Journal of Cell Science & Therapy

Skopalik, et al., J Cell Sci Ther 2014, 5:5
DOI: 10.4172/2157-7013.1000185

Research Article Open Access

Formation of Cell-To-Cell Connection between Bone Marrow Cells and Isolated Rat Cardiomyocytes in a Cocultivation Model

Josef Skopalik[1], Michal Pasek[2,5], Milan Rychtarik[3], Zdenek Koristek[1], Eva Gabrielova[4], Peter Scheer[1], Peter Matejovic[3], Martin Modriansky[4] and Martin Klabusay[1*]

[1]*Integrated Center of Cellular Therapy and Regenerative Medicine, International Clinical Research Center, St. Anne's University Hospital, Brno, Czech Republic*

[2]*Department of Physiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic*

[3]*Department of Biomedical Engineering, Faculty of Electrical Engineering and Communication, Brno University of Technology, Brno, Czech Republic*

[4]*Department of Medical Chemistry and Biochemistry, Palacky University, Olomouc, Czech Republic*

[5]*Institute of Thermomechanics - Branch Brno, Academy of Science of the Czech Republic, Brno, Czech Republic*

[*]**Corresponding author:** Martin Klabusay, Integrated Center of Cellular Therapy and Regenerative Medicine, International Clinical Research Center, Saint Anne's University Hospital Pekařská 53, Brno 656 91, Czech Republic, Tel: +420603167625; Fax: +420542217560; E-mail: m.klabusay@sky.cz

Rec date: Oct 03, 2014; **Acc date:** Nov 11, 2014; **Pub date:** Nov 13, 2014

Abstract

Aims: Limited regenerative potential of cardiomyocytes (CMs) causes irreversible changes in heart tissue during pathological processes. However bone marrow mononuclear cells (BM-MNCs) can migrate to this tissue, incorporate to the area of dead or missing myocytes, and improve the global heart function. The mechanism of BM-MSCs' incorporation and interaction with CMs is not clear. Our aim was to create an in vitro model which would enable to study the interaction of BM-MNCs with CMs and to make a microscopy description of these interactions.

Methods and Results: CMs were isolated from adult and newborn rats. BM-MNCs were isolated from bone marrow. BM-MNCs were added to the myocyte culture. Cell-to-cell adherence and Cx43 expression were evaluated by fluorescence microscopy, Ca^{2+} transients were evaluated in cardiomyocyte-BMC communication under electrical stimulation by fluo-4 fluorescence measurement. Analysis of calcein transport from BM-MNCs to CMs was performed using fluorescence microscopy.

Conclusions: The adherence of BM-MNCs to CMs occurred quickly and was stable. Cx43 was detected in contact zones between BM-MNCs and CMs; pairs which displayed Cx43 positivity represented less than 1% from all BM-MNC-cardiomyocyte pairs in the coculture. Conductive structures between CMs and BM-MNCs were formed and verified by imaging calcein transfer and synchronous Ca^{2+} transients.

Keywords: Bone marrow; Mononuclear cells; Isolated cardiomyocytes; Cocultivation; Connexins; Cell communication

Introduction

Myocardial infarction is the leading cause of heart failure and death in developed countries. It is caused by restriction of blood supply to a part of the heart muscle. The consequent ischemia may lead to necrosis of a significant part of the heart [1,2]. Adult cardiomyocytes have a very limited potential to regenerate, and the necrotic tissue cannot be reconstituted into a functional syncytium of new cardiomyocytes [3,4]. Rather, necrotic tissue is gradually replaced by a highly organized scar containing mainly fibroblasts [5]. In the majority of cases, necrosis results in heart failure, for which today there is only one known causal therapy: heart transplantation. There are well-known problems associated with allogeneic grafts, and insufficient numbers of organs are available for transplantation. Therefore, the potential for delivering stem/progenitor cells into the infarcted area is being intensely investigated. While different cell delivery methods have been used successfully in many cases [6], engraftment, subsequent regenerative effects, and the efficiencies of these processes require further study. Continued investigation is needed to develop a safe and efficient means of cell therapy.

Clinical studies and studies on experimental animal models have demonstrated the ability of delivered bone marrow mononuclear cells (BM-MNCs) or endothelial progenitor cells (EPCs) to improve the function of an ischemic heart. Nevertheless, the precise mechanism of cell homing in the affected heart and the cells' interaction with the residual functional cardiomyocytes (CMs) in the infarcted area remain unclear. While basic studies have indicated that BM-MNCs may transdifferentiate into CMs [7,8], other investigators suggest that BM-MNCs or EPCs play only a mechanical support role or act in vivo to restore the ion-conductive connection among the separated areas of heart tissue [9]. Another hypothesis states that BM-MNCs or EPCs are merely a source of paracrine factors that help CMs to function and to survive, or that eliminate pathophysiological fibroblast growth and left ventricular remodeling [10]. This divergence in published theories highlights the need for further investigation in this field, including developing in vitro experimental models. The aim of this study was to create conditions that would facilitate structural and functional connection in a model of BM-MNCs co-cultivated with isolated rat CMs and to perform its analysis. Our study used a traditional cocultivation setup which has been reported in several published works [11-13]. Genetically unmodified BM-MNCs were arranged in a microchamber with a rational concentration of neonatal or adult MCs. This arrangement permits continual microscopic analysis of tropism

by BM-MNCs to the free ends of CMs (a model for interrupted CM strands in infarcted heart), quantitative analysis of CM–BM-MNC coupling (analogous with hypothetical mechanical and conductive coupling which initiate the regenerative process in vivo), and analysis of cell survival/differentiation of all cells in the coculture. Moreover, our setup performs variable confocal scanning of cells with high spatial resolution and recording of cell activity with high temporal resolution (using an ultra-high-speed camera). An important original aspect of our analysis was quantitative comparison of the interactions with myocytes of fresh and cultured BM-MNCs. A second original aspect of our work is its parallel use and comparison of neonatal and adult MCs as a substrate in cocultivation experiments. Although neonatal MCs are standardly used in almost all published cocultivations, their use as a model of in vivo adult heart tissue is still controversial and exact comparative analyses with adult MCs are both scarce and controversial in the literature [12].

