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ORIGINAL ARTICLE

Overexpression of ornithine decarboxylase increases myogenic potential of H9c2 rat myoblasts

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Abstract Myoblast differentiation into multinuclear myotubes implies the slow-down of their proliferative drive and the expression of myogenin, an early marker of myogenic differentiation. Natural polyamines—such as putrescine, spermidine and spermine—are low molecular weight organic polycations, well known as mediators involved in cell homeostasis. Many evidences in the literature point to their role in driving cellular differentiation processes. Here, we studied how polyamines may affect the differentiation of the myogenic cell line H9c2 into the muscle phenotype. Cell cultures were committed via a 7-day treatment with insulin which induced increase in the activity of ornithine decarboxylase, the first enzyme in the polyamine biosynthetic pathway, consistent with myogenic differentiation. To evaluate the role of polyamines in the differentiation process, cells were transfected with a plasmid overexpressing a stable ornithine decarboxylase, under control of a constitutive promoter. Overexpressing cells spontaneously differentiate into myotubes, without the need for induction with insulin; multinuclear myotubes and myogenin expression were apparent within 2 days of confluency of cultures. Polyamine depletion—by means of α-difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase—abolished the differentiation process. These observations support the evidence that polyamines are a key step involved in differentiation of muscle cells.

Keywords H9c2 cells · Muscle differentiation · Ornithine decarboxylase · Polyamines · α-Difluoromethylornithine

Abbreviations

BSA	Bovine serum albumin
GM	Growing medium
DM	Differentiation medium
DFMO	α-Difluoromethylornithine
DMEM	Dulbecco's modified Eagle's medium
ODC	Ornithine decarboxylase

Introduction

Natural polyamines such as putrescine (Put), spermidine (Spd) and spermine (Spm) are positively charged aliphatic amines, ubiquitous constituents of eukaryotic cells (Pegg and McCann 1982) and involved in many biochemical processes including cell growth, cell division and cell death (Ackermann et al. 2003; Wallace et al. 2003). In addition, polyamines are also known to be able to drive cellular differentiation, as shown in literature by studies on fibroblasts (Bethell and Pegg 1981) and L6 myoblast (Erwin et al. 1983).

Muscle differentiation is a multistep process involving cell cycle arrest and withdrawal, muscle-specific protein synthesis, myoblast elongation and fusion into

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multinucleated myotubes (Andres and Walsh 1996). When myoblasts are subjected to conditions that induce differentiation, specific signaling cascades are activated and basic helix–loop–helix muscle regulatory transcription factors, such as myogenin, are subsequently induced (Buchberger et al. 1994). The myogenic process is regulated by diverse extracellular factors: basic fibroblast growth factor (FGF)-2 or transforming growth factor (TGF) β1 inhibit (Liu et al. 2001), while insulin or insulin-like growth factor (IGF)-1 and -2 stimulate (Yoshiko et al. 2002) differentiation.

Insulin and IGF-1 and -2 are indeed the most characterized stimuli inducing muscle differentiation (Molkentin and Olson 1996). Accumulating evidences show that insulin or IGFs stimulate muscle differentiation via phosphorylation of insulin receptor substrates (IRS) at multiple tyrosine residues, and subsequent activation of phosphatidylinositol 3-kinase, Akt/protein kinase B (Tureckova et al. 2001) and p38 MAPK (Cuenda and Cohen 1999) signaling cascades. In order to investigate the role of polyamines during myogenic differentiation, we studied the effect of insulin on the activity of ornithine decarboxylase (ODC), the first enzyme in polyamine biosynthetic pathway, while inducing H9c2 embryonal rat myoblasts to differentiate into myotubes. We therefore transfected the cells with a plasmid overexpressing a stable ODC, under control of a constitutive cytomegalovirus promoter, and investigated if the large amount polyamines obtained with the transfection was sufficient to address the cells towards the muscle phenotype. In both settings, experiments also were carried out during polyamine depletion, obtained with α-difluoromethylornithine (DFMO), a specific and irreversible inhibitor of ODC (Shantz and Levin 2007).

