Timing of induction of cardiomyocyte differentiation for in vitro cultured mesenchymal stem cells: a perspective for emergencies[1]

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Abstract: Mesenchymal stem cells (MSCs) have the capacity to differentiate into osteoblasts, chondrocytes, adipocytes, myocytes, and cardiomyocytes. Several established methods are presently available for in vitro isolation of MSCs from bone marrow. However, the duration necessary to culture them can be a major handicap to cell-based therapies needed for such urgent cardiovascular conditions as acute myocardial infarction and acute hindlimb ischemia. The best timing of cardiomyocyte differentiation induction after MCS isolation and expansion is still an unresolved issue. Our goal was to investigate the possibility of obtaining functional cardiomyocytes from rat MSC within a shorter time period. We examined MSCs' colony-forming capacity, CD90 and CD34 immunoreactivity during the 14 days of culturing. Cardiomyocyte differentiation was induced by 5-azacytidine. Immunohistochemic staining, together with intracellular Ca^{2+} measurement experiments, revealed that MSCs do not differentiate into any specific cell lineage but show the characteristics of MSCs on both the 9th and 14th days of the culture. To check the potential for differentiation into cardiomyocytes, experiments with caffeine application and depolarization with KCl were performed. The cells possessed some of the specific biochemical features of contracting cells, with slightly higher capacities on the 14th day. Cells from 9th and 14th days of the culture that were treated with 5-azacytidine had a higher expression of cardiac-specific markers such as troponin I, α-sarcomeric actin, and MEF2D compared with the control groups. This study illustrates that it is possible to get functional cardiomyocytes from in vitro MSC culture in a shorter time period than previously achieved. This reduction in time may provide emergency cases with access to cell-based therapies that may have previously been unavailable.

Key words: mesenchymal stem cells, cardiomyocytes, differentiation, rat, in vitro, gene expression.

Résumé : Les cellules souches mésenchymateuses (CSM) ont la capacité de se différencier en ostéoblastes, chondrocytes, adipocytes, myocytes et cardiomyocytes. Plusieurs méthodes éprouvées permettent d'isoler in vitro les CSM de la moelle osseuse. Toutefois, la durée requise pour leur culture pourrait être un désavantage majeur pour la thérapie cellulaire d'urgences cardiovasculaires comme l'infarctus du myocarde et l'ischémie des membres inférieurs. Le meilleur moment de l'induction de la différenciation des cardiomyocytes après l'isolement et l'expansion des CSM n'a toujours pas été déterminé. Nous avons étudié la possibilité d'obtenir des cardiomyocytes fonctionnels de CSM de rats sur une courte période de temps. Nous avons examiné la capacité de formation de colonies des CSM et l'immunoréactivité à CD90 et CD34 durant les 14 jours de culture. La différenciation en cardiomyocytes a été induite par la 5-azacytidine. La coloration immunohistochimique ainsi que des mesures du Ca^{2+} intracellulaire ont révélé que les CSM ne se différencient pas en une lignée cellulaire spécifique, mais montrent les caractéristiques des CSM lors des 9^{e} et 14^{e} jours de culture. Pour vérifier le potentiel de différenciation en cardiomyocytes, nous avons fait des expériences avec application de caféine et dépolarisation au KCl. Les cellules possédaient certaines des caractéristiques biochimiques spécifiques des cellules pouvant se contracter, avec des capacités légèrement supérieures le quatorzième jour. Les cellules des 9^{e} et 14^{e} jours de culture traitées avec la 5-azacytidine ont montré une plus grande expression de marqueurs spécifiques du cœur, tels que la troponine I, l'actine α-sarcomérique et MEF2D, comparativement à celles des groupes témoins. Cette étude montre qu'il est maintenant possible d'obtenir des cardiomyocytes fonctionnels d'une culture in vitro de CSM dans une courte période de temps. Cette réduction de temps pourrait fournir aux cas d'urgence un accès à des thérapies cellulaires jusqu'alors inaccessibles.

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Mots-clés : cellules souches mésenchymateuses, cardiomyocytes, différenciation, rat, in vitro, expression génique.

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Introduction

Stem cell research has gained tremendous interest in the past two decades because it provides opportunities to develop new strategies for debilitating diseases. Stem, or progenitor, cells have the potential to provide unique sources for tissue restoration in regenerative medicine and tissue engineering (Ivanova et al. 2002). Although self-renewal and differentiation are their common features, as a result of cell-to-cell communication, stem cells vary in their potential to differentiate, in their duration and pathways of self-renewal, in the places where they are mostly found, and in their divisional characteristics (Morrison et al. 1997).

