J. of Cardiovasc. Trans. Res.
DOI 10.1007/s12265-009-9144-3

Cardiac Transcription Factors Driven Lineage-Specification of Adult Stem Cells

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Received: 8 September 2009 / Accepted: 13 October 2009

Abstract Differentiation of human bone marrow mesenchymal stem cells (hBMSC) into the cardiac lineage has been assayed using different approaches such as coculture with cardiac or embryonic cells, treatment with factors, or by seeding cells in organotypic cultures. In most cases, differentiation was evaluated in terms of expression of cardiac-specific markers at protein or molecular level, electrophysiological properties, and formation of sarcomers in differentiated cells. As observed in embryonic stem cells and cardiac progenitors, differentiation of MSC towards the cardiac lineage was preceded by translocation of NKX2.5 and GATA4 transcription factors to the nucleus. Here, we induce differentiation of hBMSC towards the cardiac lineage using coculture with neonatal rat cardiomyocytes. Although important ultrastructural changes occurred during the course of differentiation, sarcomerogenesis was not fully achieved even after long periods of time. Nevertheless, we show that the main cardiac markers, NKX2.5 and GATA4, translocate to the nucleus in a process characteristic of cardiac specification.

Keywords Mesenchymal Stem Cells · Neonatal Rat Cardiomyocytes · Cardiac Specification · Cardiac Transcription Factors

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Introduction

Differentiation of stem cells into cardiomyocytes (CM) is one of the main challenges of cardiovascular regenerative medicine [1]. Signals from the environmental niche have been shown to trigger the differentiation process. To model the in vivo differentiation process, organotypic cultures and coculture of stem cells with murine visceral-endoderm-like cells, or neonatal CM have been used to stimulate cardiac differentiation [2–4]. In this context, although the molecular mechanisms that yield CM specification are not fully understood, the implication of NKX2.5 and GATA4 transcription factors is well-documented [5–9]. These factors trigger the cardiac genetic program both in embryonic stem cell (ESC) and adult stem cells recapitulating the molecular mechanism that operates during embryonic heart development [10, 11]. Their expression predicts cardiac commitment in ESC [9] and, in the developing embryo, c-kit$^+$ Nkx2.5 progenitor cells can differentiate into either smooth or cardiac muscle [12]. Moreover, progenitors expressing NKX2.5 have been identified in adult bone marrow suggesting that the bone marrow mesenchymal stem cells (BMSC) may normally have the potential for cardiac differentiation [13].

During embryonic heart development, the expression and activity of the transcription factor NKX2.5 is regulated

Published online: 21 October 2009

by members of the signaling molecules families BMP and TGF-beta [9, 14]. In turn, NKX2.5 regulates cardiac differentiation by transactivating myocardin [6]. This signaling mechanism is reproduced when stem cells are treated with a cocktail of differentiation factors [15] or after nuclear reprogramming where an early upregulation of precardiac markers like TBX5 and CXCR4 was followed by the expression of MEF2C and myocardin, providing cardiac tissue maturation [16]. This signaling machinery induces activation of several terminal cardiac differentiation genes including gap junction proteins, the α-sarcomeric actin (α-SA), the beta myosin heavy chain (β-MHC), and the atrial natriuretic peptide (ANP) [6, 17, 18]. Here, we report the time course of cardiac differentiation of hBMSC induced by co-cultivation with rat neonatal CM. This cardiac differentiation correlates with nuclear translocation of the NKX2.5 and GATA4 cardiac transcription factors in a process characteristic of cardiac differentiation.

Materials and Methods

Human Cells, Culture Condition, and Retroviral Labeling

Human BMSC were purchased (Inbiomed, San Sebastian, Guipuzcoa, Spain) and expanded following the manufacturer's instructions. Cells were retrovirally labeled using enhanced green fluorescent protein retroviral particles obtained from the PG13-PSF-GFP packaging cell line as described [11].