Materials and methods

Cell culture and induction of differentiation

H9c2 embryonal rat cells (obtained from European Collection of Cell Cultures, ECACC) were cultured in Dulbecco's modified Eagle's medium (DMEM, Biowhittaker) supplemented with 10% heat-inactivated fetal calf serum (growing medium, GM). Cells were induced to differentiate into myotube by seeding at a density of 5×10^5. One day after cell seeding, GM was replaced with differentiation medium (DM) represented by DMEM plus 200 nM insulin (Sigma). This was considered the day zero of differentiation. Full differentiation—evaluated by the morphological appearance of the cells viewed by phase contrast microscopy, by expression level of the myogenin, a typical early differentiation marker and by measuring fusion index percentage—was achieved in 14 days. Unless otherwise specified, the culture media was replaced by fresh DM every 2 days. In selected experiments, DFMO, a specific and irreversible ODC-inhibitor (Wallace and Fraser 2004) was added to cell at the seeding time.

ODC activity and polyamine content

Cells were washed with PBS and scraped in a buffer consisting of 0.1 mM EDTA, 0.02 mM pyridoxal phosphate, 2.5 mM DTT in 10 mM PBS pH 7.2. The cells were disrupted by freeze-thawing three times and then centrifuged at 11,000 rpm for 15 min. Enzyme activity in the supernatant was measured by estimation of the release of ^{14}C-CO_2 from L-1-^{14}C-ornithine during a 60-min incubation (Pignatti et al. 1999). One unit of enzyme activity corresponds to 1 nmol of decarboxylated substrate/h of incubation. Polyamine content was analyzed using reverse-phase HPLC on samples extracted using 10% trichloroacetic acid, as described previously (Shantz et al. 1992). Polyamine content is expressed as nmol/mg of protein. Polyamine depletion was obtained by culturing the cells in the presence of 100 μM DFMO from the seeding.

Western blotting

H9c2 cells were homogenized in 5 mM DTT, 2 mM EDTA, 0.1% CHAPS, 0.1% Triton X-100, and protease inhibitors in 20 mM HEPES (pH 7.5), diluted 1:1 with loading buffer (2% SDS, 5% glycerol, 0.002% bromophenol blue, 4% β-mercaptoethanol in 0.25 M Tris–HCl pH 6.8), then denatured by boiling for 3 min. Aliquots corresponding to 50 μg protein were analyzed by SDS-PAGE. After blotting, the nitrocellulose membrane was blocked with 5% non-fat dry milk for 1 h, washed with Tris-buffered saline and probed at room temperature for 60 min with specific anti-myogenin, anti-myosin heavy chain, anti-β actin primary antibodies (Santa Cruz), and

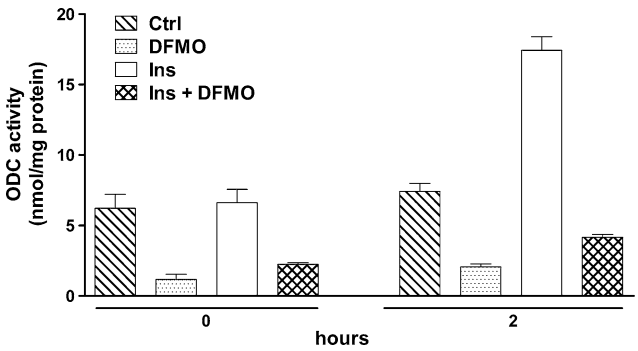

Fig. 1 Effect of insulin on ODC activity. H9c2 cells were grown for 24 h, after plating, in presence or in absence of 100 μM DFMO, then were incubated in GM (Ctrl) or DM (Ins) for 2 h. The cells were collected at the indicated times and analyzed for ODC activity. Data are mean ± SEM ($N = 3$)

anti-STAT-3 (Cell Signaling). After further washing, the membrane was incubated for 45 min with the horseradish peroxidase-conjugated secondary antibody (Santa Cruz). After washing, immunoreactive bands were visualized with an enhanced chemiluminescence kit (GE-Healthcare). Protein expression levels were obtained by a densitometric quantification with GS-800 Calibrated Densitometer and Quantity-One software (BIO-RAD).

Cell transfection

In selected experiments, a clonal H9c2 cell line over-expressing ODC, referred to as OCD^+, was used. Cells were transfected with LipofectAMINE plus reagent (Invitrogen) using a murine ornithine decarboxylase cDNA with a stop codon at position 425 cloned into pZeoCMV vector (Invitrogen) (Shantz and Pegg 1998). Sham transfected cells were used as a control. Stably transfected cells were selected in 375 μg/mL zeocin (Invitrogen).