Autologous stromal mesenchymal stem cells (MSCs) from bone marrow, first identified by Friedenstein in the 1970s (Friedenstein et al. 1974, 1976), may offer the possibility of a renewable source for the replacement of injured cells or tissues without raising ethical concerns or the possibility of immunologic reactions. MSCs are a very small fraction of the nucleated cells in the bone marrow, only 0.01%–0.1% of the total population (Pittenger et al. 1999). They have fibroblastic morphology and they are adherent spindle-shaped cells. MSCs adhere to the culture plate, leading to the formation of colonies. Usually, basal mediums with serum support are used to expand MSCs in tissue culture, and growth factors are added in order for MSCs to differentiate (Barry and Murphy 2004). In fact, their most prominent feature is their ability to differentiate into osteoblasts, chondrocytes, adipocytes, hepatocytes, neurons, and myocytes under defined conditions. They are also thought to differentiate into certain types of ectodermal and endodermal tissues, such as neurons and endothelial cells, including skeletal myocytes (Jackson et al. 2001), central and peripheral neurons (Mezey et al. 2000; Brazelton et al. 2000), and hepatic cells (Petersen et al. 1999). The master switches regulating the transitions from stem cells to differentiated cells, and the genes active in specific differentiation patterns are still not known. The reason for the difficulty in finding these regulatory genes is because of their temporal activity, the response given to inductive molecules, and the varying differentiation pathways between organisms (Baksh et al. 2004; Caplan and Bruder 2001).

Bone marrow-derived cardiac myocytes have been shown to populate rodent heart tissue after myocardial infarction (Jackson et al. 2001) or cardiac transplantation (Edelberg et al. 2002). Moreover, preclinical studies have shown that the injection of bone marrow cells directly into the myocardium enables these cells to differentiate into cardiac myocytes, smooth muscle cells, and endothelial cells. These transformations lead to the regeneration of the myocardium and improvement of cardiac function (Orlic et al. 2001; Tomita et al. 2002).

A major roadblock in the use of MSCs, however, is that MSCs are rare and the long duration of their culture limits their potential use in urgent cell-based therapies. In addition, the assessment of donor MSC functional activity at the cellular level, which is the focus of several research groups, is critically important. To our knowledge, no study has looked at the best timing to induce cardiomyocyte differentiation after MSC isolation and expansion. Therefore, we aimed to shorten the time period required for MSC culture before cardiomyocyte differentiation.

Materials and methods

Cell culture

MSCs were obtained from female, 9-week-old, 280–300 g Sprague–Dawley rats. Bone marrow heterogeneous cell population was collected from the femur and tibia by flushing with a 5-millilitre syringe containing 10% FBS (HyClone, Logan, USA) in DMEM (Invitrogen, Paisley, UK) after the rats were sacrificed by cervical dislocation. Negatively selected supernatant from CD34-labeled paramagnetic beads (Dynal, Oslo, Norway) was cultured in plastic cell-culture dishes with MesenCult medium (StemCell Technologies, Vancouver, Canada) with a 20% supplement (StemCell Technologies) and a 1% penicillin–streptomycin solution (HyClone) in a 5% CO_2 incubator at 37 °C. The next day, the media of the tissue culture plates were changed and the nonadherent cells were removed. The media of the cells were changed every 4 days, after washing with sterile 1× PBS before the change.

Our experimental study protocol was approved by the Animal Ethics Committee of Bilkent University (Bil-AEC). All the animals received care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Science, and this study protocol complied with Bilkent University's guidelines on the humane care and use of laboratory animals.

Colony-forming assay

MSCs were washed in 1× PBS and then air dried. Methanol was used for fixation and left for 5 min. The cells were washed again in a 1× PBS buffer and a giemsa-staining reagent (Carlo Erba, Milano, Italy) was added and left for 5 min. The reaction of the giemsa staining was stopped by the addition of tap water.

Induction of cell differentiation

Two different time points were used for the cardiomyocyte differentiation of MSCs. In the first group, differentiation was induced on day 9 of the culture (hereafter stated as the 9th day) and in the second group, differentiation was induced on day 14 (hereafter stated as the 14th day). The induction of cardiomyocyte differentiation was performed by

