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Difluoromethylornithine Stimulates Early Cardiac Commitment of Mesenchymal Stem Cells in a Model of Mixed Culture With Cardiomyocytes

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Abstract The efficiency of in vitro mesenchymal stem cell (MSC) differentiation into the myocardial lineage is generally poor. In order to improve cardiac commitment, bone marrow GFP^+MSCs obtained from transgenic rats were cultured with adult wild type rat cardiomyocytes for 5 days in the presence of difluoromethylornithine (DFMO), an inhibitor of polyamine synthesis and cell proliferation. The percentage of GFP^+MSCs showing cardiac myofibril proteins (cMLC2, cTnI) was about threefold higher after DFMO addition (3%) relative to the untreated control (1%). Another set of experiments was performed with cardiomyocytes incubated for 1 day in the absence of glucose and serum and under hypoxic conditions ($pO_2 < 1\%$), in order to simulate severe ischemia. The percentage of cardiac committed GFP^+MSCs was about 5% when cultured with the hypoxic/starved cardiomyocytes and further increased to 7% after DFMO addition. The contemporary presence of putrescine in DFMO-treated cells markedly blunted differentiation, while the cytostatic mitomycin C was not able to induce cardiac commitment. The involvement of histone acetylation in DFMO-induced differentiation was evidenced by the strong attenuation of cardiac commitment exerted by anacardic acid, an inhibitor of histone acetylase. Moreover, the percentage of acetylated histone H3 significantly increased in bone marrow MSCs obtained from wild type rats and treated with DFMO. These results suggest that polyamine depletion can represent a useful strategy to improve MSC differentiation into the cardiac lineage, especially in the presence of cardiomyocytes damaged by an ischemic environment. J. Cell. Biochem. 103: 1046–1052, 2008.

Key words: difluoromethylornithine; polyamine depletion; mesenchymal stem cells; cardiac differentiation; hypoxia; histone acetylation

Natural polyamines are ubiquitous compounds whose presence is related to cell proliferation [Wallace et al., 2003]. Most synthetic inhibitors of polyamine biosynthesis, such as difluoromethylornithine (DFMO), can deplete cells of putrescine and spermidine, thereby decreasing the rate of cell proliferation [Pegg et al., 1995]. Since some types of dividing cells seem to be more prone to differentiate when their process of growth is inhibited [Scott, 1997], in this study we evaluated whether mesenchymal stem cells (MSCs), after polyamine depletion, could be programmed easier to the cardiac phenotype. We previously demonstrated that the treatment of MSCs with DFMO for 24–48 h lowered intracellular putrescine and spermidine to undetectable concentrations, causing a partial, but significant, reduction in cell

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Abbreviation used: DFMO, difluoromethylornithine; cMLC2, cardiac myosin light chain 2; cTnI, cardiac troponin I; MSCs, mesenchymal stem cells; GFP^+MSCs, MSCs expressing green fluorescent proteins; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide.

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growth rate [Muscari et al., 2005]. The same treatment was also effective in counteracting both apoptotic and necrotic cytokine-induced death of MSCs. This pharmacological effect was purposed as a therapeutic strategy to blunt the excessive loss of stem cells just after their transplantation in the damaged tissues.

In the present study, a model of MSCs cultured with adult cardiomyocytes has been chosen to commit MSCs to the cardiac lineage, since this induction is quite rapid, that is within few days [Rangappa et al., 2003; Wang et al., 2006], and the presence of cardiac cells allows to create a myocardial-like environment. Although Yoon et al. [2005] demonstrated that adult cardiomyocytes can commit stem cells towards the myocardial lineage with a lesser extent than neonatal cardiomyocytes, we used the former because they are naturally present in the region of the infarcted heart which is eligible for MSC transplantation. In particular, we investigated whether energy-deprived cardiomyocytes were more prone to stimulate the process of MSC differentiation into the cardiac phenotype. Under this condition, which simulates a myocardial infarction, the differentiation potential of DFMO-treated MSCs was also investigated.

MATERIALS AND METHODS

All animal treatment was performed under guidelines on animal care and welfare determined by the Italian Bioethics Committee. Rats were anaesthetised with diethyl ether and killed by means of decapitation.