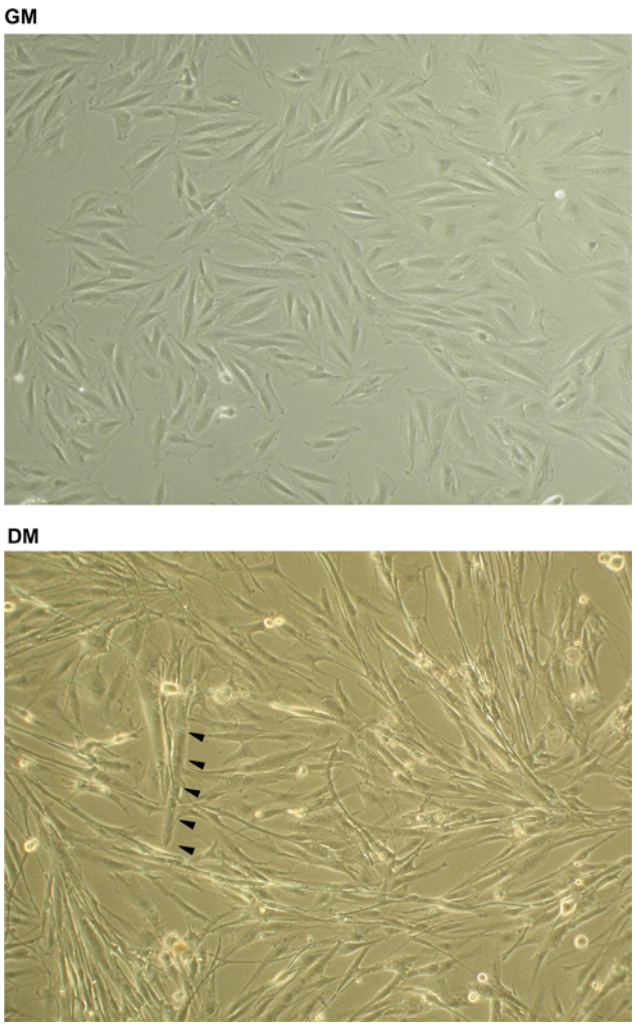

Fig. 2 Morphological changes of insulin-induced myogenic differentiation in H9c2 cells. H9c2 wild type were grown in GM for 24 h at confluence of 80–90% and the day after (day 0) were incubated for 7 days in GM or DM. *Arrows* show one of several myotubes presented in the picture. Photographs were taken at day 7

Immunofluorescence microscopy

For immunostaining analysis, cells were seeded onto glass coverslips and cultured as described above. At the end of incubation times, cells were rinsed with PBS, fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. All preparations were treated with PBS containing 4% bovine serum albumin (BSA) to saturate non-specific binding. Myogenin was visualized incubating

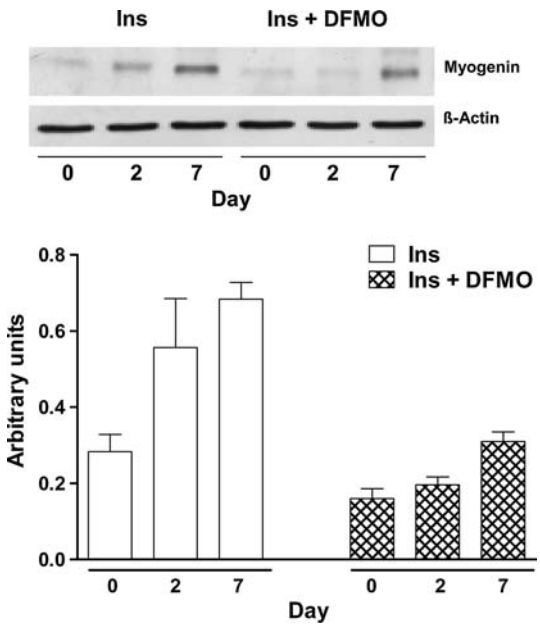

Fig. 3 Expression of myogenin as myogenic differentiation marker. H9c2 cells (*Ins*), at confluence of 80–90%, were induced to differentiate in DM for the indicated days in absence (lanes *1–3*) or in presence of 100 μM DFMO (lanes *4–6*). DFMO was added 24 h before the induction. At the end of incubation times, cell extracts were subjected to immunoblotting. *β*-actin was used as loading control. *Bar charts* show the quantification of myogenin/*β*-actin protein ratios in arbitrary units. Data are mean ± SEM ($N = 3$)