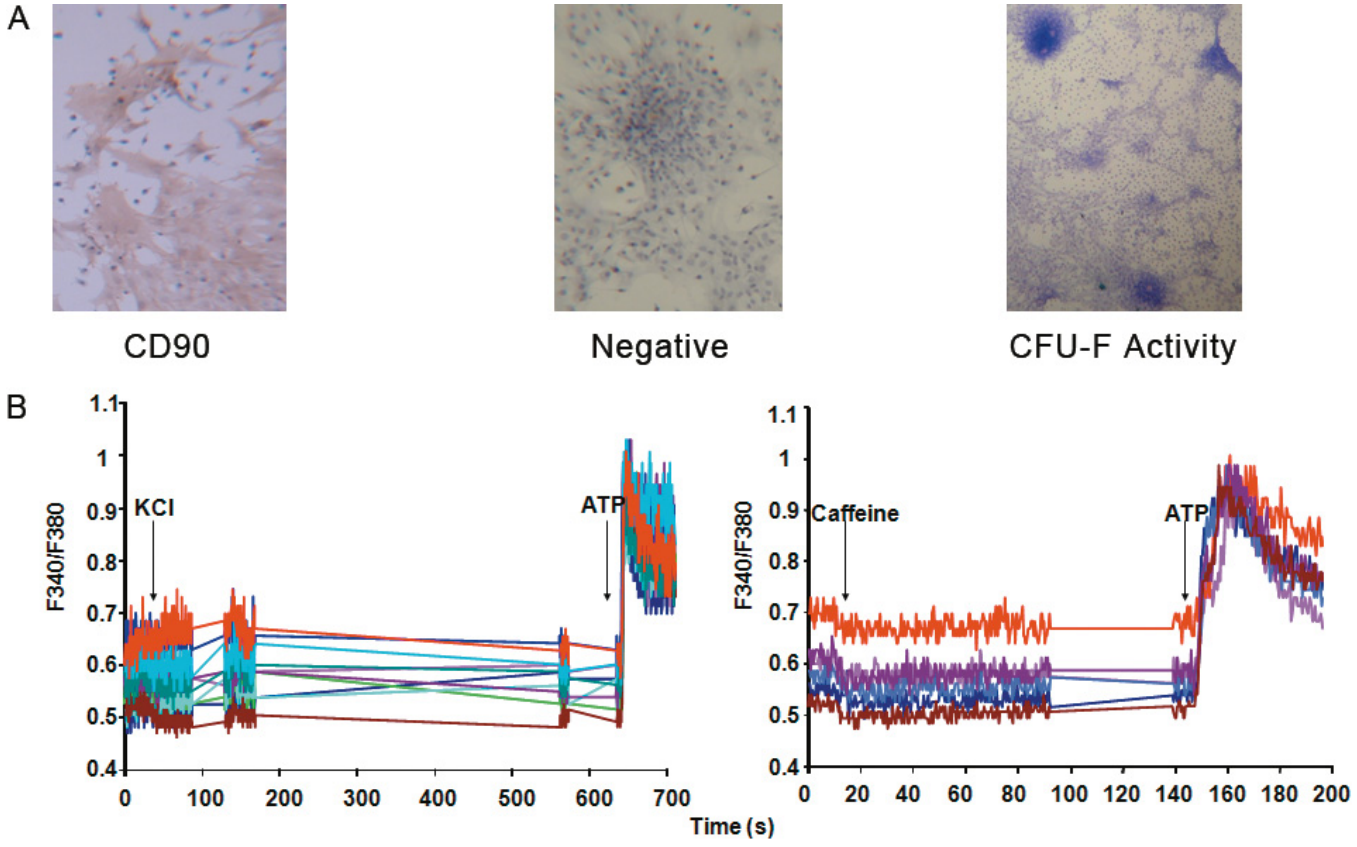
Table 1. Primers and the product size of the cardiac-specific gene markers for cDNA amplification.

Gene	Primers	Product size
Troponin I	Forward: 5′–GCGAAGCAGGAGATGGAG–3′ Reverse: 5′–TGCCACGCAGGTCATAGA–3′	250 bp
α-Sarcomeric actin	Forward: 5′–CCAGGCACTGTGGAAAGG–3′ Reverse: 5′–CACGATGGATGGGAAGAC–3′	115 bp
MEF2D	Forward: 5′–TGGGAATGGCTATGTCAGTG–3′ Reverse: 5′–CTGGTAATCTGTGTTGTAGG–3′	351 bp
GAPDH	Forward: 5′–CCTCCTCATTGACCTCAACTAC–3′ Reverse: 5′–CATGGTGGTGAAGACGCCAG–3′	219 bp

Table 2. PCR conditions for the genes.

Gene	Initial denaturation	Denaturation	Annealing	Extension	Cycles	Final extension
Troponin I	95 °C, 5 min	94 °C, 45 s	65 °C, 30 s	72 °C, 40 s	33	72 °C, 5 min
α-Sarcomeric actin	95 °C, 10 min	94 °C, 45 s	57 °C, 60 s	72 °C, 60 s	35	72 °C, 10 min
MEF2D	95 °C, 10 min	94 °C, 30 s	57 °C, 60 s	72 °C, 50 s	33	72 °C, 5 min
GAPDH	95 °C, 5 min	94 °C, 30 s	59 °C, 30 s	72 °C, 30 s	23	72 °C, 5 min

Fig. 1. Characteristics of rat mesenchymal stem cells on the 9th day of culture. (A) CD90 immunoreactivity and colony-forming capacity. No staining is observed in the negative control. (B) Cytoplasmic calcium levels in response to KCl and caffeine measured with fura-2. No response was observed with either stimulation. ATP stimulation is used as a positive control. Fura-2 signal is given as a ratio of fluorescence at 340 and 380 nm.