Neonatal Rat CM and Coculture with Human MSC

To establish cocultures, we used MSC:CM ratio of 1:25 to achieve the highest degree of MSC differentiation. Neonatal rat CM was isolated as described [11].

Immunohistochemistry and Electron Microscopy

Cells seeded onto cover slides were fixed with paraformaldehyde 2%, washed, blocked, and incubated with primary antibodies cardiac troponin (cTn I; Santa Cruz Biotechnology, CA, USA), β-MHC (Chemicon, Temecula, CA, USA), α-SA (Sigma, St Louis, MO, USA), or ANP (Chemicon). For NKX2.5 and GATA4 staining, cells were fixed, washed, and incubated 3 h at 30°C with a mixture of MeOH-DMSO. Primary antibodies against transcription factors were from Santa Cruz Biotechnology. Secondary antibodies used were Alexa Fluor 647 goat anti-mouse and Alexa Fluor 555 goat anti-rabbit (Molecular Probes, Invitrogen, USA). Cells were simultaneously labeled with 4′,6-diamidino-2-phenylindole (DAPI). For electron microscopy studies, cocultures were seeded on Lab-Tek chamber slides (NUNC, Roskilde, Denmark), washed and fixed with 3% glutaraldehyde (Durcupan, Fukla Biochemica, Rokokoma, NY, USA). The cultures were post-fixed in 2% osmium for 2 h, rinsed, dehydrated, and embedded in Araldite (Durcupan, Fluka Biochemica, Rokokoma, NY, USA). Ultrathin (0.05 μm) sections were cut with a diamond knife, stained with lead citrate, and examined under a FEI Tecnai spirit electron microscope (Hillsboro, OR, USA).

Reverse Transcription-Polymerase Chain Reaction

RNA was isolated from human heart tissue, CM, and BMSC, and reverse transcription-polymerase chain reaction (RT-PCR) was performed as described [11].

Statistical Analysis

Data were expressed as mean±SD of three independent experiments. Comparisons between 1 and 2 or 4 weeks of coculture were performed with the Willcoxon W test. Pairwise comparisons between groups at different time points were done with a Mann–Whitney U test. Statistical values were calculated using the Statistical Package for the Social Sciences software. Differences were considered statistically significant at $P<0.05$ with a 95% confidence interval.

Results

Kinetics of Cardiac-Specific Antigens Expression

Co-cultivation of human BMSC with rat neonatal CM induces initial cardiac specification of the former. To understand how this induction mechanism occurs, we performed a time course analysis of marker expression during cardiac differentiation. We focused on the timing of protein expression of cardiac differentiation markers TnI, β-MHC, ANP, and α-SA. The percentage of cells expressing α-SA reveals that after coculture with rat CM, hBMSC continuously differentiate into the cardiac lineage during at least 4 weeks (number of cells expressing α-SA at 4 weeks was 37.42±8.03% vs. 16.92±6.75% of cells expressing TnI, 16.60±2.90% of cells expressing β-MHC, and 12.43±3.03% of cells expressing ANP; $P<0.05$). Although the expression of β-MHC and TnI followed a similar pattern to that of α-SA, the number of cells expressing these antigens reached a plateau after 2 weeks of coculture (Fig. 1a). We performed RT-PCR using specific human primers and corroborated that, as detected in human heart, hBMSC cocultured with rat neonatal CM activated the expression of the terminal cardiac differentiation markers TnI and β-MHC (Fig. 1b). On the other hand, the expression of