Table 1 ODC activity and polyamine levels in ODC^+ versus control H9c2 cells

Cells	ODC activity (pmol/30 min per mg protein)	Polyamines (nmol/mg protein)		
		Put	Spd	Spm
ODC^+	515 ± 77	13.4 ± 0.5	6.2 ± 0.8	3 ± 1.2
Control	11 ± 2*	3.3 ± 0.5[§]	3 ± 0.6[#]	1.3 ± 1

Values are mean ± SEM, $n = 4$

Put putrescine, *Spd* spermidine, *Spm* spermine

* $P < 0.01$ versus ODC^+; [§] $P < 0.01$ versus ODC^+; [#] $P < 0.05$ versus ODC^+

coverslips with diluted (1:100 in 1% BSA in PBS) anti-myogenin mouse monoclonal antibody overnight at 4°C. After washing with PBS, cells were incubated for 45 min with anti-mouse secondary antibody (5 μg/ml), biotin conjugated, in PBS containing 2% BSA. The fluorescent signal was amplified using the enzymatic reaction streptavidin-phosphatase by ELF Cytological Labeling Kit (Invitrogen) according to the manufacturer's directions. Total nuclei were stained with 4′-6-diamidino-2-phenylindole (DAPI) (Sigma) at the final concentration of 1 μg/ml. After extensive washing in PBS, the coverslips were mounted with Gel Mount (Sigma) and analyzed by a IX50 Olympus inverted fluorescence microscope equipped with a digital camera.

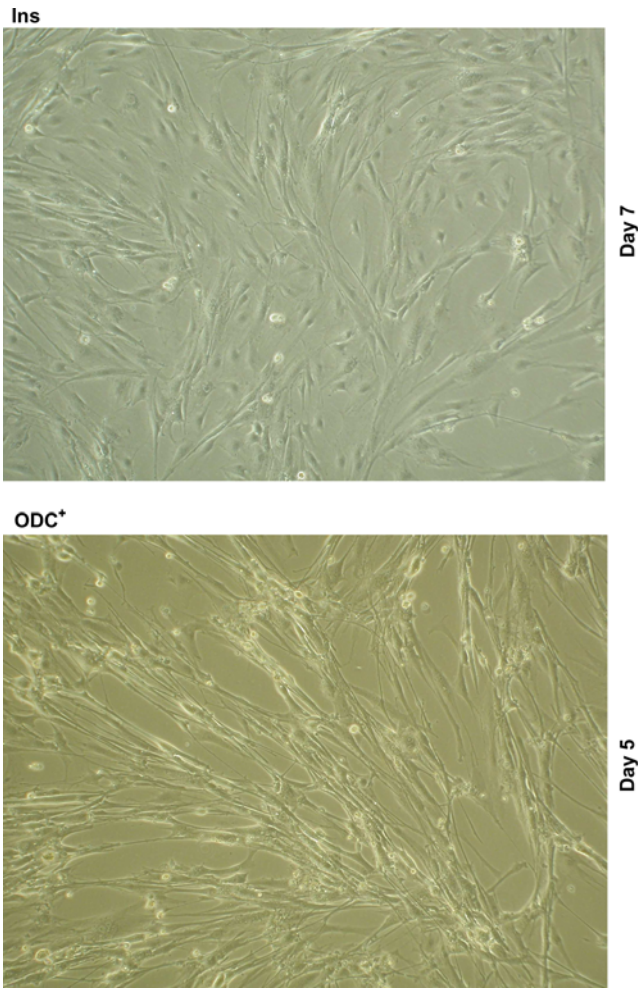

Fig. 4 Overexpression of ODC increases myogenic potential of H9c2 cells. Wild-type H9c2 and ODC-overexpressing cells were induced to differentiate, in DM and GM, respectively. Photographs were taken at day 7 for H9c2 treated with insulin and day 5 for ODC$^+$ without external stimuli

Statistical analysis

All data are expressed as means ± SEM of the indicated numbers of determinations. All the presented experiments were performed at least three times. Statistical analysis was performed using GraphPad Prism ver. 4 (http://www.graphpad.com). Comparison between two groups of measurements was performed using the Student *t* test. Differences were considered significant for $P < 0.05$.