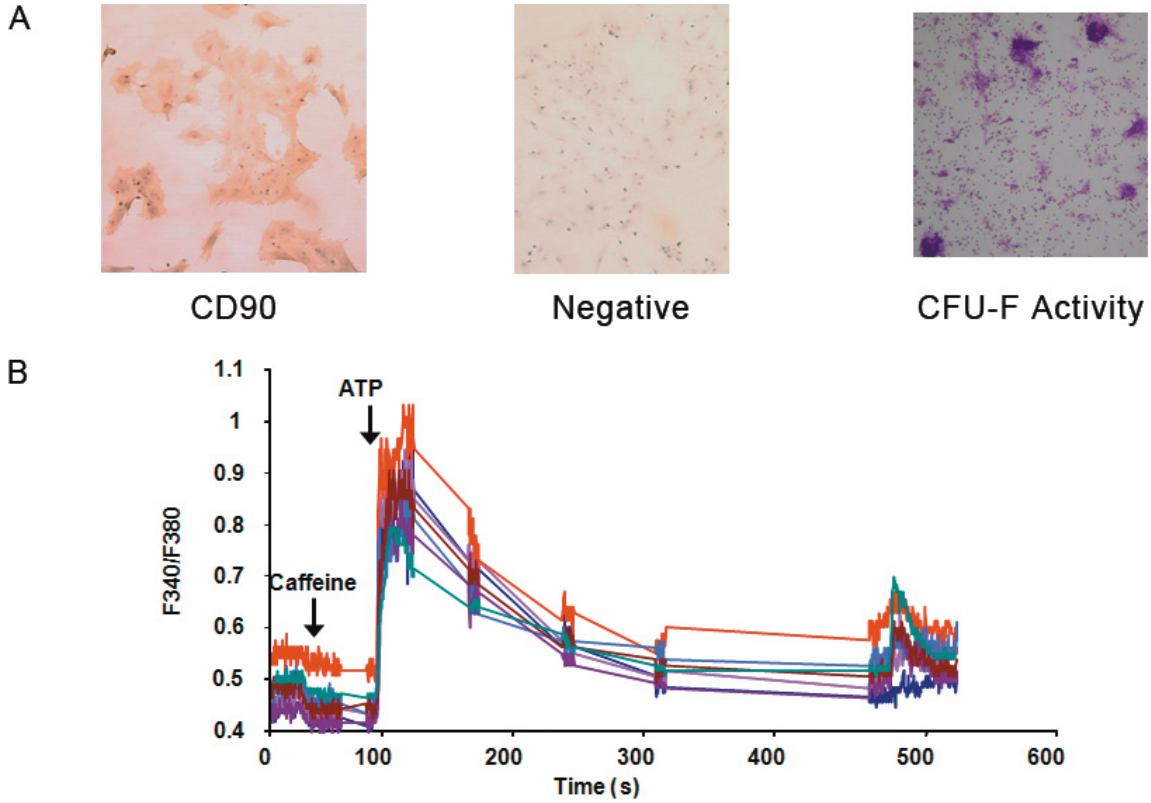


incubating cells with 10 μmol/L 5-azacytidine (Sigma, Steinheim, Germany) for 24 h. The cells were washed and expanded for another 14 days with low-glucose DMEM (Invitrogen), 20% FBS (HyClone), and 1% penicillin-streptomycin (HyClone).

Total RNA isolation and RT-PCR analysis

On the 9th and 14th day of the MSC culture and 14 days after the 5-azacytidine treatment of each group, cells were trypsinized and removed. Total cellular RNA was isolated from the precipitate by using the RNeasy Mini kit (Qiagen,

Fig. 2. Characteristics of rat mesenchymal stem cells on the 14th day of culture. (A) CD90 immunoreactivity and colony-forming capacity. No staining is observed in the negative control. (B) Cytoplasmic calcium levels in response to caffeine (or KCl, data not shown) measured with fura-2. No response was observed with either stimulation. ATP stimulation is used as a positive control. Fura-2 signal is given as a ratio of fluorescence at 340 and 380 nm.



Hilden, Germany) according to the manufacturer's protocol. The cDNAs were synthesized from the total RNA samples with the RevertAid First Strand cDNA synthesis kit (MBI Fermentas, Canada) according to the manufacturer's protocol. cDNA amplifications were performed by using oligonucleotide primers for the following genes: cardiac troponin I (cTnI), α-sarcomeric actin, MEF2D, and GAPDH (Table 1). PCR conditions of these genes are listed in Table 2.

Immunohistochemistry

Cells in the cover slips were incubated for 30 min in 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. After washing 3 times with PBS for 10 min each, slides were incubated with preblocking serum (normal goat serum 1.5%, bovine serum albumin 2%, Triton X-100 0.1%) for 1 h at room temperature. Primary antibodies of CD90 and CD34 (Chemicon, Temecula, Canada) were applied at a concentration of 1:500 dilution in preblocking solution and kept at 4 °C overnight. After washing 3 times with PBS, tissue sections were incubated for 15 min each with biotinylated anti-mouse and anti-rabbit Ig (DakoCytomation, Glostrup, Denmark) and streptavidin–horseradish peroxidase (HRP) (DakoCytomation) at room temperature. After washing for 10 min with PBS, slides were rinsed in a 0.5% solution of Triton X-100 and PBS for 30 s. Color developments were achieved by incubation with liquid DAB+ (DakoCytomation). The slides were then counterstained with hematoxylin and mounted with Faramount aqueous mounting medium (DakoCytomation).

