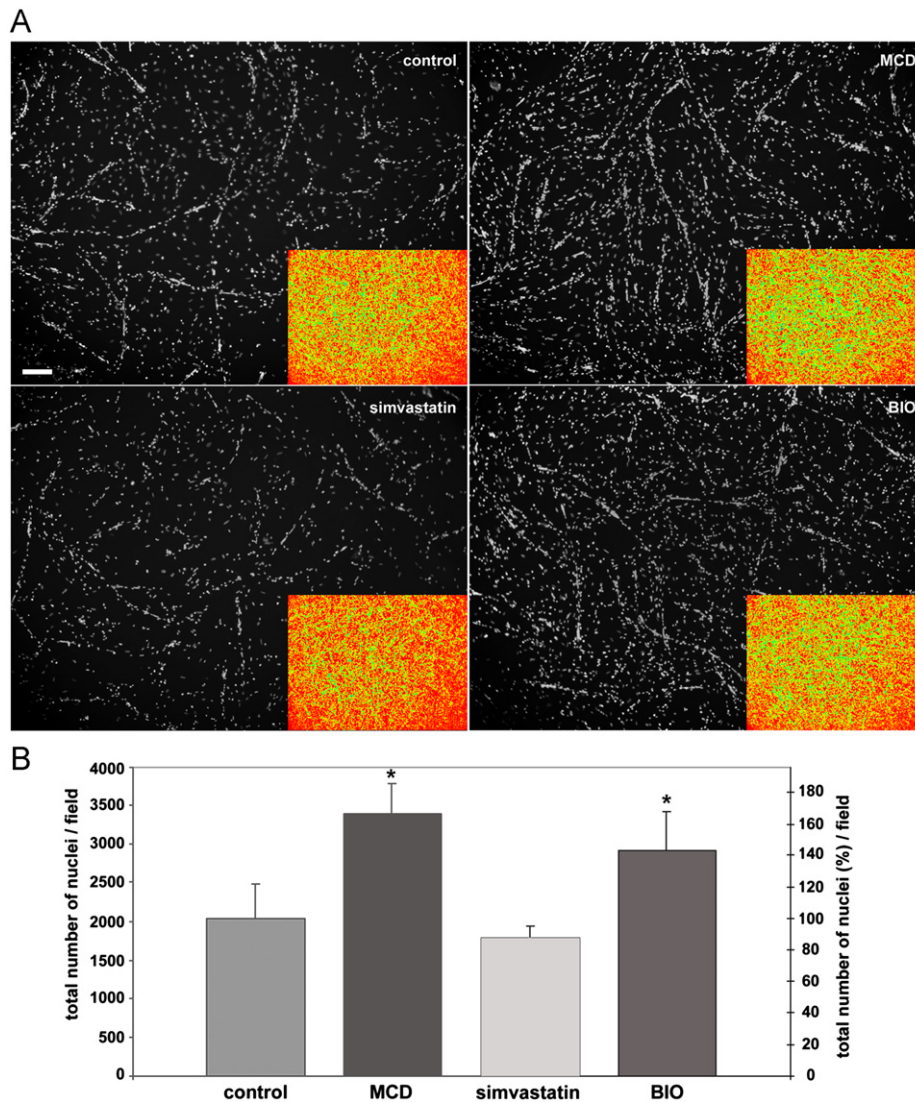

Fig. 9. Simvastatin inhibits and BIO enhances the proliferation of myogenic cells. Primary cultures of chicken myogenic cells were grown for 24 h and then treated with either 2 mM MCD, or 5 μM 6-bromoindirubin-3′-oxime (BIO) or 0.5 μM simvastatin. After 24 h, cells were fixed and stained with the nuclear probe DAPI (A). Scale bar represents 50 μm. Each inset represents the total gray levels (DAPI) of 10 random fields for each condition. The resulting 32 bit images were pseudo-colored with a spectral lookup table, where blue-violet represents the highest values (more nuclei). The total number of nuclei per field was quantified (B). (*$P < 0.05$; Student's t-test; compared with control; $n=3$). At least 50 fields for each culture condition were scored in three independent experiments.

