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Limited Plasticity of Mesenchymal Stem Cells Cocultured With Adult Cardiomyocytes

Maria Pia Gallo,[1]* Roberta Ramella,[1] Giuseppe Alloatti,[1] Claudia Penna,[2] Pasquale Pagliaro,[2] Andrea Marcantoni,[3] Francesca Bonafé,[4] Gianni Losano,[3] and Renzo Levi[1]

[1]Department of Animal and Human Biology, University of Torino, Torino, Italy
[2]Department of Clinical and Biological Sciences, University of Torino, Torino, Italy
[3]Department of Neurosciences, Division of Physiology, University of Torino, Torino, Italy
[4]Department of Biochemistry, ''G. Moruzzi'', University of Bologna, Bologna, Italy

Abstract In order to assess, in a controlled in vitro model, the differentiation potential of adult bone marrow derived stem cells we have developed a coculture procedure using adult rat cardiomyocytes and mesenchymal stem cells (MSCs) from transgenic GFP positive rats. We investigated in the cocultured MSCs the time course of cellular processes that are difficult to monitor in in vivo experiments. Adult rat cardiomyocytes and adult rat MSCs were cocultured for up to 7 days and analyzed by confocal microscopy. Several markers were studied by immunofluorescence technique. The fluorescent ST-BODIPY-Dihydropyridine was used to label calcium channels in living cells. Intracellular calcium was monitored with the fluorescent probe X-Rhod-1. Immunofluorescence experiments showed the presence of connexin-43 between cardiomyocytes and MSCs and between MSCs, while no sarcomeric structures were observed at any time of the coculture. We looked at the expression of calcium channels and development of voltage-dependent calcium signaling in cocultured MSCs. MSCs showed a time-dependent increase of labeling of ST-BODIPY-Dihydropyridine, reaching a relatively strong level after 72 h of coculture. The treatment with a non-fluorescent DHP, Nifedipine, completely abolished ST-BODIPY labeling. We investigated whether depolarization could modulate intracellular calcium. Depolarization-induced calcium transients increased in MSCs in relation to the coculture time. We conclude that MSCs cocultured with adult cardiomyocytes present preliminary evidence of voltage-dependent calcium modulation uncoupled with the development of nascent or adult myofibrils, thus showing a limited lineage specification and a low plasticity to differentiate in a full cardiomyocyte-like phenotype. J. Cell. Biochem. 100: 86–99, 2007.

Key words: cardiomyocytes; mesenchymal stem cells; calcium channels; sarcomeric proteins

Bone marrow mesenchymal stem cells (MSCs) can be easily isolated and expanded ex vivo and are relatively safe in terms of rejection reaction, thus providing a promising model for development of stem cell therapeutics. In particular they have been considered of potential clinical use for the repair of infarcted myocardium [Pittenger and Martin, 2004].

Early clinical trials on small cohorts of patients with acute myocardial infarction have generally shown that implantation of autologous unfractionated bone marrow mononuclear cells or of MSCs significantly improve cardiac function [Laflamme and Murry, 2005]. However, a recent clinical trials update from the American Heart Association [Cleland et al., 2006] reports no benefit of stem cells implantation in the ASTAMI study and a small but significant benefit in REPAIR-AMI.

Several studies have reported that animals injected after an experimental infarction with autologous or etherologous MSCs have an improved recovery [Leri et al., 2005].

However, several technical problems on the detection of successfully implanted and

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*Correspondence to: Maria Pia Gallo, Department of Animal and Human Biology, University of Torino, Via Accademia Albertina 13, I-10123, Torino, Italy.
E-mail: mariapia.gallo@unito.it

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differentiated cells are still open [Laflamme and Murry, 2005]. Due to the diverging results obtained by various investigators, the possibility of a fruitful use of MSCs has remained a matter of controversy. In particular Wang et al. [2001] found that the transplantation of undifferentiated bone marrow MSCs in the rat infarcted myocardium originated a limited amount of cardiomyocytes together with individual cells or clusters expressing fibroblastic phenotype. A further issue is the finding that a small number of donor cells might fuse with the host cells giving rise to chimeric phenotypes [Zhang et al., 2004].

On another side a full characterization of MSCs biology in vitro is still not available and several doubts remain on the phenotypes they can develop and on the conditions required [Javazon et al., 2004]. At this regard recent results from Bayes-Genis et al. [2005] report that human MSCs spontaneously express markers of cardiac phenotype in vitro.

A vast literature reports about MSCs differentiation under defined in vitro conditions into the cells of several tissues, like osteocytes, chondrocytes, adipocytes [Pittenger et al., 1999], skeletal muscle fibers [Bhagavati and Xu, 2004; Gang et al., 2004], hepatocytes [Lee et al., 2004]. In particular, regarding heart repair, they have been shown to potentially originate both endothelial vascular cells and cardiomyocytes. Among the different strategies proposed to promote in vitro cardiac differentiation of MSCs, chronic exposure to 5-azacytidine has been amply tested [reviewed by Leri et al., 2005], as well as stimulation by exogenous cytokines and growth factors [Xaymardan et al., 2004, Shim et al., 2004], and coculture with neonatal cardiac cell [Lagostena et al., 2005] or immortalized cardiac cell lines [Rangappa et al., 2003]. Cocultures between MSCs and adult cardiomyocytes were performed mainly to show functional connection between the two cell types [Valiunas et al., 2004]. Other results with adult cardiomyocytes and MSCs were controversial [Wang et al., 2005; Yoon et al., 2005] and failed to clearly show striated and functional cardiac cells during the coculture time. It is interesting that bone marrow-derived endothelial progenitor cells taken from peripheral blood have been seen to differentiate into cardiomyocytes when cocultured with neonatal rat cardiomyocytes [Badorff et al., 2003].

Recent results suggest, moreover, that the differentiation of MSCs may depend on stochastic events and the cells actually fail to acquire a truly functional status [Belema Bedada et al., 2005].

Thus, many issues remain to be clarified on the processes following MSCs culture [Javazon et al., 2004] and transplantation in the myocardium. In particular, the cellular mechanism involved in the interaction between MSCs and recipient's adult cardiomyocytes has not yet been fully described. On the basis of the above uncertainty, the present investigation aims to reproduce and study in vitro the changes induced by host's myocardial microenvironment on donor's MSCs.

As an experimental model we cocultivate adult rat MSCs and adult cardiomyocytes, while characterizing the appearance of contractile proteins, calcium channels, and calcium control changes occurring in MSCs as well as their time-course during coculture.

Our experiments showed the presence of gap-junctions both in cardiomyocytes/MSCs and in MSCs/MSCs contacts, the appearance of voltage-dependent calcium signals in cocultured MSCs but no myofibrillar structures development at any steps of coculture and therefore the absence of contractile activity. We propose that MSCs grown in vitro in a cardiac microenvironment integrate with host cells but show a limited plasticity in the differentiation process towards a functional contractile phenotype.

MATERIALS AND METHODS

Animal Care and Sacrifice

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication N° 85–23, revised 1996) and in accordance with the Italian ethical guidelines (DL 111, 27 January 1992). The local ethical committee approved the projects.