Materials and Methods

Isolation of cardiomyocytes

Adult rat cardiomyocytes were obtained from young adult Wistar rats (250 ± 50 g). Rats were anesthetized by intramuscular administration of a mixture of 1% ketamine (Narkamon inj., Spofa, Czech Republic) (80 mg/kg) and 2% xylazine (Rometar inj., Spofa, Czech Republic) (12 mg/kg) into the right femoral muscle. After the loss of reflex, the sternum was quickly cut, the ribcage opened, and the beating heart cut from circulation and extracted. We proceeded using the method of enzymatic dissociation [14]. Explanted hearts were cannulated via the aorta and perfused using a peristaltic pump for 30 s with Tyrode's solution (0.9 mmol/l $CaCl_2$) at a constant flow rate of 5 ml/min. The heart was perfused with Ca^{2+} free Tyrode's solution containing collagenase (Collagenase type II, Yakult, Japan) and protease (Protease type XIV, Sigma–Aldrich, Germany). The heart was then detached from the cannula and the ventricles were separated and dissociated into small fragments. The fragments were collected in Tyrode's–collagenase solution and gently mixed. After 30 s, the medium was replaced with standard Tyrode's solution (0.09 mmol/l $CaCl_2$) and mixed again for 10 s. The supernatant (more than 50% rod-shaped cells) was collected, filtered, and exposed to 0.9 mmol/l $CaCl_2$. Morphology of the cells was recorded for 48 h (Figure 1B).

Primary neonatal rat cardiomyocytes were isolated from Wistar rats 2–5 days old, as described previously [15]. Neonatal rats were anesthetized using diethyl ether and decapitated. Hearts were removed and dissociated in a balanced salt solution containing 20 mM HEPES, 120 mM NaCl, 1 mM NaH_2PO_4, 5.5 mM glucose, 5.4 mM KCl, and 0.8 mM $MgSO_4$ (pH 7.3-7.4). Cardiomyocytes were isolated by trypsin digestion (0.2% w/v), after which the cells were resuspended in a medium containing Iscove's modified Dulbecco's medium (IMDM) (Sigma–Aldrich, Germany) and Medium 199 (Sigma–Aldrich, Germany) in a 4:1 ratio and supplemented with horse serum (10%), fetal calf serum (5%), penicillin (100 U/mL), and streptomycin (100 µg/mL). The cells were plated for 1.5-2.0 h to allow for attachment and/or separation of non-myocardial cells (heart fibroblasts). The non-adhesive cells (cardiomyocytes) were transferred into a centrifugation tube, washed, then centrifuged at 150 × g rpm for 10 min. Suspension enriched in non-adhesive CMs was transferred to culture dishes coated with collagen I at density 5×10^4 cells/cm^2. The cells were incubated in conditions of 21% O_2 and 5% CO_2 at 37°C. Cultivation medium was removed after 72 h and replaced by a medium containing IMDM and Medium 199 (4:1) with penicillin (100 U/ml) and streptomycin (100 mg/ml). Viability and function of the neonatal cardiomyocytes were verified by various means, and viability always exceeded 95%. Viable CMs displayed contractions at a rate of approximately 100 beats per minute. Maintenance of metabolically active mitochondria was verified using JC-1 fluorescent indicator of mitochondrial membrane potential where responsiveness to uncouplers was mandatory (Figure in Supplement-1).

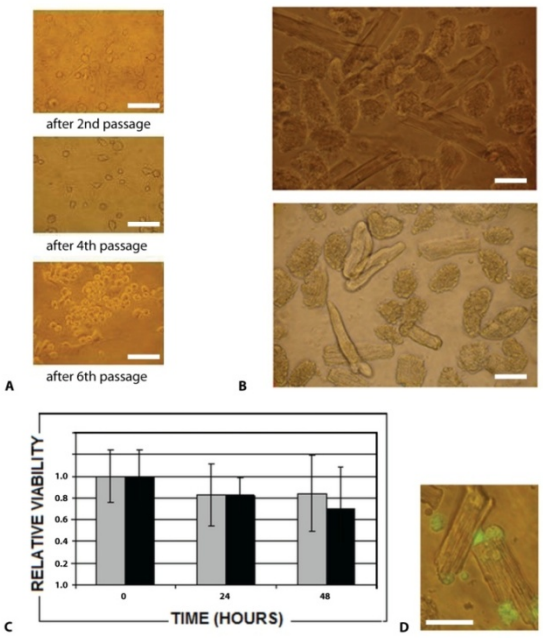

Figure 1: (A) Morphology of rat BM-MNCs adhered to the plastic bottom on the fifth day after the second, fourth and sixth passages. (B) Morphology of adult myocytes after 24 h in M1 medium (upper image) and in standard IMDM medium with10% FBS (bottom image). (C) Survival rate of adult cardiomyocytes in M1 medium (shadow bar) and standard IMDM medium (black bar). Data are presented as averages ± standard deviation. (D) BM-MNCs (green circular cells) adhere to cardiomyocytes and one of them makes mechanical connection between two cardiomyocytes, Axiovert 40C/CFL Zeiss fluorescence microscope, objective magnification 40×. Scale bars represent 40 µm.

Isolation of Bone Marrow Mononuclear Cells

Rat BM-MNCs were harvested and cultured, as previously described [12]. Briefly, cells were isolated from the bone marrow of femurs by flushing medium into the bone shaft (method of anesthesia of the rat based on ketamine and xylazine was the same as for the heart isolation protocol described above). Cell suspension was filtered through a 40 µm nylon cell strainer (BD Falcon, USA) and layered on Histopaque at 500×g for 30 min, mononuclear cell layer was plated in chambers of 24-well plates. Cells were grown in IMDM with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2.