40% increase after BIO treatment, compared to untreated cultures. These findings suggest that the Wnt/β-catenin signaling pathway is involved in the proliferation of myogenic cells observed after cholesterol depletion.

We also used a complementary approach to manipulate cholesterol levels in cells by testing the effects of simvastatin, a cholesterol-lowering drug. Simvastatin is a potent inhibitor of the 3-hydroxy 3-methylglutaryl coenzyme A reductase (HMG-CoAR), a key enzyme of the cholesterol biosynthetic pathway. 24 h-chick primary myogenic cells were treated for 30 min with simvastatin (0.5 μM). After 24 h, cells were fixed with 4% paraformaldehyde, stained with the nuclear dye DAPI and the total number of nuclei was quantified (Fig. 9). We found a 20% decrease in the total number of nuclei after simvastatin treatment, compared to untreated cultures. These results are in accordance with the work of van Vliet et al., 1996, which have shown that simvastatin and other cholesterol-lowering drugs have an anti-proliferative effect in cultured myoblasts. Together these results show that simvastatin and MCD have opposite effects in the proliferation of

myogenic cells. These two drugs differently interfere with the levels of cholesterol in the cells: MCD depletes membrane cholesterol and simvastatin inhibits its synthesis. Nevertheless, it has been shown that simvastatin can also interfere in protein glycosilation, which could be involved in the observed inhibition of myoblast proliferation inhibition.

To further evaluate the involvement of the Wnt/β-catenin pathway in the proliferation promoting effect of MCD, we decided to test the effects of Dkk1, a specific inhibitor of the Wnt/β-catenin pathway. Chick primary myogenic cells were treated for 30 min with MCD (2 mM) and then treated with either normal culture media or with recombinant mouse Dkk1 protein. After 24 h, cells were fixed with 4% paraformaldehyde, stained with an anti-desmin antibody and the nuclear dye DAPI. The total number of nuclei per field was quantified (Fig. 10). We found a 75% increase in the total number of nuclei after MCD treatment compared to control cultures. Dkk1 alone induced a decrease of 15% in the number of nuclei per field as compared to control (untreated) cultures. Interestingly, treatment with MCD followed
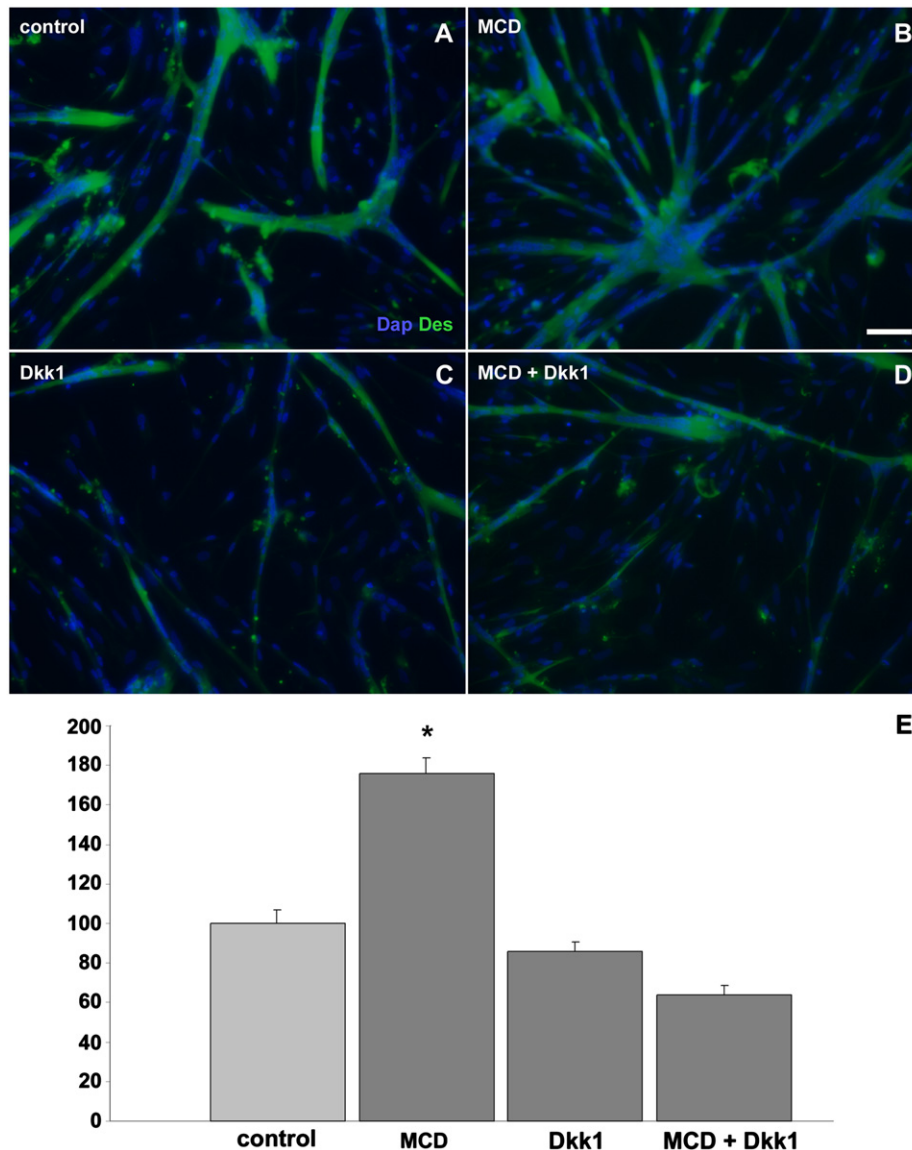