Culture Media and Solutions

MSCs culture medium contained α-MEM, 10% fetal bovine serum (FBS), 2 mmol/L glutamine, 100 U/ml Penicillin, 100 μg/ml Streptomicin, 0.25 mg/ml fungizone (BioWhittaker, Europe). Bovine aortha endothelium (BAE-1) cells culture medium contained DMEM, 10% FBS, 2 mmol/L glutamine, 100 U/ml Penicillin,

100 μg/ml Streptomicin, and 0.25 mg/ml fungizone.

Coculture medium was M1018 (Sigma), 1% FBS, 100 U/ml Penicillin, 100 μg/ml Streptomicin, 1:1,000 Insulin-Transferrin-Selenium (ITS, Sigma), 10 mmol/L butanedionemonoxime (BDM, Sigma).

The control Tyrode solution contained (mmol/L): 154 NaCl; 4 KCl; 2 $CaCl_2$; 1 $MgCl_2$; 5.5 D-glucose; 5 HEPES; pH 7.35 adjusted with NaOH.

The 0 Ca^{++} Tyrode solution was the control Tyrode without $CaCl_2$ and with 10 mM BDM, 100 U/ml Penicillin, 100 μg/ml Streptomicin; pH 7.35 adjusted with NaOH.

The depolarizing solution was modified standard Tyrode with (mmol/l): 128 NaCl; 30 KCl; 2 $CaCl_2$; 5 HEPES; 1 $MgCl_2$; 5.5 D-glucose; pH 7.35 adjusted with NaOH.

Confocal Microscopy

The inverted microscope was an Olympus IX70 with a Fluoview 200 confocal head with an Ar/Kr laser (488 and 568 nm). Image analysis and Fast Fourier Transform were performed with ImageJ (Rasband, W.S., U. S. National Institutes of Health, Bethesda, MA, http://rsb.info.nih.gov/ij/, 1997–2005) on a PowerMac G5 (Apple, Cupertino).

Adult Rat Mesenchymal Stem Cells

Rat MSCs were harvested and cultured as described by Javazon et al. [2001]. Briefly, MSCs were isolated from the bone marrow of femurs of 6–12 month—green fluorescent protein (GFP) stable transfected rats [Ito et al., 2001], by flushing medium into the bone shaft. Cell suspension was filtered through a 70-μm nylon filter (Falcon) and plated in 75-cm^2 flasks. Cells were grown in complete α-MEM (20% FBS) at 37°C and 5% CO_2. After 3 days, the medium was replaced with fresh medium (10% FBS), and the adherent cells were grown to 90% confluency to obtain samples defined as passage 0 (P0). Cells were used for experiments between passage 3 and 10.

In previous published articles [Muscari et al., 2005, Raimondo et al., 2006], we characterized the MSCs population used for their BMSCs phenotypic features and for their potential to be committed to mesenchymal lineages. In particular, MSCs were able to differentiate into both osteoblast and myogenic lineage when treated, respectively, with osteogenic medium or with 5-azacytidine. Moreover, surface antigen determination revealed no expression of the hematopoietic markers CD34 and CD45. On the contrary, it was observed a very low positivity to CD59, a Sca-1 homolog, while 53% of total MSCs were positive to CD90, a surface antigen expressed by rat MSCs.

Adult Rat Ventricular Cells

Cardiomyocytes were obtained from young adult (4–6 months) rats by enzymatic dissociation. All solutions used for dissociation contained 10 mmol/L BDM to inhibit excitation-contraction coupling. Explanted hearts were cannulated via the aorta and perfused at constant flow rate (5 ml/min) with a peristaltic pump for 5′ with 0 Ca^{++} Tyrode solution. The latter and the following operations were carried out under a laminar flow hood. The heart was then perfused for 2′ with 0 Ca^{++} Tyrode plus collagenase and finally with the same Tyrode-collagenase plus 50 μmol/L Ca^{++}, to facilitate cellular dissociation. The heart was detached from the cannula, and the ventricles cut away and minced in small fragments. The fragments were collected in Tyrode-collagenase plus 50 μmol/L Ca^{++} solution and gently mixed. After 10′ medium was replaced with Tyrode 50 μmol/L Ca^{++} and mixed again with ventricular fragments for 10′. The supernatant (more than 50% rod-shaped cells) was collected, filtered, and exposed to growing concentrations of $CaCl_2$ (from 50 to 700 μmol/L) before cells were used for cocultures.

MSCs-Cardiomyocytes Cocultures

To obtain coculture preparations, about 10,000 cardiomyocytes were plated on laminin-treated glass-bottom dishes (WillCo Wells, The Netherlands) for calcium measurements and L-type calcium channel detection or on glass coverslips (for immunofluorescence), and incubated in M1018 medium plus 10 mmol/L BDM, 100 U/ml Penicilline, 100 μg/ml Streptomicine, and ITS (1:1,000). Cardiomyocytes were then placed in a 37°C—5% CO_2 incubator for at least 2 h, until adhesion occurred. Afterwards, medium was replaced with 1.5 ml of the same medium containing about 10,000 suspended MSCs. Coculture preparations were placed in a 37°C—5% CO_2 incubator. Control MSCs without cardiomyocytes were prepared in the same conditions as cocultured MSCs.

MSCs-Bovine Aortic Endothelial Cells (BAE-1) Cocultures

For this coculture preparation MSCs and BAE-1 in a 1:1 ratio (about 10,000 cells/ml) were suspended and plated with the same medium and protocol utilized for the MSCs-cardiomyocytes coculture.

Immunofluorescence—Cocultured MSCs Analysis

Cells grown on cover slides were fixed for 30 min in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3. After three washes with sodium phosphate buffer (PBS), cells were incubated 20 min with 0.3% Triton and normal goat serum (1:100, Sigma) in PBS and stained with the primary antibody 24 h at 4°C. Cardiac specific protein detection was performed with antibodies against cardiac myosin heavy chain (MF20, 1:20) and sarcomeric-α-actinin (monoclonal mouse IgG1 isotype, clone EA-53, Sigma, 1:800). The expression of connexin-43 was demonstrated using a polyclonal antibody against connexin-43 (1:400, Sigma). Cover slides where washed twice with PBS and incubated 1 h at room temperature with the secondary antibodies (Cy3 anti-mouse IgG, Sigma) for MF20 and anti-sarcomeric-α-actinin, Cy3 anti-rabbit IgG (Sigma) for connexin-43. After two washes in PBS cover slides were mounted on standard slides with DABCO (Sigma) and observed after 24 h under confocal microscope.

Direct L-Type Calcium Channel Detection

Cells grown on glass-bottom dishes were treated with the fluorescent probe ST-BODIPY-Dihydropyridine ((4,4-difluoro-7-styryl-4-bora 3a,4a-diaza)-3-(s-indacene) propionic acid-labeled dihydropyridine, Molecular Probes, 1 nmol/L, Em = 570 nm) and placed for 10 min in a 37°C—5% CO_2 incubator. After washing, fluorescence images were taken with a confocal microscope.

In pharmacological displacement experiments, cells on glass-bottom dishes under confocal microscope were directly perfused with 10 μmol/L Nifedipine (Sigma) and then with 10 μmol/L Nifedipine plus 1 nmol/L ST-BODIPY-Dihydropyridine or, as a control, with 1 nmol/L ST-BODIPY-Dihydropyridine alone.

The perfusion system apparatus consisted of five silicone tubes connected to 5 ml syringes reservoirs with the test solutions. All tubes conveyed in a single tip placed above the selected cells. Flow from each tube was controlled by an electrovalve and a peristaltic pump maintained the solution level in the dish.