Bone marrow MSCs were isolated from wild type or green fluorescent protein (GFP)-stable transfected [Ito et al., 2001; Raimondo et al., 2006] male Wistar rats, weighing 250–300 g. The following procedures were then performed as previously described by Canossa et al. [1993] and Muscari et al. [2005]: isolation of MSCs and cardiomyocytes from adult rats, cell culture conditions, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay, and trypan blue staining. The morphology, phenotype, and potential to be committed to mesenchymal lineages of wild type and GFP^+MSCs, were previously described by Muscari et al. [2005], Raimondo et al. [2006] and Gallo et al. [2007]. Mixed cultures were performed by growing on glass coverslips 2×10^4 GFP^+MSCs with adult cardiomyocytes in a 2:1 ratio. The cultures were carried out for 5 days under standard aerobic conditions (5% CO_2, 37°C), using DMEM medium with 2 mM glucose and 10% foetal bovine serum (FBS). Simulated ischemia of cardiomyocytes was obtained by plating 1×10^4 cardiomyocytes on laminin-treated glass coverslips in DMEM medium without glucose and serum. Cardiomyocytes were also subjected to severe hypoxia ($pO_2 < 1\%$) for 24 h, using a workstation (BugBox-Jouan) saturated with N_2/CO_2 (95/5%) and thermostated at 37°C. GFP^+MSCs were then grown with the energy-deprived cardiomyocytes in their culture medium for 5 days, in a 2:1 ratio relative to the number of cardiomyocytes measured before simulated ischemia.

Immunofluorescence was performed as previously reported by Muscari et al. [2005] using monoclonal mouse anti-rat antibodies anti-cardiac myosin light chain 2 (cMLC2) and anti-cardiac troponin I (cTnI) (PharMingen). Primary antibodies and Cy3-conjugated rabbit polyclonal secondary antibodies anti-IgG (Santa Cruz Biotechnology) were diluted just before their utilisation in blocking buffer 1:100 and 1:1,000, respectively. After staining, cells were mounted with Moviol on standard glass slides and observed with a IX50 Olympus inverted microscope. Twenty fields of each sample were analysed by a software of image deconvolution (AutoQuant Imaging). Orange-stained GFP^+MSCs (merged red and green) were counted and the amount of cardiac committed cells was expressed as percentage relative to GFP^+MSCs (100%).

Flow cytometry analysis was performed using the above-mentioned anti-troponin antibody and the FC500 cytometer (Beckman Coulter Miami FL) with appropriate controls and a goat anti-mouse Ig conjugated with allophycocyanin (APC). For cell cycle analysis cells were harvested and fixed with 70% ethanol, then washed twice with phosphate buffer saline and stained with DNA Prep Kit (Beckman Coulter). Samples were analysed with FC500 Cytometer (Beckman Coulter).

Western blotting was performed as previously described by Muscari et al. [2005]. The nitrocellulose membrane was probed with the primary antibodies anti-acetylated histone H3 and anti-β-actin which was used as housekeeping gene (rabbit anti-rat polyclonal antibodies, 1:1,000 dilution in blocking buffer,

Santa Cruz Biotechnology). The secondary antibody, a horseradish peroxidase-conjugated anti-rabbit IgG, was utilised at 1:2,500 dilution in the blocking buffer (Santa Cruz Biotechnology).

RESULTS AND DISCUSSION

GFP$^+$MSCs were able to express a very early cardiac phenotype after 5 days of culture with adult cardiomyocytes. This was shown by the presence inside these cells of cMLC2 and cTnI (micrographs of Figs. 1 and 2), two specific markers of myocardial tissue. These myofibrils did not show a well-defined sarcomeric organisation, suggesting that GFP$^+$MSCs were in an initial phase of differentiation. Gallo et al. [2007] also observed that, under similar conditions of cell coculture, some GFP$^+$MSCs showed an increase in the calcium transient driven by KCl-induced depolarisation, but cell depolarisation was uncoupled from cell contraction because of the lack of myofibrillar organisation.