Fig. 10. Dkk1 inhibits the proliferation promoting effect of MCD. Primary cultures of chicken myogenic cells were grown for 24 h and then treated for 30 min with MCD (2 mM) and then treated with either normal culture media (B) or with 0.1 μg/ml of recombinant mouse Dkk1 (D). Some cells were treated only with Dkk1 (C). After 24 h, all cells were fixed with 4% paraformaldehyde, stained with an anti-desmin antibody (green) and the nuclear probe DAPI (blue) and the total number of nuclei per field was quantified (E). Scale bar (B) represents 50 μm. (*$P < 0.05$; Student's t-test; compared with control; $n = 3$). At least 50 fields for each culture condition were scored in three independent experiments.

by Dkk1 induced a decrease of 35% in the number of nuclei per field as compared to control cultures, and a decrease of 65% in the number of nuclei per field as compared to MCD-treated cultures. These results show that Dkk1 can neutralize the proliferation promoting effect of MCD and suggests that the Wnt/β-catenin pathway is involved in the enhancement of proliferation caused by methyl-β-cyclodextrin.

4. Discussion

Our current hypothesis is that cholesterol depletion by methyl-β-cyclodextrin (MCD) treatment increases myoblast proliferation (Fig. 11), which could be caused by the induction of myogenic quiescent satellite cells to enter the cell cycle. Control cells after commitment to the muscle lineage, replicate a specific number of times and express desmin. Afterwards, they elongate and fuse into myotubes. During normal myogenesis, some myoblasts do not differentiate and remain in a non-replicative stage. These satellite cells can be activated through extracellular signals, such as Wnt, to proliferate and complete their differentiation. MCD could be recruiting these quiescent cells to enter the differentiation pathway. This process increases myoblast fusion, with the formation of fully striated myotubes bearing a higher number of nuclei/cell than the observed in untreated myogenic cultures (Fig. 11).