Intracellular Calcium Measurement

Cells were incubated for 40 min with the calcium probe X-Rhod-1 (Molecular Probes, 1 μmol/L). The glass-bottom dishes containing MSCs alone (control) or MSCs + cardiomyocytes were washed and placed under the confocal microscope and perfused with standard Tyrode solution or with 30 mmol/L K^+ Tyrode (depolarizing solution), therefore washing away BDM. Frame acquisition interval was 3.3 s and the signal was expressed as (F-F0)/F0, where F0 represents the fluorescence level of the first image at the start of the measurement. For every experiment we analyzed intracellular calcium changes in all the cells in the imaging field. In every cell changes in calcium-sensitive fluorescence were monitored in the total cellular area. As the amplitude of calcium response with a non-ratiometric dye depends on a large number of variables, we did not attempt to express it in quantitative terms. We accepted as responding cells those that fulfilled the following criteria: the increase of fluorescence occurred after the application of the depolarizing solution, the background fluorescence did not significantly change, the increase was at least threefold higher than the spontaneous fluorescence fluctuation. The latter was mainly due to the solution level changes.

RESULTS

MSCs/Cardiomyocytes Coculture

First step in our study was to evaluate MSCs and cardiomyocytes proceeding in coculture. Confocal microscopy with superposition of green signal (GFP from MSCs) and conventional bright field microscopy (Nomarsky DIC) evidenced a good condition of both MSCs and cardiomyocytes after up to 7 days of coculture. Figure 1 shows MSCs and cardiomyocytes after 24, 48, 72 h, and 7 days of coculture.

Immunostaining for Connexin-43, Sarcomeric-α-actinin, and Sarcomeric Myosin

To investigate the rate of cellular integration and the presence of a typical cardiac

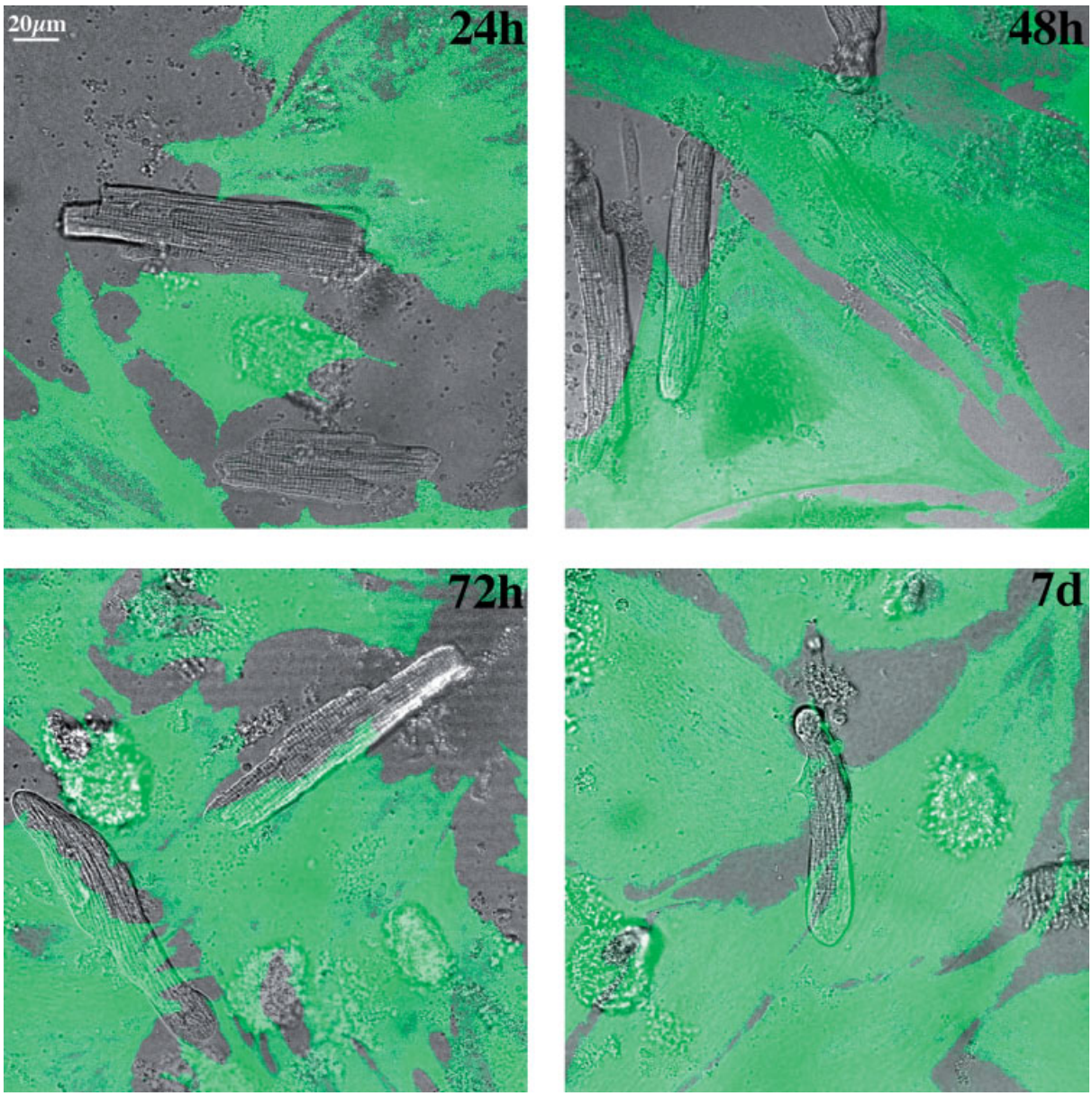


Fig. 1. Adult rat ventricular cells and adult rat marrow stromal cells in coculture. Confocal microscope images (60× magnification) of live cells from a typical coculture at 24, 48, 72 h, and 7 days; superposition of GFP (green) from MSCs and bright field microscopy (Nomarsky). Scale bar = 20 μm.

gap-junctions protein in the coculture preparations, we performed immunofluorescence experiments with a primary antibody against connexin 43 (Cx43).

Even in control MSCs without cardiomyocytes, a significative rate of Cx43 was detected (Fig. 2, panel A). In MSCs/cardiomyocytes cocultures we observed positivity for connexin Cx43 both between MSCs and between MSCs and cardiomyocytes (Fig. 2, panel B). Cx43 staining was clearly evident in the Z-axis profile from the XYZ images, as reported in panel C.

To compare the rate of MSCs integration in the cardiomyocytes coculture and in a coculture with a different cell type we performed immunofluorescence staining of Cx43 in a MSCs/BAE-1 coculture preparation. In this experiment at the contact points between MSCs and BAE-1 no Cx43 positivity was observed (supplementary data, Fig. s1).