The first group of BM-MNCs (named fresh BM-MNCs) was stained and used for cocultivation immediately (1h) after isolation. The second group of BM-MNCs (named cultivated BM-MNCs) was

incubated under the described conditions for 3 day, the medium was replaced with fresh medium (with wash-out of non-adherent cells), and the adherent cells were grown to 80% confluence before passage was made. Four passages were performed during the following 4 weeks. Cells from the second to fourth passages (morphology at Figure 1A) were used for cocultivation experiments (cocultivation of neonatal and adult CMs was performed in parallel, as detailed in the next paragraph).

Staining of BM-MNCs and cocultivation

Before coculture experiments, fresh BM-MNCs and cultured BM-MNCs after 2–6 passages were labeled with 10 μM fluorescent dye PKH26 (Sigma–Aldrich, Germany) for 5 min at room temperature in phosphate-buffered solution. The PKH26-labeled BM-MNCs were plated at density 3×10^4 cells/cm^2 (fresh) or 1.5×10^4 cells/cm^2(cultured) in one 24-well dish containing 4×10^4 neonatal CMs/cm^2 or 1×104 adult CMs. Table 1 provides an overview of cocultivation experimental variants. M1 medium was used for cocultivation with neonatal or adult CMs. (Media were based on Tyrode's solution and IMDM media produced by Sigma–Aldrich, Germany. The complete composition of the M1 medium is available in Supplement 2. A comparison of the viability of CMs in IMDM [with addition of 10% FBS] and M1 medium was performed, as detailed in Results) All culture solutions were supplemented with penicillin (100 U/ml) and streptomycin (100 mg/ml). All four sets of cocultivation variants (with exceptions named below) underwent basic measurement of cell-to-cell adhesion and three different types of cell-to-cell connection analysis 24 h after initialization of cocultivation, as described immediately below.

Setup 1	Setup 2	Setup 3	Setup 4
Neonatal	Neonatal	Adult	Adult
Cardiomyocytes	Cardiomyocytes	Cardiomyocytes	Cardiomyocytes
+	+	+	+
Fresh BM-MNCs	Cultured BM-MNCs	Fresh BM-MNCs	Cultured BM-MNCs
In M1 medium	In M1 medium	In M1 medium	In M1 medium

Table 1: Setups for cocultivation experiments

Basic detection of BM-MNCs adherence to CMs

The liquid inside the each cultivation chamber was gently resuspended by 100 uL automatic pipette (decoupling of cells in pairs which did not create real mechanical adherence but only short time adhesion), minimally 10 wide fields contained minimally 40 CMs was immediately identified in each cultivation chamber and basic visual analysis was used for quantification of the BM–MNCs with strong adherence to CMs. The percentage of adhered BM-MNC was quantified in 160 wide fields for each of 8 independent adult animal experiments and in 160 wide fields for each of 6 independent neonatal animal experiments.

Detection of Cx43 intracellular connection

BM-MNCs and CMs grown on cover slides on the bottom of dishes were fixed for 30 min in 4% paraformaldehyde. After three wash-outs with phosphate buffered saline, BM-MNCs and CMs were incubated 20 min with 0.3% Triton and stained with the primary IgG antibody anti-connexin43 for 2 h (1:400, C8093, Sigma–Aldrich, Germany). BM-MNCs and CMs were then washed twice with phosphate buffered saline and incubated for 1 h at room temperature with the secondary antibodies (1:100, F 9006 Sigma–Aldrich, Germany). To detect Cx43, fluorescence imaging was performed (confocal microscopy).

Detection of CM – BM-MNC coupling based on measurement of Ca^{2+} transients

After 24 h, the coculture was washed with Tyrode's solution and incubated at 37°C and 5% CO_2 with fluo 4-AM (5 μM, Molecular Probes, Eugene, OR, USA) for 30 min. Cells were then transferred to the microscope chamber and overflowed with Tyrode's solution (pH 7.4, at 37°C). Two silver electrodes connected to a stimulator (type AT50, Chirana, Czech Republic) were used for electrical stimulation (1 Hz, 10 mA/cm^2). Images of beating CMs and surrounding BM-MNCs were obtained using an FITC filter set (excitation 488 nm, emission 512 nm) together with Moticam 2000 (Motic, China) and i-Speed 3 (Olympus, Japan) CCD cameras to obtain a set of images with 50 ms time crop. Synchronous fluo-4 fluorescence signals in CMs and neighboring BM-MNCs were recorded. Periodic increasing and decreasing intensity of fluorescence signals with the same time periods for both cells were considered as synchronic flashes. Sets of frames with 50 ms time crop were converted to pseudocolor images (transformation of intensity to rainbow color scale, using Olympus i-Speed lab software, Olympus, Japan).

Detection of CM – BM-MNC coupling based on measurement of calcein diffusion

BM-MNCs were incubated with 2.5 μM calcein-AM for 60 min at 37°C instead of PKH26 before cocultivation. BM-MNCs were washed out and used for cocultivation (the same setup as described in Table 1). Detection of calcein diffusion from BM-MNC to CM was performed using fluorescence microscopy.

Analysis of basic markers of cardiomyocyte transdifferentiation – recording of cell contractility and development of alpha-actinin sarcomeric structure

Occurrence of spontaneous active contraction of BM-MNCs was evaluated using a CCD camera on a fluorescence microscope to capture a set of images with 50 ms time crop. Contractions were also recorded during and after application of electrical stimulation. Anti-actinin antibodies (primary antibodies A7732, Sigma–Aldrich, Germany) were used for staining the BM-MNCs. Anti-mouse IgG1 conjugated with FITC was used as a secondary antibody (1:100, F 9006 Sigma–Aldrich, Germany). FITC-positive intracellular structures of BM-MNCs were compared with positive controls for sarcomeric actin structure in freshly isolated adult and neonatal CMs.