In order to reproduce in vitro an ischemic micro-environment, another set of cardiomyocytes was subjected to simulated severe ischemia by 24 h exposure to hypoxia, without glucose and serum. This condition provoked the death of about 70% of cardiomyocytes, as determined by the MTT assay (see supplementary online material, Fig. A). GFP$^+$MSCs were then grown with the ischemic-like cardiomyocytes, in the presence or absence of 1 mM DFMO. After 5 days, cells showing the presence of GFPs and cardiac myofibrils (orange fluorescence) were counted and their amount expressed as percentage relative to total GFP$^+$MSCs (Figs. 1 and 2). In the mixed cultures carried out under control condition (absence of DFMO, aerobic cardiomyocytes) the two types of myofibrils were present only in 1% of GFP$^+$MSCs, in accordance with similar findings described by Fukuhara et al. [2003]. A higher number of aerobic cardiomyocytes in the mixed cultures did not increase the percentage of GFP$^+$MSCs expressing sarcomeric proteins (data not shown). On the contrary, when cardiomyocytes were subjected to simulated ischemia, the percentage of GFP$^+$MSCs showing the cardiac markers significantly increased of about fivefold relative to control.

To evaluate whether a general cause of cell death, different from hypoxia and energy deprivation, could be sufficient to stimulate the expression of an early cardiac phenotype in GFP$^+$MSCs, a mixed culture with sonicated cardiomyocytes was also performed (about 70% of dead cells evaluated by the MTT assay; unpublished results). Under this condition the presence of cTnI was also observed, but only in 1.7% of GFP$^+$MSCs (see supplementary online material, Fig. B), a percentage which was lower than that obtained after simulated ischemia. Thus, an ischemia-like environment

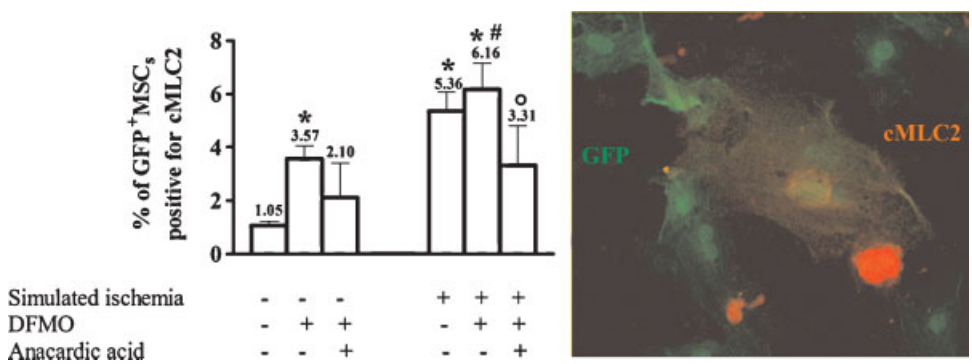

Fig. 1. Effect of DFMO on cMLC2 expression in GFP$^+$MSCs cultured with cardiomyocytes. GFP$^+$MSCs were cultured in a 2:1 ratio with cardiomyocytes which were subjected or not to simulated conditions of severe ischemia (see Materials and Methods Section). Mixed-cultured cells were grown for 5 days in the presence or absence of 1 mM DFMO, an inhibitor of polyamine biosynthesis, and of 5 μM anacardic acid, an inhibitor of histone acetyltransferase. The micrograph on the right side shows a representative field where a GFP$^+$MSC emits orange fluorescence (merged fluorescence between green proteins and Cy3-conjugated antibodies against cMLC2). Undifferentiated GFP$^+$MSCs (green) and necrotic cardiomyocytes (red) are also present. Magnification = 20×. Statistical analysis was performed by ANOVA followed by Bonferroni's test. Values of bar graphs are expressed as percentage ± SEM (number of experiments for each bar graph = 4–8). $^*P < 0.05$ versus normoxia without DFMO and anacardic acid; $^{\#}P < 0.05$ versus normoxia plus DFMO without anacardic acid; $^{\circ}P < 0.05$ versus simulated ischemia plus DFMO without anacardic acid.