Further experiments were aimed to investigate the appearance of sarcomeric proteins in cocultured MSCs, with immunostaining against sarcomeric-α-actinin and sarcomeric

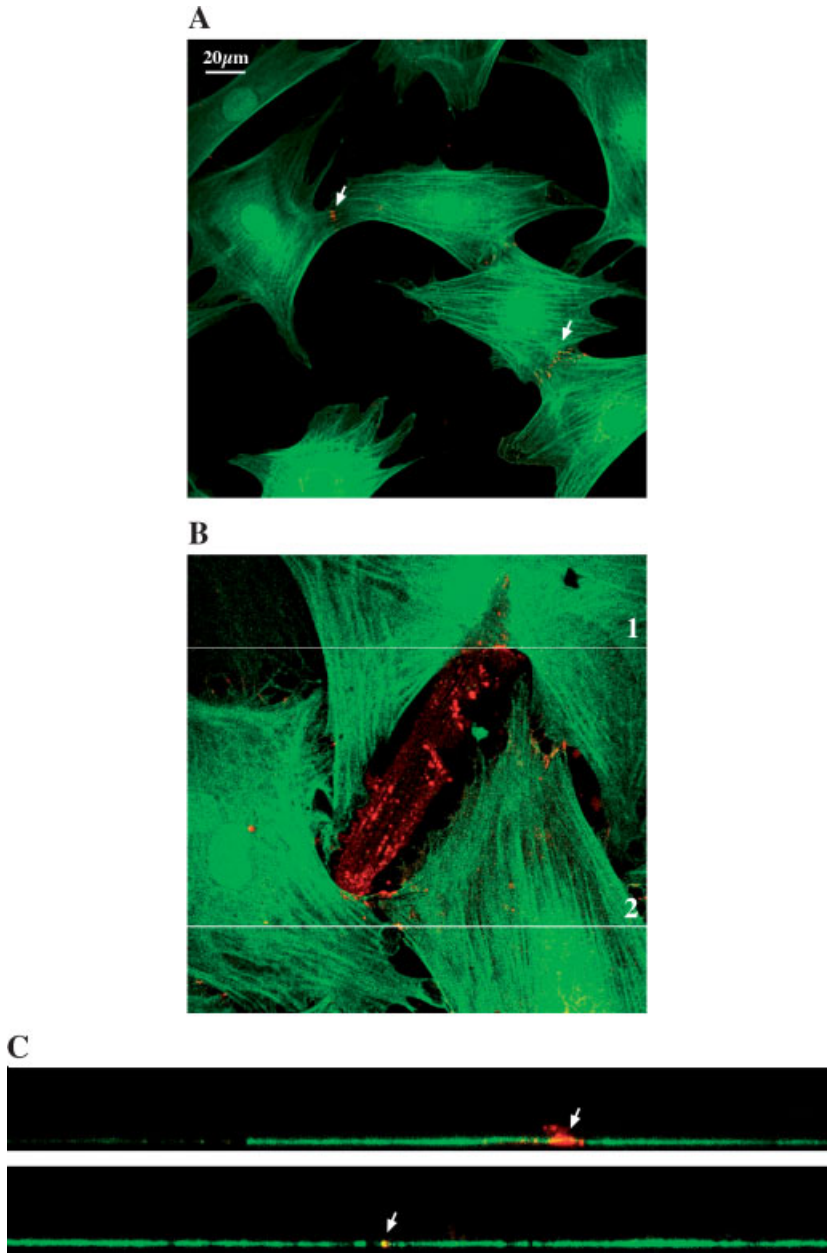


Fig. 2. Immunofluorescence detection with confocal microscope of connexin-43 (red spots) in a 48 h coculture preparation. **A**: Control MSCs. **B**: 48 h coculture. **C**: Vertical reslices correspondent to the white lines in the confocal image stack in B, showing connexin-43 detection between the central cardiomyocytes and a neighboring MSCs (line 1) and two adjacent MSCs (line 2). 60× magnification. Scale bar = 20 μm.

myosin at different time of coculture, precisely 48 h and 7 days. All these experiments demonstrated the absence of sarcomeric structures in cocultured MSCs.

Figure 3 shows a typical time-course of sarcomeric-α-actinin immunostaining in cocultured and in control MSCs: we observed a detectable red fluorescent signal on stress fibers and adhesion structures both in control (A) and cocultured MSCs (B, C) but the appearance of a sarcomeric organization after coculture was not detected. This result was confirmed by the Fast Fourier Transform analysis performed on immunodecorated stacks that excluded the presence of periodic structures in both control (D, 1) and cocultured MSCs (E, 3) compared to the periodic signals evidenced in the cardiomyocytes (E, 2).

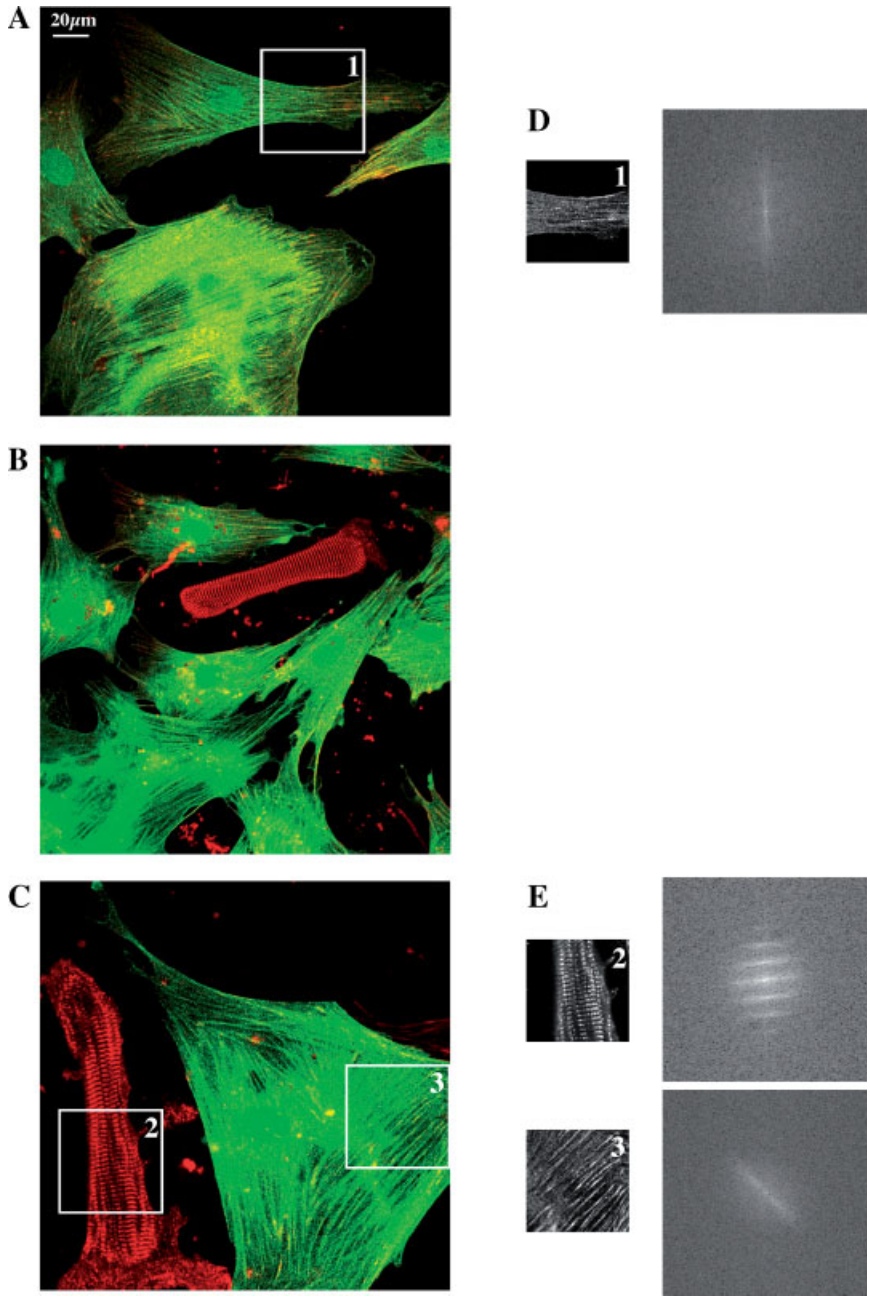
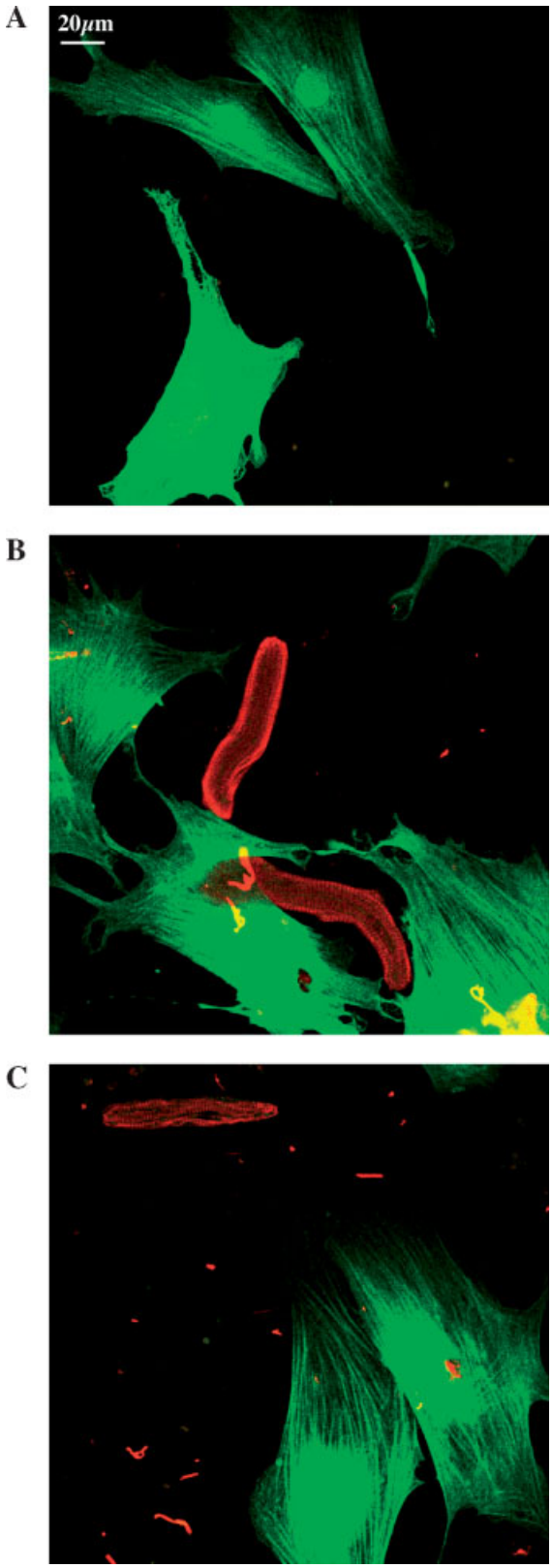