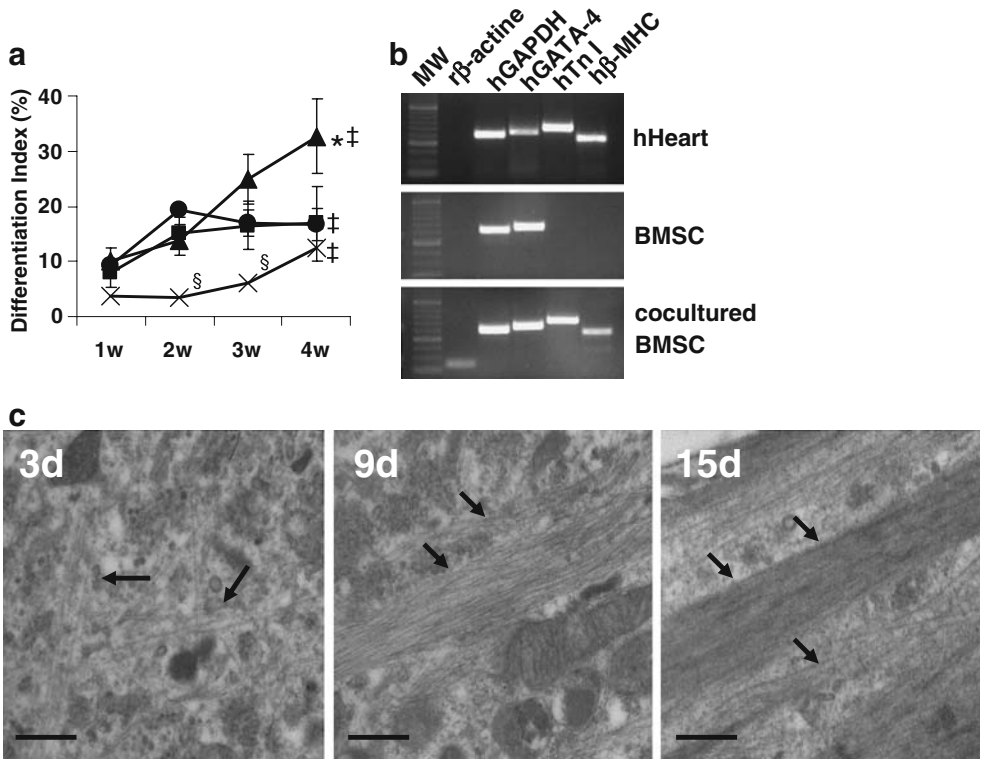

Fig. 1 Differentiation of BMSC in coculture with neonatal rat CM. **a** Graph showing the percentage of BMSC positive for α-SA (*filled triangle*), TnI (*filled square*), β-MHC (*filled circle*), and ANP (*multiplication sign*) at the times indicated (§$P<0.05$ in ANP vs. α-SA, Tn I, and β-MHC at 2 and 3 weeks; *$P<0.05$ in α-SA vs. Tn I, β-MHC and ANP at 4 weeks; ‡ $P<0.05$ in each antigen at 4 weeks vs. 1 week). **b** Amplification of human GAPDH, GATA4, TnI, β-MHC, and rat β-actin sequences by semiquantitative PCR from human heart tissue and BMSC cultured or not with CM for 15 days. Representative reactions for each specific set of primers are shown. **c** Ultrastructural analysis of BMSC after 3, 9, or 15 days of coculture with CM. *Arrows* point myofibers in cytoplasm of BMSC. *Scale bar*=0.5 μm

ANP was activated after 3 weeks of coculture (number of cells expressing ANP was 3.78±0.18% at 1 week, 3.53±0.34% at 2 weeks, and 6.03±2.94% at 3 weeks). These results indicate that during differentiation of hBMSC into the cardiac lineage, there is a temporally differential activation of cardiac markers.

To confirm cardiac differentiation, we analyzed the ultrastructural characteristics of the differentiated hBMSC. We performed electron microscopy on cocultures after 3, 9, and 15 days (Fig. 1c). Myofibers in hBMSC were dispersed in the cytoplasm of early cocultured hBMSC and were progressively enlarged and thickened after 9 days of coculture. After 15 days, differentiated BMSC were completely filled of dense clusters of filaments of 0.5-2 μm thickness organized parallel to the cell axis. This indicates that coculture with rat CM induces cardiac marker activation and myofibers formation in the hBMSC.