To test if the cells induced to proliferate by MCD are quiescent cells, we analyzed the expression of M-cadherin in myoblast cultures treated with MCD (Fig. 12). M-cadherin is expressed in quiescent satellite cells and thus is considered as an early marker of skeletal muscle lineage (Krauss et al., 2005). Cell culture extracts were analyzed by 10% SDS-PAGE followed by immunoblotting. Quantification of immunoblots revealed an 80% increase in the levels of M-cadherin expression when compared to control cells (Fig. 12). Our results show that cholesterol depletion by MCD induces an increase in the expression of the quiescent myogenic cell marker M-cadherin.

Recently, we have shown that a soluble and active form of Wnt-3a is released in the culture media of chick myogenic cells after cholesterol depletion (Portilho et al., 2007), and that the canonical Wnt/β-catenin signaling pathway is activated in these cells (Mermelstein et al., 2007). Wnt-3a molecules have been shown to have a proliferative role in chick somites (Galli et al., 2004). Thus, it is likely that cholesterol depletion by MCD promotes a release of active and soluble Wnt molecules to the culture medium where it can bind to Frizzled receptors in the surrounding cells and activate the Wnt/β-catenin signaling cascade, which will then regulate the expression of several genes, including β-catenin, cell cycle regulators, and striated muscle-specific differentiation markers (Mermelstein et al., 2005, 2007; Portilho et al., 2007).
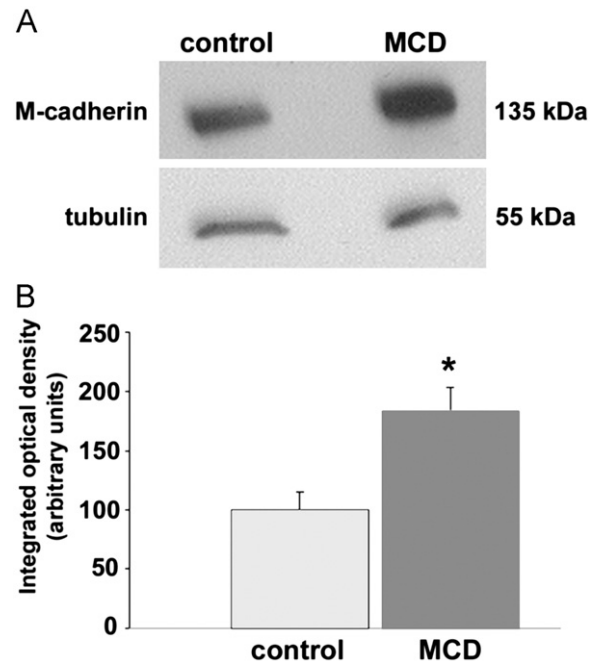

Fig. 12. Cholesterol depletion enhances the expression of M-cadherin. Primary cultures of chicken myogenic cells were grown for 24 h, treated with MCD (2 mM) for 30 min and after 3 h cell culture extracts were analyzed by Western blot using antibodies against M-cadherin. Quantification of immunoblots revealed an 80% increase in M-cadherin levels in MCD treated-cells when compared to control cells. ($^*P < 0.05$; Student's t-test; compared with control; $n=3$). One representative image of the experiment is shown (A) and the semi-quantitative analysis (B) represents the mean of three independent experiments.
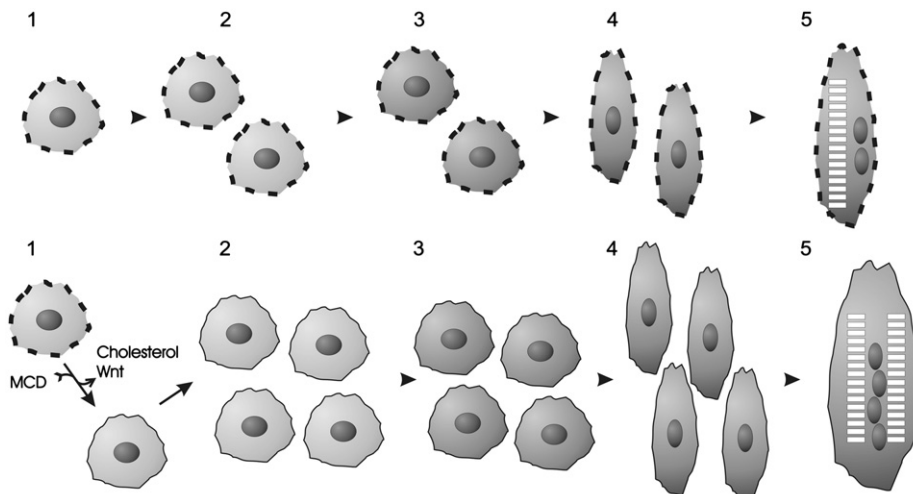