Fig. 3. Confocal microscope images of immunofluorescence detection for sarcomeric-α-actinin (red) in control MSCs and MSCs, and cardiomyocytes at different coculture times (48 h and 7 days). **A**: Control MSCs. **B**: 48 h coculture. **C**: 7 days coculture. **D, E**: Fast Fourier Transformation (FFT) of the regions of interest (ROI), correspondent to the numbered squares, from the stack images in A and C of the red signal, showing the absence of periodic events in a control MSC (1) and in a 7 day cocultured MSC (3) compared with the signal in a cardiomyocyte (2). 60× magnification. Scale bar = 20 μm.

Cardiac myosin immunostaining experiments showed no positivity of cocultured MSCs, as illustrates in Figure 4: in control (A) and cocultured (B, C) MSCs only the green GFP signal is detectable.

Time-Dependent and Specific Fluorescent Dihydropyridine Labeling of Cocultured MSCs

It has been observed in human MSCs in primary culture, on rounded cells resulting



from cell detachment and short term (15 min) reattachment, the presence of calcium current in a small percentage of cells and of mRNA for the α1-subunit of L-type calcium channels [Heubach et al., 2004]. As reported in the same work and also from our unsuccessful attempts, no electrophysiological measurement could be performed on adherent cells. Moreover, in c-kit$^+$ stem cells from adult mice bone marrow in coculture with neonatal cardiomyocytes no mature or functional voltage-dependent calcium channels were observed [Lagostena et al., 2005].

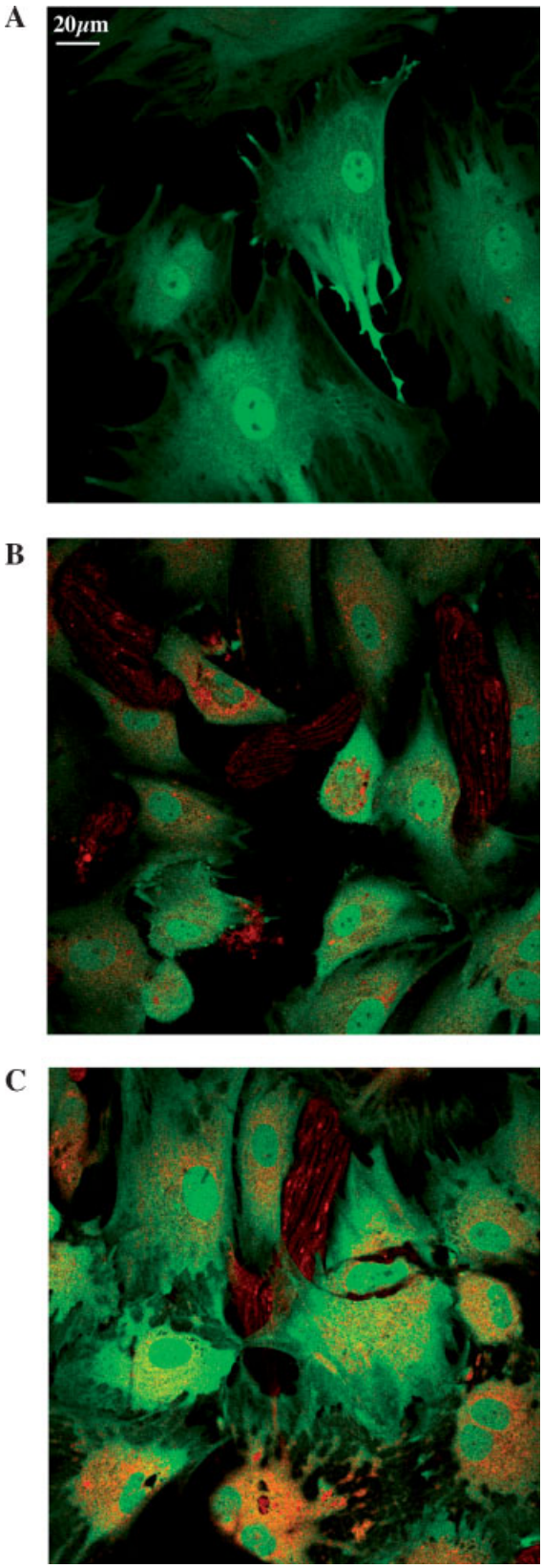
We analyzed the expression of L-type calcium channel in MSCs during coculture using a red fluorescent-tagged Ca^{2+} channel blocker, ST-BODIPY-Dihydropyridine. Living cells were incubated for 10 min with 1 nmol/L ST-BODIPY-Dihydropyridine, then washed in Tyrode solution and observed with confocal microscopy. Fluorescence was not observed in control MSCs (Fig. 5A), but was present in MSCs from 48 h in coculture (Fig. 5B,C), and cardiomyocytes were also labeled.