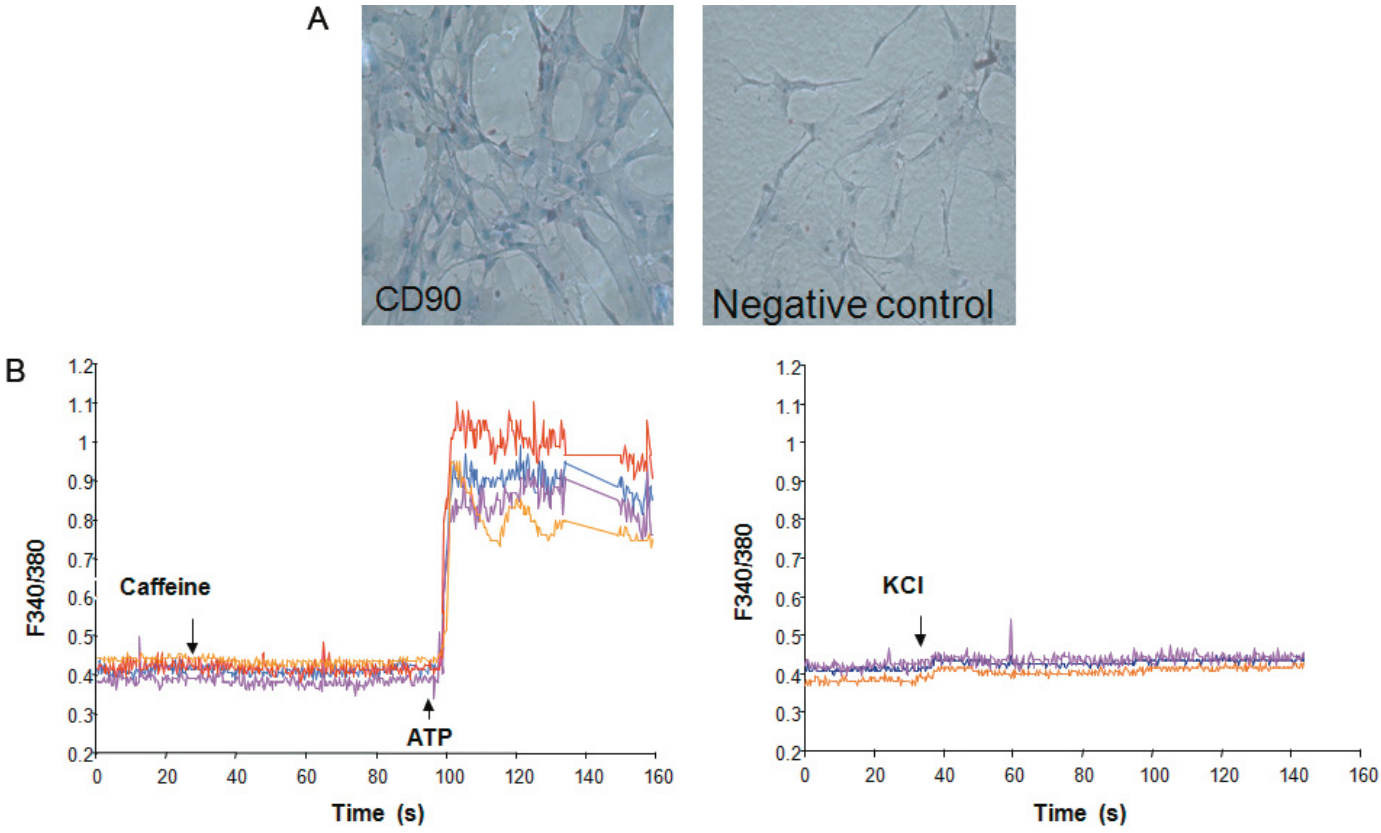
Intracellular Ca^{2+} measurement

Cells were grown on round cover slips in 6-well plates and loaded with fura-2 AM at room temperature (21–24 °C) for 50 min. Cells were then washed before the experiments with Hepes-buffered physiologic NaCl bathing solution containing 1.2 mmol/L Ca^{2+} and 1 mmol/L Mg^{2+}. Cells were challenged either with caffeine to stimulate ryanodine receptors or with ATP to stimulate P2Y purinergic receptors. In addition, we applied high-KCl bathing solutions and monitored intracellular calcium concentrations to see whether depolarization could induce any intracellular Ca^{2+} transients. Cover slips were fixed to a Teflon chamber and mounted on an inverted fluorescent microscope (Nikon TE-300, Japan). Fluorescence ratios were obtained by exciting cells at 340 and 380 nm with a microscope-based spectrofluorimeter with a temporal resolution of one ratio every 0.5 s. Emission signals were collected at 510 nm with an IC-300 ICCD (intensified charge-coupled device) camera with Delta Ram, (Photon Technology International, USA). Data was collected and analyzed by Image Master software (Photon Technology International).

Statistical analysis

All data were expressed as means ± SD. Data were ana-

Fig. 3. Differentiation into cardiomyocytes was induced by applying 5-azacytidine to MSCs on the 9th day of culture. (A) No CD90 immunoreactivity is present after induction. (B) Cytoplasmic calcium levels in response to KCl and caffeine measured with fura-2. ATP stimulation is used as a positive control. Fura-2 signal is given as a ratio of fluorescence at 340 and 380 nm.



lyzed by performing paired *t* test using Minitab Statistical Software (State College, USA). A value of $p < 0.05$ was considered to be statistically significant.