All protocols of work with laboratory animals were approved by the Masaryk University Animal Care Committee (adult rats) and Palacky University Animal Care Committee (neonatal rats). A total of 26 adult rats were used, 16 of which were used for CMs isolation (6 of which were excluded from statistical summary due to low viability of isolated CMs at the beginning of the experiments), 10 of which were used for allogenic bone marrow cells isolation. 160 neonatal rats were used for CMs isolation (an average 20 per experiment).

Results

Analysis of BM-MNCs adherence to CMs and CM – BM-MNC coupling (detection of Cx43 created between BM-MNCs and CMs)

Fluorescence microscopy analysis of the CMs cocultures with and BM-MNCs showed strong adherence of some BM-MNCs to CMs (Figure 1C and 1D). The proportions of fresh and cultured BM-MNCs that strongly adhered to adult or neonatal CMs are shown in Table 2.

The adherence of cell pairs was stable and additional resuspendation approved no decoupling of cell pairs during 30 s of intensive cell resuspension in more than 80% of cases. BM MNC – adult CM adherence developed very quickly. Additional experiments showed that the first relatively strong adherence between BM-MNCs and adult CMs can be detected as soon as 5 min after the beginning of the cocultivation (Supplement 3).

Connexin 43 (Cx43) was detected by immunostaining in contact zones of cultured BM-MNCs and adult CMs as well as in contact zones of cultured BM-MNCs (Figure 2) and neonatal CMs (Figure 3). On the other hand, fresh BM-MNCs displayed contact zones with neonatal and adult CMs that after 24 h of coculture were negative for Cx43. In agreement with other authors [12], we found that cultured BM-MNC – CM pairs that display this Cx43 positivity constitute only less than 1% of all BM-MNC – CM pairs in the coculture dish. These results implied from 8 independent adult animal experiments and 6 independent neonatal animal experiments (based on evaluation ≥200 pairs of CM – BM-MNC in each independent experiment).

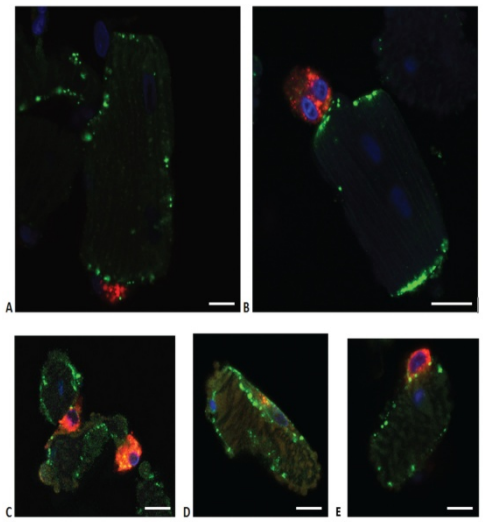

Figure 2: BM-MNCs (red) adhered to adult myocytes after 24 h coculture. Cx43 (green lines) was observed in some contact zones of BM-MNCs and cardiomyocytes (A, B, D, E). Some BM-MNCs can also create a connection between two different myocytes (C). Zeiss LSM 700 confocal microscope, objective magnification 63×. Scale bars represent 40 μm.

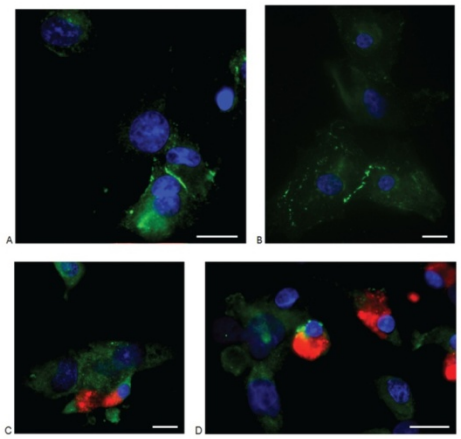

Figure 3: Control positive staining of Cx43 between neonatal cardiomyocytes 3 days old (A, B). BM-MNCs (red cells stained with PKH26) adhered to adult myocytes after 24 h coculture (C, D). Cx43 (green lines) was observed in some contact zones of BM-MNCs and CMs (D). Zeiss LSM 700 confocal microscope, objective magnification 63×. Scale bars represent 40 μm.

Setup 1	Setup 2	Setup 3	Setup 4
Neonatal	Neonatal	Adult	Adult
cardiomyocytes	cardiomyocytes	cardiomyocytes	cardiomyocytes
+	+	+	+
Fresh BM-MNCs	Cultured BM-MNCs	Fresh BM-MNCs	Cultured BM-MNCs
4.5 ± 1.2%	6.2 ± 1.5%	1.3 ± 1.1%	3.2 ± 1.1%

Table 2: Proportions of BM-MNCs adhered to CMs in different cocultivation setups. Setup1 and Setup 2: statistical results computed from 6 × 160 wide fields (6 independent neonatal animal experiments); Setups 3 and 4 computed from 6×160 wide fields (6 independent adult animal experiments). One wide field contained minimally 40 CMs and BM-MNCs.

Detection of CM – BM-MNC coupling based on measurement of calcein molecules diffusion

The analysis of CMs which had contact with calcein-positive BM-MNCs showed that some CMs became calcein positive during the time of cocultivation. An illustrative image from fluorescence microscopy is shown in Figure 4. This suggests a possibility of calcein transfer through gap junctions or other connection structures between CMs and BM-MNCs. We found that percentage of adult BM-MNC – CM pairs that display this calcein transfer positivity was 3.3 ± 1.2 for adult CM and 4.7 ± 0.8 for neonatal cardiomyocyte. These results implied from 8 independent adult animal experiments and 6 independent neonatal animal experiments (based on evaluation ≥200 pairs of CM – BM-MNC in each experiment).