No labeling was instead observed when MSCs where cocultured with bovine aortic endothelial cells (supplementary data, Fig. s2).

To further assess the specificity of the labeling, we treated the cultures with a larger concentration of a different non-fluorescent DHP, Nifedipine (10 μmol/L), before exposing cells to ST-BODIPY-Dihydropyridine (1 nmol/L) directly by perfusion under confocal microscope observation. In Figure 6A significant frames from the image sequence (1 frame every 1.24 min) were extracted from an XYT acquisition (3.3 s frame interval) of a 48 h coculture perfused with control Tyrode solution, with 10 μmol/L Nifedipine and with Nifedipine plus 1 nmol/L ST-BODIPY-Dihydropyridine. Neither MSCs nor the cardiomyocyte were marked with the fluorescent DHP.

As a final control of the procedure, we performed the same experiments in the absence of Nifedipine to show that ST-BODIPY-Dihydropyridine labeling is rapid. Figure 6B shows an experiment on a 48 h coculture perfused with control Tyrode solution and with 1 nmol/L

Fig. 4. Confocal microscope images of immunofluorescence detection for sarcomeric myosin (red) in control MSCs and MSCs, and cardiomyocytes at different coculture times (48 h and 7 days). **A**: Control MSCs. **B**: 48 h coculture. **C**: 7 days coculture. 60× magnification. Scale bar = 20 μm.



ST-BODIPY-Dihydropyridine. The fluorescent probe shows similar MSCs labeling as observed in the experiments presented in Figure 4, where ST-BODIPY-Dihydropyridine was applied by preincubation and washout.

Voltage-Dependent Calcium Modulation in Control and Cocultured MSCs

To investigate the coupling between the voltage-dependent calcium channel expression observed in the previous experiments and the appearance of a voltage-dependent calcium modulation in the cocultured MSCs, we performed calcium measurements in 24, 48, 72 h, and 7 days cocultures and in control MSCs. As the X-Rhod-1 probe has been described to have a tendency to compartimentalize into mitochondria, we performed control experiments on cardiomyocytes using simultaneously X-Rhod-1 and Fluo-3. The two dyes gave very similar signals (data not shown).

As a control of the potassium-induced depolarization at least one cardiomyocyte was included and analyzed in each acquisition field, and its contraction was visually observed. In order to show the preserved functionality of cocultured cardiomyocytes we present in the supplementary data (Fig. s3) a typical experiment of calcium measurement in a 7 days coculture that simultaneously displays the cardiomyocyte contraction after depolarization and the time course of the intracellular calcium changes upon two potassium stimulations. As after 4 day the cardiomyocytes are strongly attached to the dish and to the neighboring cells only a small but unequivocal contraction could be observed. In several observations some neighboring MSCs could be seen to be slightly pulled by the myocyte.

Bar graphs in Figure 7 summarizes calcium measurements after K^+ depolarization in 24, 48, 72 h and 7 days cocultures and in control MSCs. Cocultures were carefully observed with the confocal microscope before potassium stimulation to avoid artifacts due to partial superposition of MSCs with cardiomyocytes. GFP

Fig. 5. Time-dependent increase of labeling with fluorescent dihydropyridine (ST-BODIPY-Dihydropyridine) of cocultured MSCs. In these experiments living cells were incubated 10′ with 1 nmol/L ST-BODIPY-Dihydropyridine, washed in control Tyrode solution and then observed in confocal microscopy. **A**: Control MSCs. **B**: 48 h coculture. **C**: 7 days coculture. 60× magnification, superposition of ST-BODIPY-Dihydropyridine (red) and GFP (green) fluorescence. Scale bar = 20 μm.