Nuclear Translocation of Cardiac Transcription Factors NKX2.5 and GATA4

To further characterize the mechanism of differentiation, we analyzed the behavior of the cardiac transcription factors NKX2.5 and GATA4. These two factors induce cardiac differentiation by translocating to the nucleus. We performed immunohistochemistry for anti-NKX2.5 and anti-GATA4 on cocultures of hBMSC/rCM at 3 and 15 days to examine the localization of these transcription factors in hBMSC (Fig. 2). hBMSC expressed GFP as a result of previous retroviral infection to differentiate them from the rat CM. Cells were simultaneously stained with α-SA (yellow) to determine whether hBMSC had initiated the differentiation process and DAPI to detect all cell nuclei. After 3 days in coculture, hBMSC (green) showed a diffuse pattern of GATA4 and NKX2.5 (red) expression in the cytoplasm, and there was no expression of cardiac antigens. However, 15 days after coculture, both NKX2.5 and GATA4 transcription factors accumulated in the hBMSC nuclei and these cells expressed the terminal cardiac differentiation marker α-SA. These results indicate that as early as 15 days after coculture with rCM, hBMSC have initiated differentiation into CM.

Discussion

Isolation of cardiac precursors or induction of cardiomyogenic differentiation of adult stem cells is the object of an intense debate. The use of committed cardiac precursors may circumvent teratogenic problems and cardiac precursors will presumably integrate better in a differentiated cardiac tissue than undifferentiated stem cells in cell-based therapies [19, 20]. Microenvironment interactions have been shown to play an essential role in cardiac differentiation of stem cells in vivo and in vitro [4, 21]. For this reason, we used the coculture method with neonatal CM to