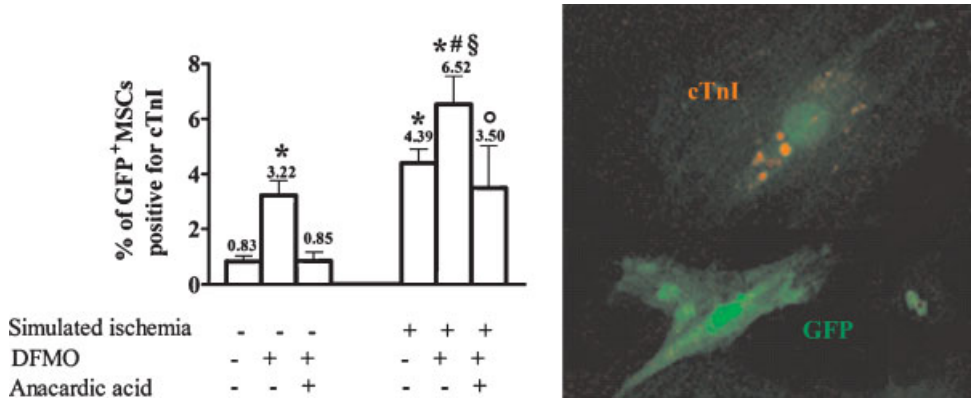

Fig. 2. Effect of DFMO on cTnI expression in GFP$^+$MSCs cultured with cardiomyocytes. The micrograph on the right side shows the presence of cTnI, as orange fluorescence, in a GFP$^+$MSC (merged fluorescence between green proteins and Cy3-conjugated antibodies against cTnI). An undifferentiated GFP$^+$MSC is also present and can be identified as a fully green cell. Other conditions are as described in the legend of Figure 1. Statistical analysis was performed by ANOVA followed by Bonferroni's test. Values of bar graphs are expressed as percentage ± SEM (number of experiments for each bar graph = 4–8). $^*P < 0.05$ versus normoxia without DFMO and anacardic acid; $^{\#}P < 0.05$ versus normoxia plus DFMO without anacardic acid; $^{\circ}P < 0.05$ versus simulated ischemia plus DFMO without anacardic acid. $^{\S}P < 0.05$ versus simulated ischemia without DFMO and anacardic acid.

with hypoxic and energy deprived cardiomyocytes seems to better stimulate the commitment of GFP$^+$MSCs to the myocardial lineage rather than cardiomyocytes which are damaged by an aspecific lethal insult.

The nature of molecules derived from cardiomyocytes which are responsible for induction of GFP$^+$MSC differentiation remains to be explored. Most works suggest that normal cardiac cells can stimulate differentiation by a direct contact with cocultured MSCs [Fukuhara et al., 2003; Rangappa et al., 2003; Yoon et al., 2005; Wang et al., 2006], but factors which are involved have not been found yet. On the contrary, only few researches state that soluble compounds released from cardiomyocytes can induce differentiation of MSCs under coculture conditions [Li et al., 2007]. The present study suggests that both mechanisms could be invoked, but with different effectiveness depending on whether cardiac cells are viable or severely damaged. When we cultured aerobic cardiomyocytes with MSCs in a transwell system, in order to take the two cell populations separated but soaked in the same medium, cardiac differentiation was not stimulated (data not shown), indicating that cell-to-cell contact was needed to commit MSCs under these conditions. By contrast, when we prior injured the cardiomyocytes, the number of committed MSCs increased, suggesting that other differentiating compounds could be released from the permeabilised/disrupted cells.

The high mobility group box 1 (HMGB1), an ubiquitous transcription factor, has been recently described to be released from damaged cardiomyocytes and to stimulate stem cell differentiation into the cardiac phenotype [Germani et al., 2007]. HMGB1, after its release from necrotic but not apoptotic cells, binds the receptor of advanced glycation end products (RAGE), a receptor that we detected in rat MSC plasma membrane (data not shown), activating a specific signal transduction pathway into the target cell [Sorci et al., 2004]. Other findings support the notion that cytoplasmatic and nuclear extracts are able to reprogram somatic cells, that is human MSCs derived from adipose tissue can differentiate into cardiac muscle cells when exposed to cell-free extracts of cardiomyocytes [Gaustad et al., 2004].

Among these putative compounds released from cardiomyocytes and involved in MSC differentiation, other differences should be underlined depending on whether cells are damaged by an aspecific or ischemic-like injury, since we demonstrated that the latter condition was even more effective for stem cell commitment. As a matter of fact, recent works show that cardiomyocytes stressed by severe ischemic conditions increase the production and the release of a large number of growth factors which improve myocardial regeneration

[Torella et al., 2007]; among them, the hepatocyte growth factor and the insulin-like growth factor-1 have been demonstrated to promote survival and proliferation of stem cells, as well as their differentiation towards cardiac lineages under both in vitro and in vivo conditions [Urbanek et al., 2005; Forte et al., 2006].