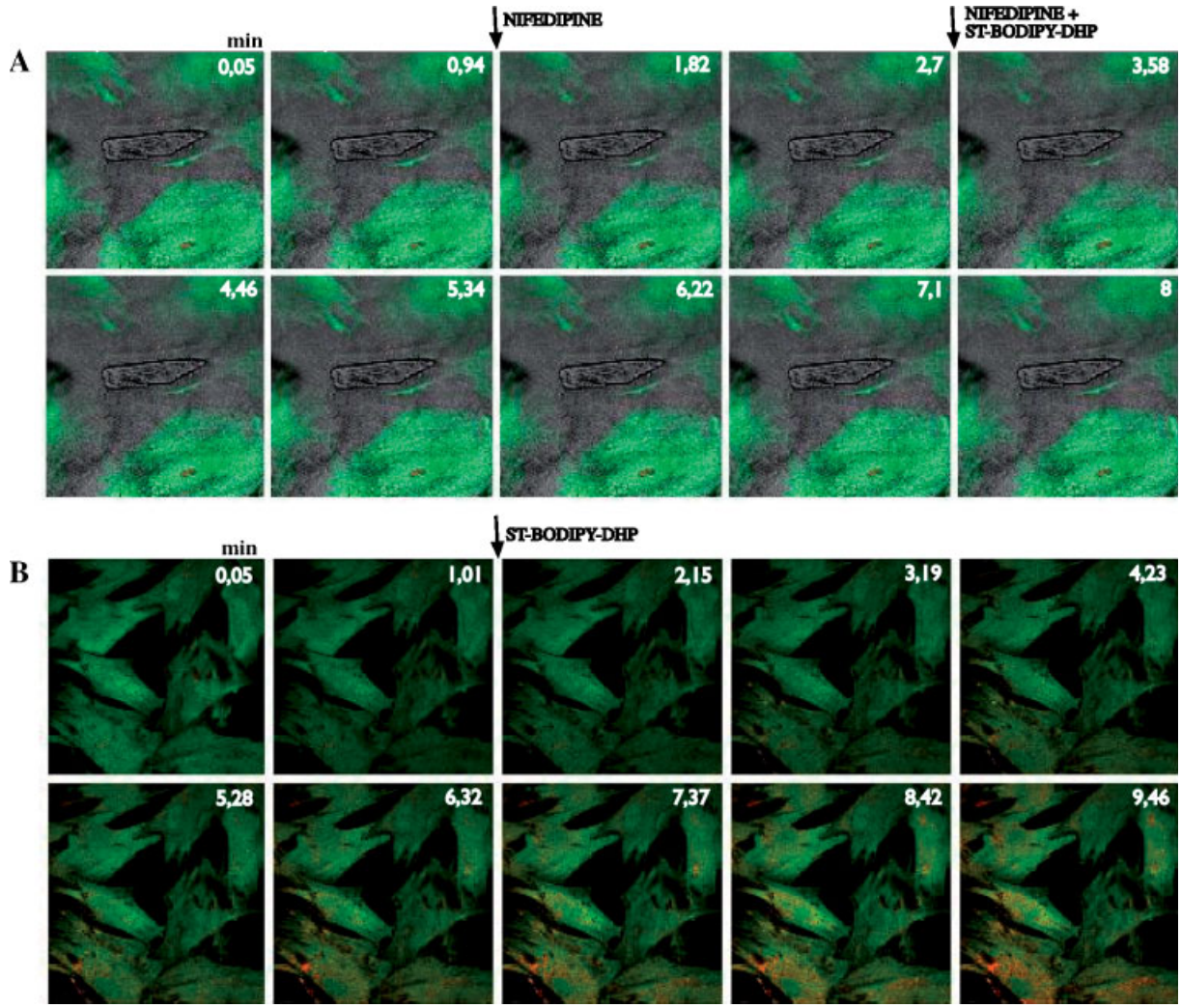


Fig. 6. ST-BODIPY-Dihydropyridine block by Nifedipine. Living cells on glass-bottom dishes were successively perfused with 10 μm Nifedipine and subsequently with 10 μm Nifedipine plus 1 nmol/L ST-BODIPY-Dihydropyridine (**A**) or directly perfused with 1 nmol/L ST-BODIPY-Dihydropyridine (**B**). Frame acquisition was every 3.3 s and 10 images (1 every 1.24 min) were extracted from the total stacks sequence. The arrows indicate drug applications. A: 48 h coculture perfused with control Tyrode solution, 10 μm Nifedipine and with 10 μm Nifedipine plus 1 nmol/L ST-BODIPY-Dihydropyridine. Superposition of red fluorescence (ST-BODIPY-Dihydropyridine), green fluorescence (GFP), and bright field shows the cardiomyocyte unlabeled with the fluorescent dihydropyridine after Nifedipine pretreatment. B: 48 h coculture perfused with control Tyrode solution and with 1 nmol/L ST-BODIPY-Dihydropyridine. 60× magnification.

fluorescence was used as a control of the stability of the laser source, and all fields that showed marked instability either in the GFP signal or in background signal in areas devoid of cells were discarded.

We observed a growing percentage of responsive cells along the period of coculture, in particular after 48 h: 4% in control MSCs (6/166), 6% in 24 h cocultures (3/54), 26% in 48 h (19/72), 37% in 72 h (19/51), 39% in 5–7 days (17/44).

Figure 8 shows a representative experiment of intracellular calcium measurements in a 24 h coculture. The image in Figure 8A, (X-Rhod-1 and GFP fluorescence) shows two cardiomyocytes and several MSCs. The line graph in panel B represents the time course of the normalized fluorescence for a responsive MSC, as indicated in the upper legend. Panel C shows the responses of the two cardiomyocytes. Application of depolarizing solution is indicated by the horizontal bars in the line graphs.

DISCUSSION

A vast literature has presented the potential of MSCs to differentiate into cardiomyocytes

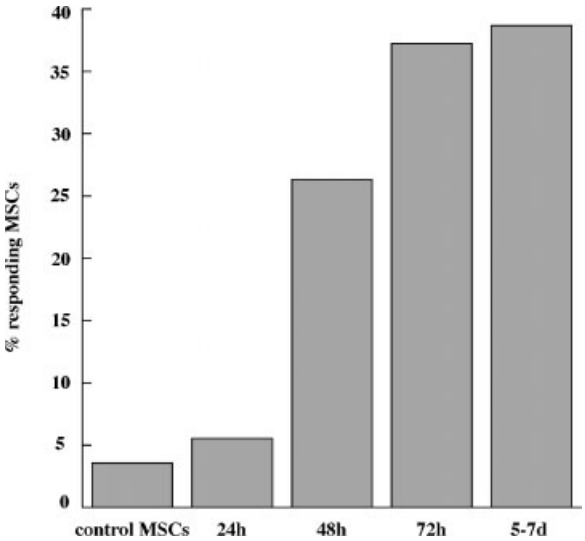


Fig. 7. MSCs responses to depolarization. Bar graph showing the percentage of MSCs responding to high K^+ depolarization with a calcium concentration change: 4% in control MSCs (6/166), 6% in 24 h cocultures (3/54), 26% in 48 h (19/72), 37% in 72 h (19/51), 39% in 5–7 days (17/44).

in both in vitro and in vivo models [Haider and Ashraf, 2005]. Even with the amount of experiments performed, many details on the mechanisms and limits of this process are still not clear. Controversial results have emerged even in comparable conditions, stimulating speculations on basic physiological and developmental issues, such as the potential of bone marrow derived stem cells to fuse with resident cells [Zhang et al., 2004] or which pathways may lead to cell dedifferentiation and redifferentiation. A further problem is the lack of a clear definition of which markers are evidence for differentiation [Belema Bedada et al., 2005]. The in vivo experiments are faced with the difficult issue to find the relatively few injected cells in the organ and to properly identify them among the vast amount of non-myocyte cells that form the heart. Moreover, recent results introduced the hypothesis that adult stem cells may represent loose ends of progenitor cells that stopped serving an important physiological role and failed to acquire a fully functional phenotype [Belema Bedada et al., 2005].

A basic issue is also related to the puzzling question posed by the discovery that some gastrointestinal tumors may be due to mobilized MSCs that migrate to the inflamed tissue [Houghton et al., 2004]. There is, nevertheless, a substantial agreement that some benefits upon MSCs transplantation may be achieved, even if it is questioned whether it arises directly from the regeneration of cardiac tissue or from more general improvements of the healing process, derived by increase of vascularization or decrease in fibrotic scar [Pittenger and Martin, 2004].

The in vitro assessment of the differentiation potential of MSCs has been mainly monitored in cultures containing either MSCs alone treated with 5-azacytidine [Xu et al., 2004] or with a cocktail of defined growth factors [Shim et al., 2004; Xaymardan et al., 2004]. Cell treatment with 5-azacytidine has a long scientific history, as it has been at first proposed to induce differentiation in several cell lines, mainly in an attempt to reduce the aggressivity of transformed cells. It apparently acts by modifying methylation [Momparler, 2005]. Other experiments studied coculture model of MSCs with neonatal cardiomyocytes [Lagostena et al., 2005] or cardiomyocyte-cell lines [Rangappa et al., 2003; Beeres et al., 2005], but not extensively with primary adult cardiomyocytes. We are presenting a study in controlled conditions of the effect of adult cardiomyocytes on the expression of markers of differentiation in MSCs. Previous data on MSCs/adult cardiomyocytes coculture [Wang et al., 2005; Yoon et al., 2005] have been presented but apparently did not clearly show the presence of cardiomyocytes that retain a normal functional morphology along the culture period.

After establishing a culture protocol, we have then examined and found connexin-43 expression both between MSCs and between cardiomyocytes and MSCs. The formation of gap junctions is a marker of cell–cell interaction and is required to allow electrical signal propagation, a prerequisite for any functional grafting in the myocardium. While even in MSCs alone expression of Cx43 is present in coculture conditions we observed a clear evidence of this signal only in the MSCs/cardiomyocytes contacts but not in the MSCs/BAE-1 contacts (Fig. s1).

Some authors have showed evidence of a complete establishment of muscular protein networks in MSCs upon clonal selection in the presence of 5-azacytidine [Xu et al., 2004] and the expression of markers typical of cardiac myocytes, but not functional differentiation in MSCs cocultured with neonatal cardiomyocytes [Lagostena et al., 2005].