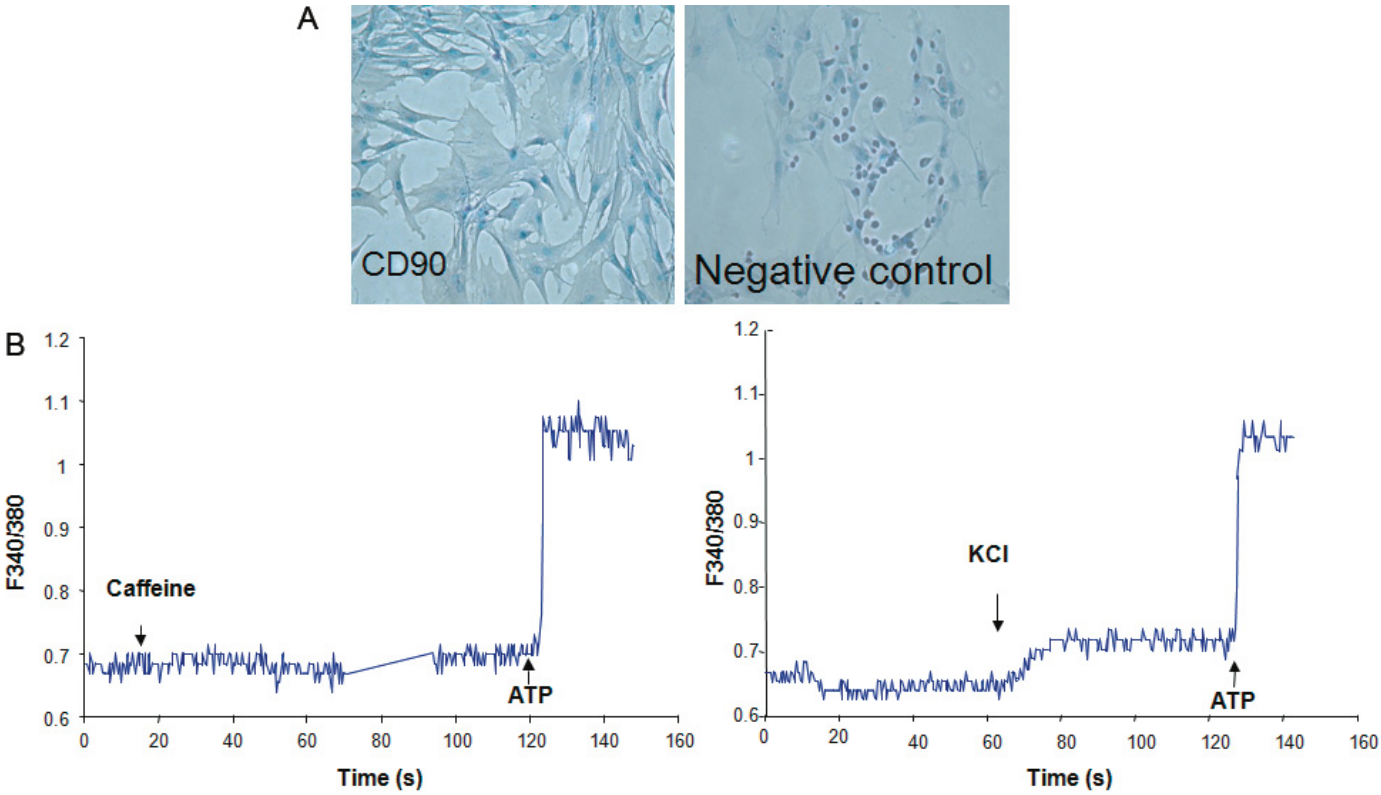
Results

We first investigated the characteristics of MSCs on the 9th and 14th days of the culture by their CD90 expressions and CFU-F (colony-forming unit – fibroblast) activities (Figs. 1A and 2A). We also compared their free intracellular Ca^{2+} concentrations (Figs. 1B and 2B). On the 9th day of the culture, rat MSCs were stained positive for CD90 (Fig. 1A), started to form colonies (Fig. 1A), and did not evoke any Ca^{2+} response upon application of caffeine and depolarization with KCl (Fig. 1B). These results were similar to those on the 14th day of the culture, confirming that both cells were undifferentiated (Figs. 2A and 2B). On the other hand, they responded to (0.5×10^{-4} mol/L) extracellular ATP application with a clear Ca^{2+} transient (Figs. 1B and 2B). These cells stained negative with the CD34 antibody (data not shown).

Next, MSCs from the 9th and 14th days of the culture were treated with 5-azacytidine to induce cardiomyocyte differentiation. Fourteen days after this treatment, we checked their CD 90 expressions (Figs. 3A and 4A) and their responses to extracellular KCl and caffeine as an increase in cytosolic Ca^{2+} was noted after the cells were loaded with fura-2 (Figs. 3B and 4B). We applied 10 mmol/L caffeine and 45–60 mmol/L extracellular KCl to the cells. Cells treated with 5-azacytidine on the 9th and 14th days of the culture did not respond to 10 mmol/L caffeine. On the other hand, cells treated with 5-azacytidine on the 14th day of the culture responded to the KCl application with a small increase of cytosolic Ca^{2+}. Cells treated with 5-azacytidine on the 9th day also responded with a very small Ca^{2+} increase. These results may suggest that voltage-activated Ca^{2+} channel expression is starting in both groups of cells 14 days after the 5-azacytidine treatment; however, there is no indication of the presence of functional ryanodin receptors. All cells responded to ATP (0.5×10^{-4} mol/L) application with a sudden cytoplasmic Ca^{2+} increase.