Results

Effect of insulin on ODC activity

In order to address whether polyamines are involved in myogenic differentiation, H9c2 cells were cultured in GM or in DM, in absence or in presence of DFMO. Figure 1 shows that ODC activity in H9c2 cells increases more than twofold after 2 h of insulin treatment. The induction of ODC activity was completely blocked in the cells pre-treated with DFMO, an irreversible and specific inhibitor of ODC; DFMO is also able to reduce ODC activity as in basal cells, as well in H9c2 cells treated with insulin.

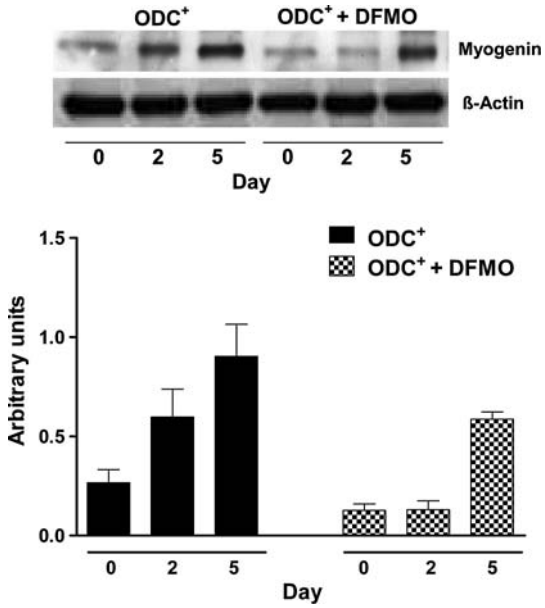

Fig. 5 Western blot of myogenin expression in ODC$^+$. Cells were induced to spontaneously differentiate, in GM for the indicated days; either in absence (lanes *1–3*) or in presence of 100 μM DFMO (lanes *4–6*) added at the seeding time. At the end of incubation times, cell extracts were subjected to immunoblotting to detect myogenin protein expression and *β*-actin was used as loading control. *Bar charts* show the quantification of myogenin/*β*-actin protein ratios in arbitrary units. Data are mean ± SEM ($N = 3$)

Insulin induces myogenic differentiation of H9c2 cells

Once confluence of H9c2 cells occur, they spontaneously withdraw from cycling and differentiate into non-proliferating cells, characterized by morphological changes such as myoblast alignment, elongation and a typical fusion into multinucleated myotubes. This spontaneous differentiation is completed 14 days after confluency (data not shown). H9c2 cells, exposed to insulin, completely differentiate into myotubes after 7 days, as shown in Fig. 2, where morphological differences (black arrows indicate multinucleated cells) are prominent in insulin-treated cells versus control. Differentiation was confirmed by determining the amount of the transcription factor myogenin, an early marker of myogenic differentiation, by immunoblotting. We observed that insulin increases myogenin expression in H9c2 cells (Fig. 3). DFMO, consistent with its ability to reduce ODC activity induced by insulin in H9c2 cells, as shown in Fig. 1, also delays the increase in myogenin expression driven by insulin treatment (Fig. 3).

Overexpression of ODC induces myogenic differentiation of H9c2 cells

To evaluate if polyamines themselves may drive the differentiation process, cells were transfected with a plasmid overexpressing ODC under control of a constitutive cytomegalovirus promoter. As shown in Table 1, H9c2 cells overexpressing ODC activity (ODC^+) show a significant increase in polyamine synthesis with respect to control. Moreover, differentiation of overexpressing cells into myotubes occurs without the need for any external stimulus and faster (day 5) than in the wild-type cells treated with insulin (day 7) (Fig. 4). To confirm the differentiative role