Fig. 11. Schematically, in chick myogenic cultures, round myoblasts change in 24 h into a bipolar morphology and fuse over 48 h to form multinucleated myotubes (top scheme). In the presence of methyl-β-cyclodextrin (MCD), a higher number of myogenic cells are recruited to the formation of multinucleated myotubes with centrally localized nuclei (bottom scheme). Cholesterol depletion by MCD enhances the secretion of Wnt molecules from myogenic cultured cells, inducing the activation of the Wnt/β-catenin pathway. Wnt signaling activates the expression of downstream genes, such as Wnt3, beta-catenin and desmin, that in turn enhances myogenic cell proliferation. The discontinuous lines in the plasma membrane represent the presence of membrane microdomains and the darker cytoplasm in some cells represents the expression of muscle specific proteins.

Our results show that MCD induces an enhancement in proliferation of muscle cells. We had previously shown that MCD also has an effect in muscle differentiation (Mermelstein et al., 2007). We assume that MCD initially enhances proliferation of cells, which will differentiate into muscle fibers afterwards. The decision between proliferation or differentiation varies among different cell types. Serum withdrawal is necessary to induce the switch from proliferation into differentiation in several cell lines. Other cell types, such as hepatocytes, replicate while fully differentiated. Chick primary cultures of skeletal muscle cells necessarily have to go through a proliferative phase followed by a differentiation phase. This switch is autonomous, genetically programed, and independent of changes in the amount of serum present in the growth media.

Methyl-β-cyclodextrin has been widely used as a cholesterol-depleting and raft-disorganizing agent in the last years. Importantly, our results show that MCD also induces myoblast proliferation, and therefore care should be taken when using MCD for the extraction of membrane cholesterol without looking for other consequences, such as cell replication.

5. Conclusions

In summary, the present study provides evidence that cholesterol depletion by methyl-β-cyclodextrin induces the proliferation of desmin-positive myoblast cells. These effects are attributable to the activation of the Wnt/β-catenin pathway. These findings indicate that rapid changes in the cholesterol content in cell membranes of myoblasts can induce cell proliferation.

The induction of myogenic cell proliferation by MCD opens the exciting possibility that MCD might become an attractive therapeutic tool to treat skeletal muscle degenerative pathologies, such as muscular dystrophies, where muscle cell loss is an important concern.

References

Andrés, V., Walsh, K., 1996. Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis. J. Cell Biol. 132, 657–666.

Bertino, B., Knape, W.A., Pytlinska, M., Strauss, K., Hammou, J.C., 1994. A comparative study of DNA content as measured by flow cytometry and image analysis in 1864 specimens. Anal. Cell Pathol. 6, 377–394.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Costa, M.L., Escaleira, R.C., Cataldo, A., Oliveira, F., Mermelstein, C.S., 2004. Desmin: molecular interactions and putative functions of the muscle intermediate filament protein. Braz. J. Med. Biol. Res. 37, 1819–1830.