To illustrate the cardiomyocyte commitment of these cells, we performed RT-PCR analysis to investigate the expression pattern of cardiac-specific markers before and after the induction with 5-azacytidine. RT-PCR experiments showed that cardiac-specific troponin1, α-sarcomeric actin, and MEF2D expression increased after the 5-azacytidine induction compared with that of the cells that were cultured with the control media both on the 9th day (Fig. 5A) and the 14th day of the culture (Fig. 5B). The expression of cardiac-specific troponin1 and α-sarcomeric actin were statistically significant on the 9th day compared with that of the

Fig. 4. Differentiation into cardiomyocytes was induced by applying 5-azacytidine to MSCs on the14th day of culture. (A) No CD90 immunoreactivity is present after induction. (B) Cytoplasmic calcium levels in response to KCl and caffeine measured with fura-2. ATP stimulation is used as a positive control. Fura-2 signal is given as a ratio of fluorescence at 340 and 380 nm.



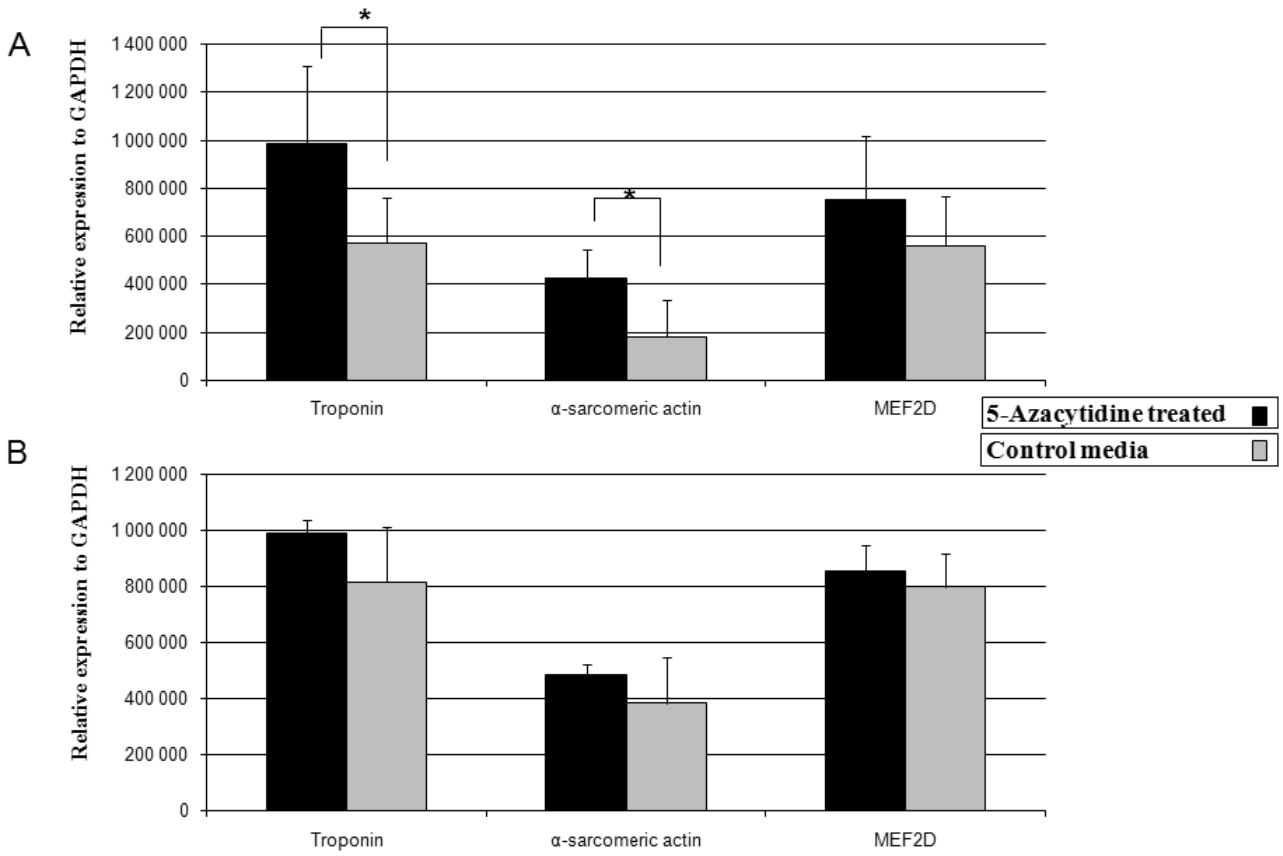
control group. The values shown in Figs. 5A and 5B were obtained by normalizing the expression values to that of the loading control, GAPDH.