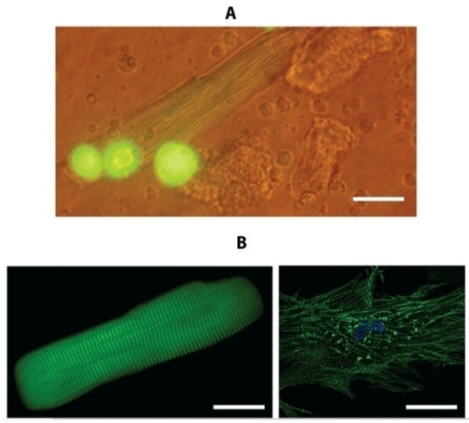

Figure 4: (A) Calcein-positive BM-MNCs and calcein-positive rod-shaped cardiomyocytes after 24 h coculture. Zeiss Axiovert 40C/CFL epifluorescence microscope, objective magnification 40×. (B) Control positive staining of alpha-actininsarcomeric structure inside adult cardiomyocyte (left) and neonatal cardiomyocyte (right). Stained by anti-alpha-actinin antibody and secondary anti-mouse IgG1–FITC antibody. Blue points are nuclei stained with DAPI. Generated by Zeiss LSM700 confocal microscope, objective magnification 63×. Scale bars represent 40 μm.

Detection of CM – BM-MNC coupling based on measurement of Ca^{2+} transients

Ion communication between cultured BM-MNCs and adult CMs (800 evaluated pairs in total, 4 independent animal experiments) and between cultured BM-MNCs and neonatal CMs (700 pairs in total, 4 independent animal experiments) was analyzed (Fresh BM-MSC – MC pairs could not be precisely evaluated, because of small diameter of the cells and misrepresenting noise/signal in used microscopy set up). From these evaluated samples, only 12 BM-MNCs displayed temporal changes of Fluo-4 fluorescence in their cytoplasm. However, eight of these cases were not synchronous with fluo-4 fluorescence transients observed in the surrounded CM. Three BM-MNC – adult CM pairs displayed Fluo-4 fluorescence flashes which were synchronous (Figure 5A and Supplement 4 video). Only one BM-MNC – neonatal CM pair displayed Fluo-4 fluorescence flashes synchronous with fluo-4 flashes in the surrounded neonatal CM. While the amount of BM-MNCs adhered to neonatal CMs was greater than the amount of BM-MNCs adhered to adult CMs, only few BM-MNCs with oscillating Fluo-4 signals were found adhering to neonatal CMs.

During these analyses, cases were observed when the fluorescence of adhered BM-MNCs was influenced by the fluorescence of the beating neonatal CM. Total fluorescence recorded from BM-MNCs could thus be artificially enhanced and misleading (Figure 5B, left) in such case as when a BMC adheres within the optical axis of the microscope to the neonatal CM. Therefore, approximately 10 such potentially artificially influenced cases of BM-MNC – CM pairs were excluded from the statistical analysis. Observation of BM-MNCs adhering to adult CMs did not exhibit similar problems. Adult CMs are cuboid in shape and not as flat as neonatal CMs. Therefore, BM-MNCs adhered mostly to the vertical wall of adult CMs, perpendicular to the optical axis of the microscope (Figure 5C, right). Very rarely did these BM-MNC cells attach to the front side of CMs.

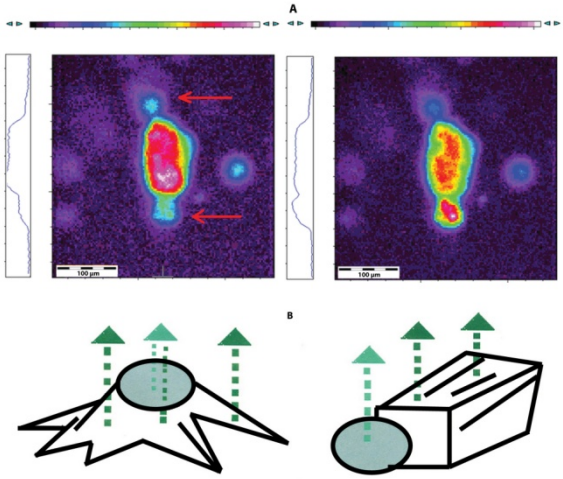

Figure 5: (A) Fluo-4 loaded BM-MNCs (red arrows) adhered to adult myocytes. Two frames from a video sequence show changes of intensity Fluo-4 fluorescence over time (marker of time changes of intracellular Ca^{2+} concentration) in BM-MNCs and cardiomyocytes. The video sequence shows synchronous changes in intracellular concentration of Ca^{2+} in BM-MNCs and surrounded cardiomyocytes. The histogram on the left side of the frames shows actual relative intensity of fluorescence from the cells. An Olympus QCL epifluorescence microscope, with objective magnification 60× and camera i-Speed 3 (Olympus, Japan) were used. Pseudocolor transformation was performed (lab software Olympus i-Speed 3). (B) Comparison of BM-MNC adhered to neonatal cardiomyocyte (left picture) and BM-MNC adhered to adult cardiomyocyte (right picture); green arrows represent fluorescence originated from Fluo-4 in cytoplasm of the cells. In the case of neonatal cardiomyocytes, part of the fluorescence from CMs goes through the BM-MNC and the fluorescence can be detected as artificial fluorescence from the BM-MNC.

Analysis of basic markers of cardiomyocyte transdifferentiation – recording of cell contractility and development of alpha-actinin sarcomeric structure BM-MNCs in coculture displayed neither spontaneous active contraction nor contraction after application of electrical pulses. BM-MNCs did not display sarcomeric actinin-like structures in comparison with isolated adult and neonatal CMs (Figure 4B). These results implied from 4 independent adult animal experiments and 4 independent neonatal animal experiments, each result of setup 1, 2, 3 and 4 from each independent experiment was based on evaluation ≥100 BM-MNCs.