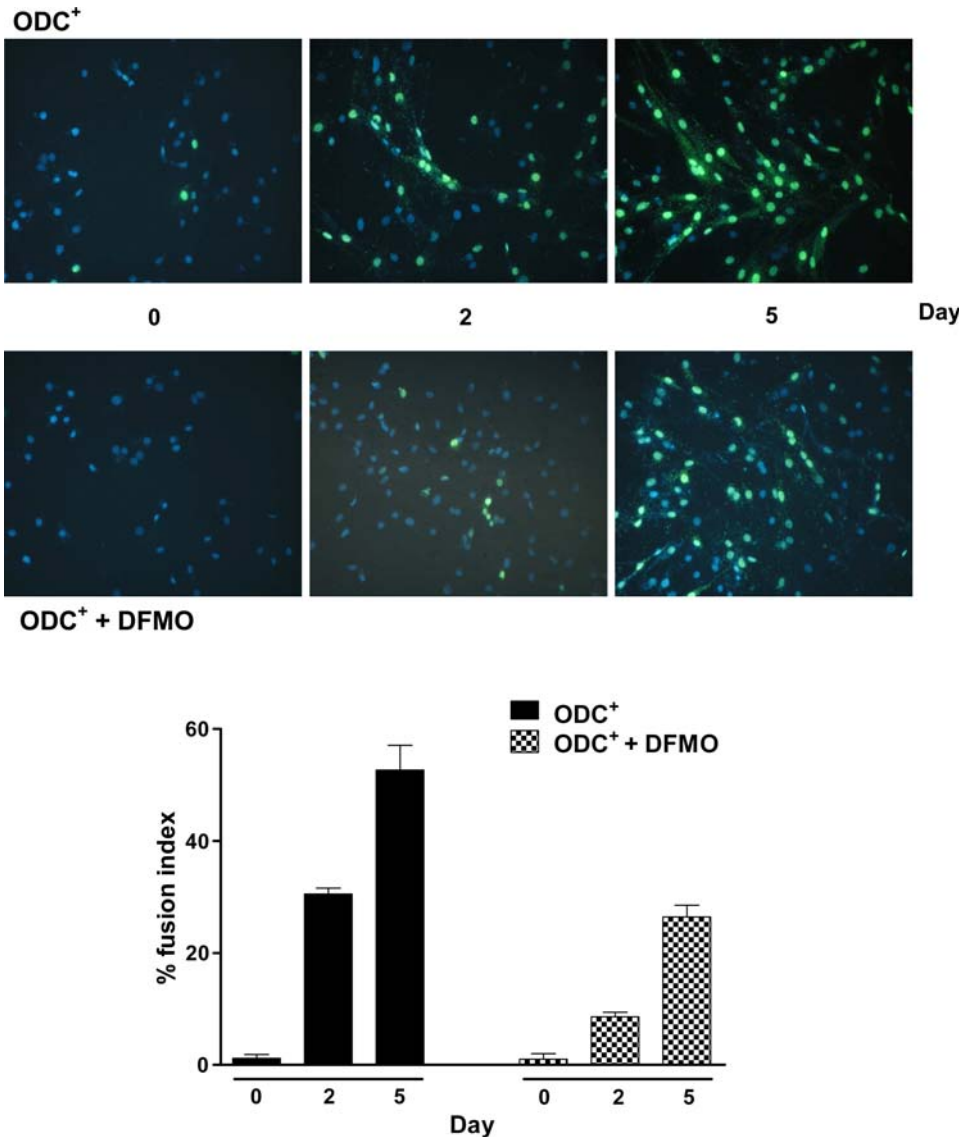

Fig. 6 Effect of DFMO inhibition of myogenic differentiation in ODC^+. Cells grown for 24 h after seeding, in the presence or absence of 100 μM DFMO, were induced to spontaneously differentiate in GM for the indicated days. DFMO was added 24 h before the induction. At the end of the incubation times, cells were immunostained with anti-myogenin mouse monoclonal antibody. Total nuclei were stained with DAPI. Pictures were taken after seeding (day 0) and at days 2 and 5. Histogram shows the fusion index percentage [100 × (myogenin-positive nuclei number/total nuclei number)] used to quantify the differentiation process. Data are mean ± SEM ($N = 4$)

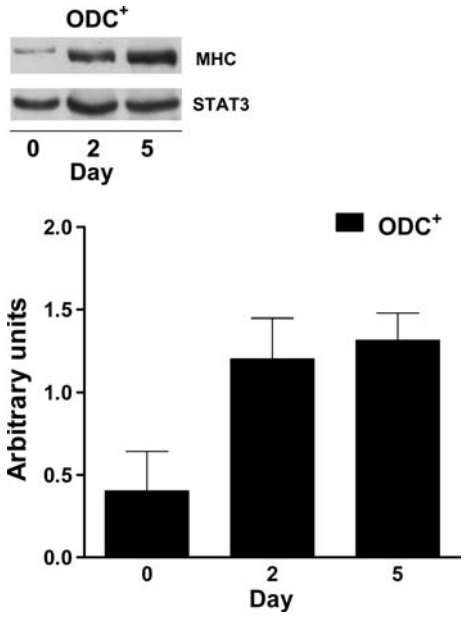

Fig. 7 Expression of myosin heavy chain (MHC) protein in ODC$^+$. Cells were induced to spontaneously differentiate into GM for the indicated days. At the end of incubation times cell extract were prepared and subjected to western blot to detect MHC protein expression. STAT-3 was used as loading control. *Bar charts* show the quantification of MHC/STAT-3 protein ratios in arbitrary units. Data are mean ± SEM ($N = 3$)