The addition of DFMO to the mixed culture was also able to increase the expression of myocardial proteins in GFP^+MSCs (Figs. 1 and 2). In particular, after DFMO treatment, cTnI was present in more than 3% of the GFP^+MSCs grew with aerobic cardiomyocytes and in about 7% of the GFP^+MSCs cultured with the hypoxic/starved cardiomyocytes.

We also tested whether the addition of polyamines to DFMO-treated GFP^+MSCs was able to prevent the observed stimulation of differentiation. Figure 3 shows that the percentage of GFP^+MSCs positive for cTnI, evaluated by FACS analysis, was threefold lower in cells that were contemporary treated with putrescine and DFMO with respect to cells exposed only to DFMO. These results demonstrated that differentiation was mainly dependent on polyamine depletion and not to other effects eventually exerted by DFMO. However, putrescine addition was not able to inhibit differentiation at all. This was presumably due to the fact that putrescine concentration was 10-fold lower than that of DFMO in order to avoid the generation of reactive oxygen species derived from polyamine oxidation in the medium.

The differentiating effect of DFMO was then compared with that of mitomycin C, another cytostatic compound which does not affect polyamine metabolism. To inhibit proliferation, GFP^+MSCs were exposed to 10 μg/ml mitomycin C for 2 h and washed three times with media prior to plating [Engler et al., 2006]. This treatment was effective to arrest GFP^+MSCs in the G2-M phase of cell cycle, as evaluated by flow cytometry analysis (data not shown). Under this condition, the coculture with adult cardiac cells did not significantly increase the percentage of GFP^+MSCs positive for cTnI (Fig. 3). This suggest that DFMO stimulates cardiac commitment through a metabolic change induced by polyamine depletion which is different from the inhibition of cell cycle progression.

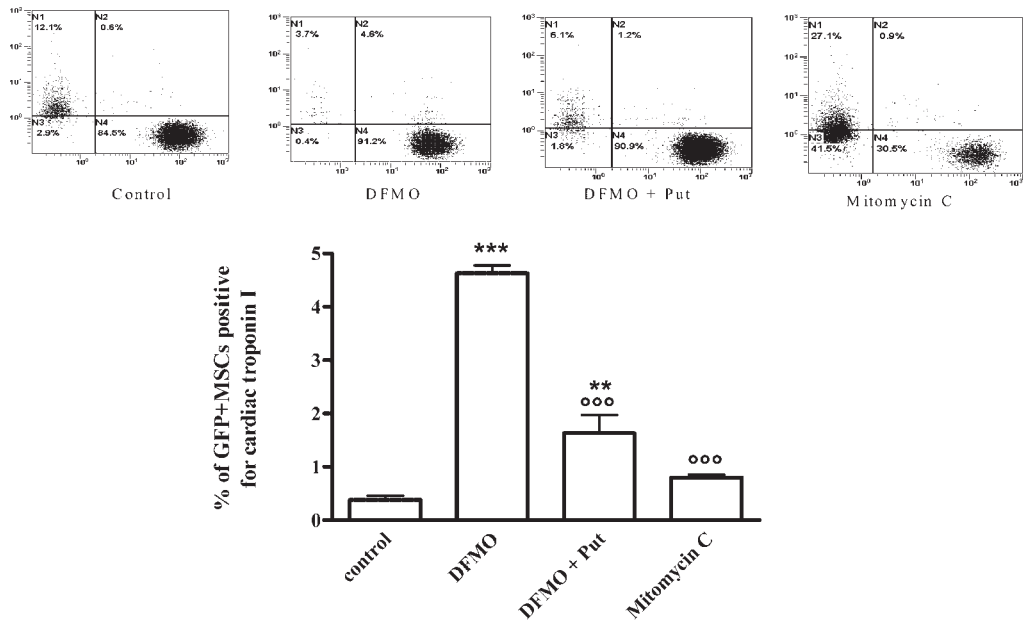

Fig. 3. Effect of different treatments on GFP^+MSC differentiation cultured with cardiomyocytes. GFP^+MSCs were treated for 5 days with: 1 mM DFMO (DFMO), 1 mM DFMO plus 100 μM putrescine (DFMO + Put), or 10 μM mitomycin C (Mitomycin C). One mM aminoguanidine, an inhibitor of serum amino oxidases, was added to the medium of cells treated with both DFMO and putrescine to limit the production of reactive oxygen species derived from putrescine oxidation. The percentage of cells positive for cTnI was evaluated by flow cytometry analysis. The upper panel shows representative fluorescence cell scattering, where *x*- and *y*-axis report the number of fluoresceine and allophycocyanin positive cells, respectively. Statistical analysis was performed by ANOVA followed by Bonferroni's test. $N = 3$; $^{**}P < 0.01$ versus control; $^{***}P < 0.001$ versus control; $^{°°°}P < 0.001$ versus DFMO.