Discussion

Clinical studies and studies on experimental animal models have demonstrated the ability of delivered bone marrow mononuclear cells (BM-MNCs) or endothelial progenitor cells (EPCs) to settle down into the affected area of myocardium and to some extent improve the function of an ischemic heart [16]. The commonly acknowledged scheme of BM-MNC homing consists of three steps: 1) chemo-

attractant factors are released by the infarcted area, 2) BM-MNCs migrate from the vessel through the endothelium, and 3) BM-MNCs adhere to CMs and can make conductive junctions with the CMs or eventually start transdifferentiation processes. Nevertheless, the precise mechanisms of the BM-MNCs homing in on the affected myocardium – and especially their interaction with the residual CMs in the infarcted area – remain unclear.

Aspects of BM-MNCs' interaction with the infarcted tissue (changes in morphology of the BM-MNCs, quantification of BM-MNCs' integration into the muscle, adhesion to endothelial cells and to the extracellular matrix) have been analyzed using electron and fluorescence microscopy on heart samples extracted and fixed postmortem [17-19]. The junctions created between BM-MNCs and CMs have also been analyzed [12] (application of cells to an extracorporeal rat beating heart model and subsequent analysis of fixed slices of the heart tissue). Direct analysis of BM-MNC – CM interaction in situ would be ideal, but this has not yet been achieved. The first intravital-microscopy setups for MSC tracking have been developed [20,21], but the problems with resolution and long-time invasiveness have not yet been settled. Until intra-vital microcopy will be perfected, research must rely on other techniques that have been developed to mimic the microstructure of infarcted heart in vivo and to allow the simulation and systematic observation of interaction between uncoupled myocytes and progenitor/stem cells. This type of experiment is known as a cocultivation experiment, and it is used also in this work.

The number of similar cocultivation experiments which simulate interaction of cardiac cells with BM-MNCs is very limited in the literature. We found no more than 15 original publications from the past 8 years, as well as several subsequent upgrades which were based on heterotypic cell-pair interactions in microchambers and a cell strands coculture setup on opto-electronical microchips (most of which are from the past 4 years; [22-24]). There also have been reports of state-of-the-art upgrades, including tissue bundles examination using external stimulatory forces [25] and micro-bioreactors with large ventricular slice [26,27]. To the best of our knowledge, however, almost all of these state-of-the-art cultivation settings have utilized modified MSCs (typically transfected for upgrade of ion channel) or another atypical type of stem/progenitor cells (iPS, ESC, genetically modified fibroblast) and these experiments principally did not analyze the simple interaction of fresh or cultured BM-MNCs.

Moreover, if we consider especially cocultivation experiments with unmodified BM-MNCs or MSCs, it can be seen that most of the published cocultivation experiments use only neonatal CMs as a substrate and the interactions are not well compared (or comparable) with those of adult CMs as these actually occur in infarcted heart. Our work is among the first in which neonatal and adult CMs are used in parallel for cocultivation experiments and whereby both myocyte types are handled under the same conditions (growth medium, concentration of the cell mass in the coculture related to probability of the cell interaction, experiment timeframe).

Cell survival in coculture and BM-MNC – CM adherence

The cultivation media influence the viability and reaction of the cells. This study shows that the M1 medium (details in Materials and Methods section) is better for cultivation of adult CMs than IMDM with 20% FBS (routinely used for the cultivation of BM-MNCs). M1 medium has ingredients similar to those of Tyrode's solution, which is routinely used in short-time experiments with freshly isolated CMs [14,28], but FBS contents and some ion concentrations are different. Despite relatively high viability, changes in shape from rectangular to elliptic were observed in 40–60% of adult viable CMs during the first 24 h. These changes had been described previously [29] and they remain a main reason why we limited the time frame for our analyses to 24 h. These changes of shape are similar to those seen in FBS-free Tyrode's solution.

Our results show that 2–5% of fresh BM-MNCs and 3–6% of cultured BM-MNCs strongly adhered to the CMs after 24 h. The concentrations of BM-MNCs and CMs in coculture used in this study produce very similar results in terms of the ability for and amounts of BM-MNC adherence as reported in previously published papers [12,13,30]. Decrease of BM-MNCs' concentration in coculture leads to a decreasing rate of adhered BM-MNCs, as we found during detailed analysis (data not shown). We hypothesize that the observed strong adherence of cells may play a role in vivo in a mechanical stabilization of tissue in the infarcted zone.

Communication between cells

Another aim was to evaluate the conductive function of the Cx43-positive structures between BM-MNCs and CMs. Microphotographs from fluorescence microscopy show that the fluorescence probe calcein was transported from BM-MNCs to CMs. The results also proved synchronous Ca^{2+} transients between BM-MNCs and CMs. BM-MNCs displayed periodic decrease and increase of cytosolic Ca^{2+} concentration, and the Ca^{2+} transients were synchronized with Ca^{2+} transients in the neighboring CM.

Conducting structures were formed between CMs and cultured BM-MNCs, and calcein transfer occurred through these, where the fraction of positive cells was about 3% in BM-MNC – adult CM coculture and 5% in BM-MNC – neonatal CM coculture. Some other authors have observed more than 50% of cocultured cells displaying calcein-conducting positivity based on fluorescence-activated cell sorting [11,31]. Nevertheless, nonautomated precise optical fluorescence microscopy analysis did not show a similar 50% positivity, because flow cytometry may in this case suffer from overestimation and artificial results for this type of experiment [3]. Ion-conductive structures between BM-MNCs and CMs have been closely observed on neonatal CMs and cultured BM-MNCs in several published studies [30,32]. Results have shown that 24 h are sufficient for establishing the ion-conductive structure between BM-MNCs and neighboring CMs [30,33,34]. Analyses of adult rat CMs (as a cocultivation anchor) combined with fresh and cultured BM-MNCs (as fraction progenitor injected into the coculture) from the same species have not yet been published (only [11] described one principally similar experiment), and our results may thus point to a feasible path for further research.