played by polyamines, DFMO treatment slowed down ODC$^+$ cell differentiation. Immunoblotting data (Fig. 5) show the anti-differentiative effect caused by DFMO treatment, evaluated as a reduced myogenin expression. As a further evidence of myogenic differentiation driven by ODC overexpression, the fusion index percentage—taken as the proportion of myogenin-positive nuclei in polynucleated cells relative to the total number of nuclei—was evaluated in ODC$^+$ cells. In accordance with immunoblotting results, immunostaining data show that early after seeding (day 2) ODC$^+$ cells show a spontaneous induction of the differentiative process. Again, treatment with DFMO is able to blunt this effect (Fig. 6).

Finally, western blotting evaluation of marker of advanced cell differentiation into the skeletal muscle phenotype, i.e. the sarcomeric myosin heavy chain (MHC), also showed that ODC overexpression was sufficient to induce the cells to show a large increase of their signal within day 2 after seeding (Fig. 7).

Discussion

The role of polyamines in regulating cell cycle, cell division, tissue growth and differentiation is prominent. Several evidences in the literature point at this latter functional effect of theirs. For instance, ODC activity and putrescine levels are correlated with myeloid cell differentiation induced by retinoic acid treatment (Stabellini et al. 2004) and polyamine depletion caused by DFMO, an irreversible inhibitor of ODC, prevents adipocyte differentiation (Vuohelainen et al. 2009). Concerning muscle cell differentiation, a recent paper demonstrated that the impaired skeletal muscle function in male mice with genomic androgen receptor knockout, was consistent with a reduced expression of genes encoding polyamine biosynthetic enzymes, suggesting that androgen-driven muscle development also involves the regulation of polyamine biosynthesis (MacLean et al. 2008). This work corroborates early results by Erwin et al., who showed in 1983 that polyamine depletion inhibits the differentiation of L6 myoblast cells. Starting from these evidences, and insulin being among the most characterized stimuli inducing muscle differentiation (Molkentin and Olson 1996), in this work we studied the effect of insulin on the activity of ornithine decarboxylase (ODC), the first enzyme in polyamine biosynthetic pathway, while inducing H9c2 embryonal rat myoblasts to differentiate into myotubes. ODC activity increased in H9c2 cells upon insulin treatment, consistent with their differentiation into myotubes; as assessed by morphological evaluation and western blotting detection of the amount of the transcription factor myogenin, an early marker of myogenic differentiation. DFMO, consistent with its ability to reduce ODC activity induced by insulin in H9c2 cells, also delayed the increase in myogenin expression driven by insulin treatment. This prompted us to evaluate if polyamines themselves may drive the differentiation process into the muscle phenotype. To this aim, we transfected H9c2 cells with a plasmid overexpressing ODC under the control of a constitutive cytomegalovirus promoter. These cells with a 50-fold increase in ODC activity (ODC$^+$) obviously showed a significant increase in polyamine synthesis with respect to control, and differentiated into myotubes without the need for any external stimulus and faster (day 5) than the wild-type cells treated with insulin (day 7). Morphological analysis, fusion index percentage and myogenin expression consistently indicate that early after seeding (day 2) ODC$^+$ cells show spontaneous induction of the differentiative process. In addition, treatment with DFMO slowed down ODC$^+$ cell differentiation, showing the specific role played by polyamines. In addition to the early markers of differentiation studied, also the MHC protein was induced in ODC$^+$ cells within day 2 after seeding, suggesting that polyamines may drive H9c2 cells towards their full differentiation into the muscle phenotype. These results confirm and extend the evidences about the pivotal role of polyamines in myogenic process and address a prospect suggestion for genetic engineering of signals to be turned on in muscle stem cells for muscle regeneration purposes.

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