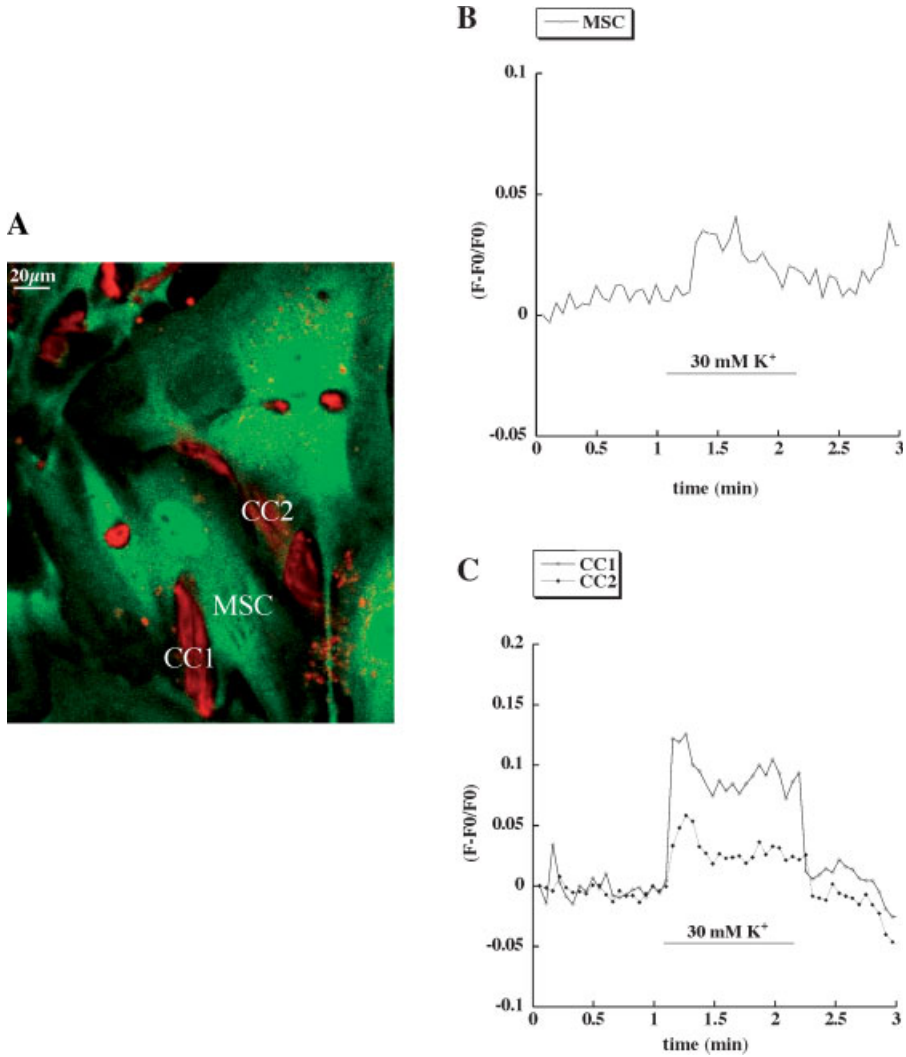


Fig. 8. Representative experiment of intracellular calcium measurement with high K^+ depolarization in a 24 h coculture. **A**: confocal image showing the 24 h coculture. 60× magnification, superposition of Xrhod-1 and GFP fluorescence. **B**: Time course of calcium concentration changes (normalized fluorescence) from the responsive MSC in A. **C**: Time course of calcium concentration changes (normalized fluorescence) from the two cardiomyocyes (CC1 and CC2) in A. The horizontal bars indicates the time of application of depolarizing (30 mM K^+) Tyrode.

In our results even after 1 week of coculture, we have not seen any ordered sarcomeric structure that can indeed be easily identified in cardiac myocytes by a ordered pattern in the Fourier transform of the confocal images. In particular, sarcomeric-α-actinin immunostaining revealed the same pattern in control and cocultured MSCs, with an evident staining of stress fibers and focal adhesion. In agreement with our immunofluorescence results Bayes-Genis et al. [2005] recently shown that in human MSCs sarcomeric-α-actinin, but not sarcomeric myosin, was spontaneously expressed.

Furthermore, the appearance of such structures could not be considered by itself a specific marker of muscular differentiation, as many different cell types in culture as well as in situ present stress fibers with a periodic sarcomere-like organization [Peterson et al., 2004] and myofibroblast cell lines of mesenchymal origin express sarcomeric proteins [Mayer and Leinwand, 1997].

Our coculture procedure is affected by clear limitations, such as a restriction to 1 week of the observation and the presence of an excitation-contraction uncoupler, that allows a longer

survival period of the cardiac myocytes. One might speculate that even longer time might lead to a new destiny of the MSCs or at least of a subpopulation. Still, most in vitro experiments that have described differentiations have been performed for even shorter times. The excitation-contraction uncoupling drug does not by itself in our hands induce persistent changes in the cardiac myocyte as cells resume contraction rapidly upon washout of the drug even after several days in culture, as demonstrated by the presence of contractions upon potassium stimulation. In control experiments (data not shown) cardiac myocytes have been field stimulated during rapid washout of BDM and recovery of contraction occurred within minutes.

We acknowledge that our approach is still rudimentary, as we do not maintain the coculture under continuous electrical stimulation. Therefore, during coculture the cardiomyocytes may have lost their properties which provide the "cardiac niche." To avoid this possibility we carefully controlled during potassium depolarization experiments that all cardiomyocytes present in the observed field showed a clear contractility (supplementary data, Fig. s3), and this test was used to validate both the depolarization and the cardiomyocytes functionality.

A sound hypothesis could nevertheless be that differentiation of MSCs into cardiac myocytes could require at least a stage of periodic contraction, in a way resembling the embryonic to adult development in the heart.

ST-BODIPY-Dihydropyridine specific labeling of live cocultured MSCs indicates an increase in voltage-gated calcium channels (VGCC) expression. Moreover the absence of labeling in the MSCs/BAE-1 coculture suggests that cardiac microenvironment could be necessary in this process.

However this behavior is not necessarily a marker of differentiation toward an excitable cell, as many different cell types express VGCC for many different functions, including growth factor induced fibroblast cell migration [Yang and Huang, 2005].

Our observation of depolarization-induced calcium transients in MSCs in proximity with myocytes is in agreement with ST-BODIPY-Dihydropyridine specific labeling of cocultured MSCs. Moreover, in a similar experiment [Beeres et al., 2005], it has been observed the electrotonic propagation of action potentials across MSCs implanted in an in vitro lesion of a layer of beating myocytes. In both cases the result could be in perspective clearly beneficial, even if the MSCs themselves are incapable of action potentials and contraction. We have been unable to observe any clear-cut contraction in MSCs upon depolarization and in the presence of calcium increases. The only movements we have detected were clearly related to close-by myocytes that were pulling the MSCs.

As a summary our results agree with the hypothesis that it is difficult to obtain in vitro a complete differentiation of bone marrow derived stem cells, at least in the absence of a complete reprogram as can be achieved with 5-azacytidine. The limited plasticity could anyway explain the improvement observed in in vivo experiments and in patients, as the formation of gap-junctions could restore conduction that is blocked instead by fibroblasts, and the presence of MSCs could reduce the formation of scar tissue.

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