Evaluation of transdifferentiation

No spontaneous contractility of BM-MNCs or contraction of BM-MNCs upon electrical stimulation by electrodes has been observed in cases of 24 h of cocultivation of BM-MNCs with neonatal or adult myocytes. In addition, no BM-MNCs displayed typical sarcomeric alpha-actinin structures. Similar conclusions were presented in a case of contact with adult CMs [12]. Transdifferentiation of BM-MNCs after coculture with neonatal CMs remains controversial. Our short-term experiments produced no direct evidence of such transdifferentiation.

Limitations of the study

A basic limitation of our in vitro experimental setup consists in the simplified access of BM-MNCs to CMs. In vivo, the path for BM-MNCs to CMs consists of three steps: 1) chemo-attractant factors are released by the infarcted area, 2) BM-MNCs migrate from the blood vessel through the endothelium, and 3) BM-MNCs adhere to the CMs in tissue [35]. In our in vitro model, there are no vessels and no transendothelial border. BM-MNCs are present in culture directly next to the cardiomyocytes.

Another limitation of our experimental results derives from the contractile properties of adult CMs. Adult isolated CMs with non-affected ion balance in their cytoplasm do not exhibit spontaneous contractility (only about 5% of CMs with affected ion balance in the cytoplasm exhibited periodic or non-periodic spontaneous contractions). On the other hand, neonatal CMs display spontaneous contractions with a high rate, approximately 100 beats per minute. Permanent contractions of CMs in vivo (or at least permanent periodic polarization of CMs) can be a necessary positive stimulus supporting adherence of BM-MNCs to CMs and formation of ion-conductive structures between BM-MNCs and CMs, as well as possible transdifferentiation of BM-MNCs [36].

Conclusions

Fresh BM-MNCs and cultivated BM-MNCs are able to survive under specific conditions for 24 h in coculture with neonatal or adult CMs. About 5% of fresh BM-MNCs and cultured BM-MNCs in our in vitro setup strongly adhered to neonatal cardiomyocytes, and about 1.5% or 3% of fresh BM-MNCs or cultured BM-MNCs in our in vitro setup adhered to adult CMs. Our results show this relatively strong mechanical connection between BM-MNCs and CMs and very rapid formation in the first minutes and hours after first accession of the cells. This should be an important mechanical premise for following electrophysiological coupling of the cells.

Contact regions between cultured BM-MNCs and CMs (both adult and neonatal) displayed structural connection (Cx43 positivity) and functional conduction structure (vital dye intercellular transport and ion intercellular transport) in our coculture experiments. Nevertheless, the relative number of BM-MNC-CM pairs with functional conductive structure was limited. No transdifferentiation of BM-MNC was observed and no fresh BM-MNCs or cultured BM-MNCs developed cardiomyocyte-like differentiation during the 24 h coculture experiment.

Acknowledgements

This work was financially supported by the Ministry of Health of the Czech Republic; Contact grant (project number: NS9670-4), Czech Science Foundation (project number GACR 301/11/0662), European Regional Development Fund (project FNUSA-ICRC, CZ. 1.05/1.1.00/02.0123) and institutional fund RVO: 61388998.

References

1. Cleutjens JP, Blankesteijn WM, Daemen MJ, Smits JF (1999) The infarcted myocardium: simply dead tissue, or a lively target for therapeutic interventions. Cardiovasc Res 44: 232-241.
2. Thygesen K, Alpert JS, White HD (2007) Joint ESC/ACCF/AHA/WHF Task Force for the Redefinition of Myocardial Infarction. Universal definition of myocardial infarction. Eur Heart J 28: 2525-2538.
3. Gruh I, Beilner J, Blomer U, Schmiedl A, Schmidt-Richter I, et al. (2006) No evidence of transdifferentiation of human endothelial progenitor cells into cardiomyocytes after coculture with neonatal rat cardiomyocytes. Circulation 113: 1326-1334.
4. Kajstura J, Leri A, Finato N, Di Loreto C, Beltrami CA, et al. (1998) Myocyte proliferation in end-stage cardiac failure in humans. Proc Natl Acad Sci U S A 95: 8801-8805.
5. Weber KT, Sun Y, Díez J (2008) Fibrosis: a living tissue and the infarcted heart. J Am Coll Cardiol 52: 2029-2031.
6. Martin-Rendon E, Brunskill SJ, Hyde CJ, Stanworth SJ, Mathur A, et al. (2008) Autologous bone marrow stem cells to treat acute myocardial infarction: a systematic review. Eur Heart J 29: 1807-1818.
7. Orlic D, Hill JM, Arai AE (2002) Stem cells for myocardial regeneration. Circ Res 91: 1092-1102.
8. Xaymardan M1, Tang L, Zagreda L, Pallante B, Zheng J, et al. (2004) Platelet-derived growth factor-AB promotes the generation of adult bone marrow-derived cardiac myocytes. Circ Res 94: E39-45.
9. Badorff C, Brandes RP, Popp R, Rupp S, Urbich C, et al. (2003) Transdifferentiation of blood-derived human adult endothelial progenitor cells into functionally active cardiomyocytes. Circulation 107: 1024-1032.
10. Tang YL, Zhao Q, Qin X, Shen L, Cheng L, et al. (2005) Paracrine action enhances the effects of autologous mesenchymal stem cell transplantation on vascular regeneration in rat model of myocardial infarction. Ann Thorac Surg 80: 229-236.
11. Yoon J, Shim WJ, Ro YM, Lim DS (2005) Transdifferentiation of mesenchymal stem cells into cardiomyocytes by direct cell-to-cell contact with neonatal cardiomyocyte but not adult cardiomyocytes. Ann Hematol 84: 715-721.
12. Gallo MP, Ramella R, Alloatti G, Penna C, Pagliaro P, et al. (2007) Limited plasticity of mesenchymal stem cells cocultured with adult cardiomyocytes. J Cell Biochem 100: 86-99.
13. Valiunas V1, Doronin S, Valiuniene L, Potapova I, Zuckerman J, et al. (2004) Human mesenchymal stem cells make cardiac connexins and form functional gap junctions. J Physiol 555: 617-626.
14. Bébarová M, Matejovic P, Pásek M, Simurdová M, Simurda J (2005) Effect of ajmaline on action potential and ionic currents in rat ventricular myocytes. Gen Physiol Biophys 24: 311-325.
15. Chlopcíková S, Psotová J, Miketová P (2001) Neonatal rat cardiomyocytes--a model for the study of morphological, biochemical and electrophysiological characteristics of the heart. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 145: 49-55.
16. Gaebel R, Furlani D, Sorg H, Polchow B, Frank J, et al. (2011) Cell origin of human mesenchymal stem cells determines a different healing performance in cardiac regeneration. PLoS One 6: e15652.
17. Abbott JD, Huang Y, Liu D, Hickey R, Krause DS, et al. (2004) Stromal cell-derived factor-1alpha plays a critical role in stem cell recruitment to the heart after myocardial infarction but is not sufficient to induce homing in the absence of injury. Circulation 110: 3300-3305.
18. Klabusay M, Scheer P, Doubek M, Rehakova K, Coupek P, et al. (2009) Retention of nanoparticles-labeled bone marrow mononuclear cells in the isolated ex vivo perfused heart after myocardial infarction in animal model. ExpBiol Med (Maywood) 234: 222-231.
19. Rota M, Kajstura J, Hosoda T, Bearzi C, Vitale S, et al. (2007) Bone marrow cells adopt the cardiomyogenic fate in vivo. Proc Natl Acad Sci U S A 104: 17783-17788.
20. Toma C, Wagner WR, Bowry S, Schwartz A, Villanueva F (2009) Fate of culture-expanded mesenchymal stem cells in the microvasculature: in vivo observations of cell kinetics. Circ Res 104: 398-402.
21. Kikuta J, Ishii M (2012) Recent advances in intravital imaging of dynamic biological systems. J Pharmacol Sci 119: 193-197.
22. Ma Z, Liu Q, Liu H, Yang H, Yun JX, et al. (2012) Laser-patterned stem-cell bridges in a cardiac muscle model for on-chip electrical conductivity analyses. Lab Chip 12: 566-573.