Galli, L.M., Willert, K., Nusse, R., Yablonka-Reuveni, Z., Nohno, T., Denetclaw, W., Burrus, L.W., 2004. A proliferative role for Wnt-3a in chick somites. Dev. Biol. 269, 489–504.

Hawke, T.J., Meeson, A.P., Jiang, N., Graham, S., Hutcheson, K., DiMaio, J.M., Garry, D.J., 2003. p21 is essential for normal myogenic progenitor cell function in regenerating skeletal muscle. Am. J. Physiol. Cell Physiol. 285, C1019–1027.

Holtzer, H., Dilullo, C., Costa, M.L., Lu, M., Choi, J., Mermelstein, C.S., Schultheiss, T., Holtzer, S., 1991. Striated myoblasts and multinucleated myotubes induced in non-muscle cells by MyoD are similar to normal *in vivo* and *in vitro* counterparts. In: Ozawa, E, Masaki, T, Nabeshima, Y (Eds.), Frontiers in Muscle Research. Elsevier, Amsterdam, pp. 187–207.

Kaufman, S.J., Foster, R.F., 1988. Replicating myoblasts express a muscle-specific phenotype. Proc. Natl. Acad. Sci. USA 85, 9606–9610.

Krauss, R.S., Cole, F., Gaio, U., Takaesu, G., Zhang, W., Kang, J.S., 2005. Close encounters: regulation of vertebrate skeletal myogenesis by cell-cell contact. J. Cell Sci. 118, 2355–2362.

Macleod, K.F., Sherry, N., Hannon, G., Beach, D., Tokino, T., Kinzler, K., Vogelstein, B., Jacks, T., 1995. p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. Genes Dev. 9, 935–944.

Mermelstein, C.S., Portilho, D.M., Medeiros, R.B., Matos, A.R., Einicker-Lamas, M., Tortelote, G.G., Vieyra, A., Costa, M.L., 2005. Cholesterol depletion by methyl-β-cyclodextrin enhances myoblast fusion and induces the formation of myotubes with disorganized nuclei. Cell Tissue Res. 319, 289–297.

Mermelstein, C.S., Portilho, D.M., Mendes, F.A., Costa, M.L., Abreu, J.G., 2007. Wnt/β-catenin pathway activation and myogenic differentiation are induced by cholesterol depletion. Differentiation 75, 184–192.

Portilho, D.M., Martins, E.R., Costa, M.L., Mermelstein, C.S., 2007. A soluble and active form of Wnt-3a protein is involved in myogenic differentiation after cholesterol depletion. FEBS Lett. 581, 5787–5795.

Qin, L., Yang, Y.B., Tuo, Q.H., Zhu, B.Y., Chen, L.X., Zhang, L., Liao, D.F., 2009. Effects and underlying mechanisms of curcumin on the proliferation of vascular smooth muscle cells induced by Chol:MbetaCD. Biochem. Biophys. Res. Commun. 379, 277–282.

Soddu, S., Blandino, G., Scardigli, R., Coen, S., Marchetti, A., Rizzo, M.G., Bossi, G., Cimino, L., Crescenzi, M., Sacchi, A., 1996. Interference with p53 protein inhibits muscle differentiation. J. Cell Biol. 134, 193–204.

van Vliet, A.K., Nègre-Aminou, P., van Thiel, G.C., Bolhuis, P.A., Cohen, L.H., 1996. Action of lovastatin, simvastatin, and pravastatin on sterol synthesis and their antiproliferative effect in cultured myoblasts from human striated muscle. Biochem. Pharmacol. 52, 1387–1392.

Wei, Q., Paterson, B.M., 2001. Regulation of MyoD function in the dividing myoblast. FEBS Lett. 490, 171–178.

Wodarz, A., Nusse, R., 1998. Mechanisms of Wnt signaling in development. Annu Rev. Cell Dev. Biol. 14, 59–88.