Discussion

The possibility of using stem cell-based therapies has opened a new therapeutic era for cardiovascular diseases, which claim roughly as many lives as cancer, chronic lower respiratory diseases, accidents, diabetes mellitus, influenza, and pneumonia combined (1999–2002 National Health and Nutrition Examination Survey, available from www.americanheart.org, "Heart disease and stroke statistics, 2005 update"). Applications involving the use of stem cells in humans, which might have been considered 'science fiction' fewer than 20 years ago, are now being utilized with a great success rate (Akar et al. 2006). Different types of stem or progenitor cells have been used to target cardiac regeneration, including MSCs (Laflamme and Murry 2005; Tousoulis et al. 2008; Wang and Li 2007; Atsma et al. 2007; Ohnishi and Nagaya 2007). Aside from their differentiation potential, there are other aspects that make MSCs valuable for new therapeutic strategies. MSCs actively inhibit T-cell proliferation and as a result are considered nonimmunogenic or hypoimmunogenic, which is important for a host response to allogenic MSC therapy. MSCs can also be frozen to preserve them, and when they are thawed they function apparently normally, thus allowing for future 'off-the-shelf' therapy approaches. However, the time required for isolation, expansion, and differentiation of specific cell types can be a major handicap to the optimal timing of stem-cell delivery to acutely injured tissues or organs. This issue is of paramount importance in cardiovascular medicine (Bartunek et al. 2006). The assessment of donor MSC functional activity at the cellular level, which is the focus of several research groups, is also critically important. There are no available data from experimental studies or clinical trials that show the exact timing of the induction of cardiomyocyte differentiation before MSC delivery to an injured heart or ischemic limb.

Our study demonstrated that rat MSCs did not differentiate but exhibited characteristics of MSCs by the 9th day of the culture similar to those on the 14th day of the culture. As expected, these cells started to form colonies on day 7 (data not shown) and these colonies became positive for CD90 staining by day 9. We observed that caffeine (10 mmol/L) application and depolarization with KCl (45–60 mmol/L) did not evoke any Ca^{2+} responses in MSCs on the 9th and 14th days of the culture, which confirmed that they were still undifferentiated cells. On the other hand, they responded to extracellular ATP (0.5×10^{-4} mol/L) application with clear Ca^{2+} transients. These results indicate the absence of any functional ryanodine receptor in rat MSCs and also demonstrate that these cells do not differentiate and clearly exhibit characteristics of MSCs by the 9th day of the culture. Therefore, 9 days of in vitro isolation and expansion of MSCs can be adequate for the application of any differentiating agents to these cells. To our knowledge, this is the shortest time yet that obtains functional MSCs in rat models. Shortening the culture time by nearly

Fig. 5. Expression profile of cardiac-specific markers in 5-azacytidine-treated MSCs on the (A) 9th and (B) 14th day of culture. Expression of the genes was normalized by using GAPDH as the loading control and comparing with the cells cultured in the control media. The increase in the expression of cardiac-specific troponin1 ($p = 0.04$) and α-sarcomeric actin ($p = 0.01$) on the 9th day was statistically significant. Black bars, 5-azacytidine-treated; grey bars, control media.



5 days could be a very useful development for patients awaiting urgent cell-based therapies.

In this study we also demonstrated that MSCs could be differentiated into cardiomyocyte-like cells by applying 5-azacytidine in vitro (Makino et al. 1999) and in vivo (Toma et al. 2002; Shake et al. 2002), as shown previously. However, the exact mechanism of MSCs' differentiation into cardiomyocyte-like cells is not clear. Several groups have reported that in vitro differentiation of MSCs into cardiomyocytes depends on different factors, including the number of passages or the combination of certain growth factors or molecules (Zhang et al. 2005; Antonitsis et al. 2007; Muscari et al. 2008).

To further characterize the cardiomyocyte differentiation, MSC cultures from day 9 and day 14 were treated with 10 μmol/L 5-azacytidine to induce cardiomyocyte differentiation (Figs. 3 and 4). Fourteen days after the 5-azacytidine treatment, we observed that these cells, both on the 9th (Fig. 3A) and 14th (Fig. 4A) days of the culture, did not express the mesenchymal stem cell marker CD90. Both groups responded, albeit slightly, to extracellular KCl with an increase in cytosolic Ca^{2+}; however, this response appeared to be more evident on the 14th day of the culture. On the other hand, cells from both cultures did not respond to 10 mmol/L caffeine application, but responded to ATP (0.5×10^{-4} mol/L) application with a sudden cytoplasmic Ca^{2+} increase (Figs. 3B and 4B), probably due to the stimulation of endogenous P2Y receptors. These results may suggest that voltage-activated Ca^{2+} channel expression starts 14 days after the 5-azacytidine treatment on the 9th and 14th days of the culture, but there is no indication of the presence of functional ryanodin receptors. Further experiments are needed to verify this point. It is noteworthy that compared with the controls, both the 9th- and 14th-day 5-azacytidine-treated cells expressed increased levels of cardiomyocyte-specific genes (Figs. 5A and 5B). The increase in the expression of cardiac-specific troponin1 ($p = 0.039$) and α-sarcomeric actin ($p = 0.012$) on the 9th day was statistically significant. These findings suggest that MSCs from day 9 may be used for the in vitro differentiation into cardiomyocytes.

In conclusion, our study demonstrated that MSCs from adult rat bone marrow are able to differentiate into cardiomyocyte-like cells as early as 9 days after isolation and expansion after being induced by 5-azacytidine, evidenced by expressing cardiomyocyte-specific genes and losing the expression of mesenchymal stem cell marker genes. These results indicate that the culture time for MSCs can be shortened by nearly 5 days, which could be useful for patients awaiting urgent cell-based therapies.

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