In order to investigate the mechanisms involved in the cardiac differentiation sustained by DFMO, we studied the process of histone acetylation. Indeed, this covalent modification has been described by Fu et al. [2004] to promote gene transcription and cell differentiation. Also, Pollard et al. [1999] suggested that histone hyperacetylation can antagonize the ability of polyamines to stabilize highly condensed states of chromosomal fibres which represses transcription. In our study, the addition of anacardic acid, an inhibitor of histone acetyl transferase activity [Balasubramanyam et al., 2003], quite completely abolished the differentiating effect of DFMO under both aerobic and ischemia-like conditions (Figs. 1 and 2).

Wild type bone marrow rat MSCs were also treated for 9 days with the same concentration of DFMO used for mixed cultures (1 mM) and the time course of histone-H3 acetylation was investigated by Western blot analysis (Fig. 4). The percentage of acetylated histone H3 in DFMO-treated MSCs progressively increased relative to the corresponding untreated control, reaching a value of about 170% at the sixth day. This process was limited in time since at day 9 the percentage of acetylated histone H3 was the same of that observed just before treatment. Although we can not explain this behaviour, it is possible that the initial phase of cardiac commitment can be characterised by a very transient switch in gene expression priming MSC transdifferentiation. The delayed effect of DFMO on histone acetylation likely reflected the period that is necessary to deplete MSCs of polyamine. Taken together, the findings that histone H3 is hyperacetylated and the cardiac commitment inhibited by anacardic acid in DFMO-treated MSCs, suggest that histone acetylation regulates in a relevant manner the master genes driving myocardial differentiation in polyamine-depleted MSCs. However, further studies are needed to better understand how a reduction in the cellular content of polyamines can increase the differentiation potential of MSCs.

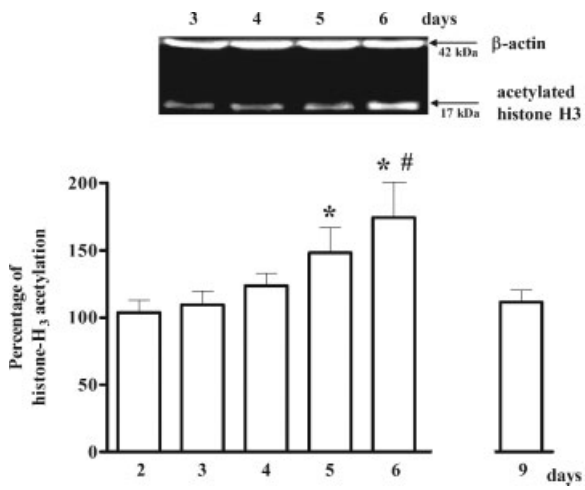

Fig. 4. Time course of histone-H3 acetylation in MSCs treated with DFMO. The levels of the acetylated histone H3 in MSCs, cultured in the presence of 1 mM DFMO, were evaluated by Western blotting. Values are expressed as percentage ± SEM of the optical density referred to DFMO-treated MSCs relative to control MSCs evaluated at the same time of culture. Statistical analysis was performed by ANOVA followed by Bonferroni's test. The number of experiments for each bar ranges between 4 and 10. $P < 0.05$ versus day 2 (*) and versus day 3, 4, and 9 (#).

In conclusion, we demonstrated that DFMO can stimulate the early differentiation of MSCs to the myocardial lineage in a model of mixed culture with adult cardiomyocytes and this effect is accompanied by histone acetylation. Moreover, cardiac commitment can be amplified by conditions simulating severe ischemia of cardiomyocytes. Studies are in progress to assess whether polyamine-depleted MSCs can promote in vivo myocardial regeneration, taking into account that DFMO is not toxic for humans and eventual side effects appear only after high-dosage or long-term treatments [Meyskens and Gerner, 1999].

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