23. Askar SF, Ramkisoensing AA, Atsma DE, Schalij M, de Vries AA, et al. (2013). Engraftment Patterns of Human Adult Mesenchymal Stem Cells Expose Electrotonic and Paracrine Pro-Arrhythmic Mechanisms in Myocardial Cell Cultures. Circulation: Arrhythmia and Electrophysiology, CIRCEP-113.
24. Pedrotty DM, Klinger RY, Badie N, Hinds S, Kardashian A, et al. (2008). Structural coupling of cardiomyocytes and noncardiomyocytes: quantitative comparisons using a novel micropatterned cell pair assay. American Journal of Physiology-Heart and Circulatory Physiology, 295: H390-H400.
25. Vandenburgh H, Shansky J, Benesch-Lee F, Barbata V, Reid J, et al. (2008) Drug-screening platform based on the contractility of tissue-engineered muscle. Muscle Nerve 37: 438-447.
26. Pillekamp F, Reppel M, Dinkelacker V, Duan Y, Jazmati N, et al. (2005) Establishment and characterization of a mouse embryonic heart slice preparation. Cell Physiol Biochem 16: 127-132.
27. Habeler W, Pouillot S, Plancheron A, Pucéat M, Peschanski M, et al. (2009) An in vitro beating heart model for long-term assessment of experimental therapeutics. Cardiovasc Res 81: 253-259.
28. Pásek M, Simurda J, Orchard CH, Christé G (2008) A model of the guinea-pig ventricular cardiac myocyte incorporating a transverse-axial tubular system. Prog Biophys Mol Biol 96: 258-280.
29. Banyasz T, Lozinskiy I, Payne CE, Edelmann S, Norton B, et al. (2008) Transformation of adult rat cardiac myocytes in primary culture. Exp Physiol 93: 370-382.
30. Beeres SL, Atsma DE, van der Laarse A, Pijnappels DA, van Tuyn J, et al. (2005) Human adult bone marrow mesenchymal stem cells repair experimental conduction block in rat cardiomyocyte cultures. J Am CollCardiol 46: 1943-1952.
31. Plotnikov EY, Khryapenkova TG, Vasileva AK, Marey MV, Galkina SI, et al. (2008) Cell-to-cell cross-talk between mesenchymal stem cells and cardiomyocytes in co-culture. J Cell Mol Med 12: 1622-1631.
32. Ma Z, Yang H, Liu H, Xu M, Runyan RB, et al. (2013) Mesenchymal stem cell-cardiomyocyte interactions under defined contact modes on laser-patterned biochips. PLoS One 8: e56554.
33. Garbade J, Schubert A, Rastan AJ, Lenz D, Walther T, et al. (2005) Fusion of bone marrow-derived stem cells with cardiomyocytes in a heterologous in vitro model. Eur J CardiothoracSurg 28: 685-691.
34. Pijnappels DA, van Tuyn J, de Vries AA, Grauss RW, van der Laarse A, et al. (2007) Resynchronization of separated rat cardiomyocyte fields with genetically modified human ventricular scar fibroblasts. Circulation 116: 2018-2028.
35. Rose RA, Jiang H, Wang X, Helke S, Tsoporis JN, et al. (2008) Bone marrow-derived mesenchymal stromal cells express cardiac-specific markers, retain the stromal phenotype, and do not become functional cardiomyocytes in vitro. Stem Cells 26: 2884-2892.
36. Iijima Y, Nagai T, Mizukami M, Matsuura K, Ogura T, et al. (2003) Beating is necessary for transdifferentiation of skeletal muscle-derived cells into cardiomyocytes. FASEB J 17: 1361-1363.