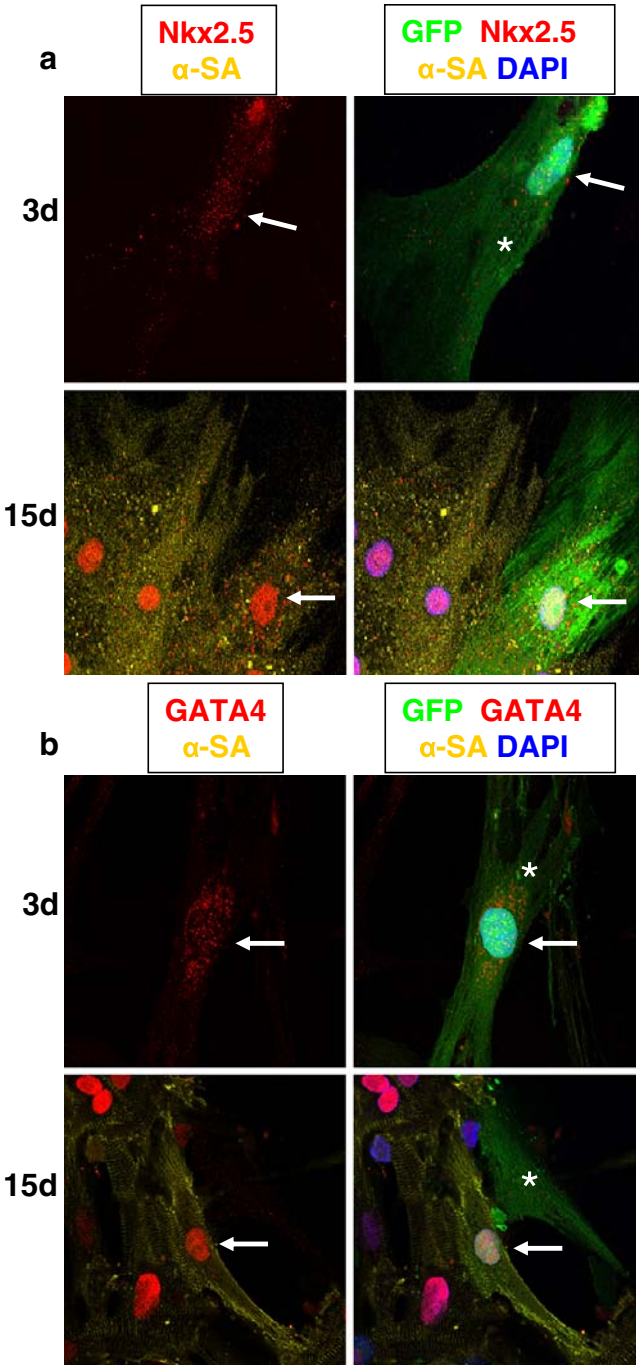

Fig. 2 Nuclear translocation of NKX2.5 and GATA4 transcription factors in BMSC. **a** Immunocytochemistry with antibodies anti-NKX2.5 (*red*) and anti-α-SA (*yellow*) at 3 and 15 days of coculture. **b** Immunocytochemistry with antibodies anti-GATA4 (*red*) and anti-α-SA (*yellow*) at 3 and 15 days of coculture. BMSC were visualized by the green epifluorescence. Nuclei were stained with Dapi (*blue*). *Arrows* point the nucleus of BMSC. *Stars* point undifferentiated BMSC. Images were acquired at ×400 magnification

generate the appropriate microenvironment to induce cardiac differentiation of hBMSC. We and others have reported a direct correlation between nuclear translocation of cardiac-specific transcription factors and cardiac specification of different stem cell types [11, 22, 23]. This nuclear translocation of factors is sufficient to induce cardiomyogenic transformation in ESC [24], however, after 4 weeks of coculture with neonatal rat CM, it did not trigger full cardiac determination of hBMSC. Under the experimental conditions used, hBMSC cells formed gap junctions with neighboring CM and expressed several muscle specific markers like β-MHC, Tn I, or α-SA after coculture with CM [11, 25–27]. However, they did not form sarcomers with the characteristic z-lines or show significant electrical coupling with CM. These processes required additional stimuli, such as forcing alignment of MSC with CM [28]. Additionally, cardiac differentiation of hBMSC can be induced using a set of recombinant factors. After several days of culture with cardiopoietic factors, treated hBMSC underwent nuclear translocation of cardiac transcription factors NKX2.5 and MEF2C which promoted expression of cardiac markers, sarcomerogenesis, and calcium transient activity [29].

Using the coculture method with rCM, we have detected differences in the kinetics of several cardiac markers. While β-MHC and Tn I reached the maximum levels of expression after 2 weeks of coculture that were maintained two more weeks, expression of α-SA and ANP increased by the time of coculture, doubling the expression at 4 weeks versus 2 weeks. Interestingly, ANP antigen required prolonged stimulus in comparison with the other antigens. Although transcription of ANP is also regulated by NKX2.5 and GATA4, they act synergistically and this cooperative regulation may require longer periods of time [5]. We also show that differentiating hBMSC form organized myofibers which may be the initial rudiments of cardiac sarcomeric organization. Additionally, after 15 days in coculture, hBMSC translocate the NKX2.5 and GATA4 factors to the nucleus and express markers of CM terminal differentiation. Further studies based on transcriptional dynamics during cardiac specification are needed to elucidate the complex mechanisms of cardiac differentiation. Coculture techniques provide initial cardiac specification of hBMSC, and full cardiac determination may be accomplished with longer periods of coculture and additional stimuli. In the future, this issue will be addressed and a combination of secreted factors will be added to increase the efficiency of the process.

Acknowledgments Supported by grants from the Instituto de Salud Carlos III for the Regenerative Medicine Program of Valencian Community, the RETICS program and from the FIS (PI07/784, CP08/80). PS is a research Bellow (Miguel Servet Program) at Fundación para la Investigación Hospital la Fe. AA and CG are postdoctoral fellows from the Centro de Investigación Principe Felipe.

We are indebted to Dr. A Chapel for the gift of pSF-G13 cell line. We thank A. Hernández and E. Lafuente from the Service of Confocal microscopy at CIPF for technical assistance, and M. Soriano for acquisition of electron microscopy micrographs.

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