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Induction of Cardiac Myogenic Lineage Development Differs between Mesenchymal and Satellite Cells and is Accelerated by Bone Morphogenetic Protein-4

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Abstract

Our aim was to further elucidate the cardiac lineage development of bone marrow-derived mesenchymal stem cells (MSC) and to identify cells which had the potential for cardiac myogenic differentiation when compared to skeletal muscle satellite (Sk-sat) myogenesis. Unlike Sk-sat, MSC expressed the early cardiac markers Nkx2.5 and GATA4. Their expression was significantly increased by culturing MSC with Bone Morphogenetic Protein 4 (BMP4). Enhanced cardiac myogenic lineage differentiation and loss of stem cell characteristics induced by BMP4 were further confirmed by flow cytometry of cells stained for Nkx2.5 and Sca-1 expression. MSC also expressed skeletal genes (MyoG, ssTnI, Sk-Act) early in culture but their expression was suppressed when BMP4 was added from day 0–6 ($p < 0.05$). BMP4 treated MSC also exhibited a 6-fold increase in cTnI expression by day 12 in culture. The average MSC action potential time duration at 90% (APD90) was 32.3 ± 4 ms, with some cells exhibiting action potentials closer to Sk-sat APD90 of 13.7 ± 0.9 ms. After treatment with BMP4, MSC significantly increased their APD90 to 54.4 ± 7.6 ms, shifting from the shorter skeletal-like signature, towards a longer action potential duration more characteristic of a cardiomyocyte signature. Our results show that MSC and Sk-sat exhibit similarities in myogenic lineage development early in culture but that BMP4 clearly enhances cardiac myogenic development, suppresses skeletal myogenesis, and leads to loss of “stemness” in MSC. These findings provide novel information regarding the use of BMP4 to accelerate cardiac myogenic development in harvested MSC and further support the use of MSC in cardiac regenerative therapy.

1. INTRODUCTION

Bone marrow-derived mesenchymal stem cells (MSC) are easily accessible, display high proliferation in culture, and are multipotent and immunoprivileged [1, 2]. MSCs used for regenerative therapy of the heart or skeletal muscle, significantly reduce scar formation and improve the functional capacity of muscle compared to non-treated muscle [3, 4]. However, differentiation of these same cells into myocytes, although existent, is not well defined [5,

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6]. Previously, we evaluated murine MSC calcium cycling characteristics and preliminary gene and protein expression during myogenic differentiation [7]. We observed increased myogenic expression in differentiated cultures when MSC were enriched for the surface protein CD117 and selected for the $\alpha 2\delta 1$ subunit of the dihydropyridine receptor (DHPR) increasing significantly the number of cTnT⁺ cells from 14% to 50% [7]. However, our follow-up studies showed that some of the cTnT⁺ cells co-expressed ssTnI, skeletal-actin, and formed myotubes characteristic of skeletal muscle cells.

Our purpose in the present study was to further elucidate the myogenic development of MSC and to delineate cells which had a greater potential for developing into cardiac cells compared to a skeletal muscle lineage. We performed temporal studies of cardiac- and skeletal-specific gene and protein expression and compared their expression to those of skeletal satellite (Sk-sat) cells. We reasoned that, the Sk-sat cells population contained early skeletal muscle progenitor cells; thus comparing their temporal myogenic gene expression with those observed in MSCs would help us understand MSC myogenic differentiation. We hypothesized that during MSC myogenic differentiation, these heterogeneous cells differentiate along at least two possible pathways, the skeletal and cardiac lineage. Thus some of the cells in the culture would express the genes and proteins similar to Sk-sat, whereas other cells would exhibit gene expression patterns similar to early cardiac muscle cells. Based on our data and that previously published by other investigators, we also subjected MSCs and Sk-sat cells to reduced levels of serum in culture to accelerate the process of myogenic differentiation.

We conclude that MSCs contain a mixed population of cells that follow both a skeletal myogenic lineage, characteristic of the pattern observed with Sk-sat cells and a cardiac myogenic lineage. Furthermore, our results show that subjecting this mixed population of MSCs to bone morphogenetic protein-4 (BMP4) and low serum conditions enhances the expression of cardiac developmental genes (e.g., GATA4, Nkx-2.5) and suppresses the early skeletal muscle genes. In parallel with these changes in gene expression, MSC action potential time durations at 50% (APD₅₀) and 90% (APD₉₀) significantly increased after BMP4 treatment. This represented a shift away from the shorter skeletal-like action potential time durations towards longer action potential durations more characteristic of a cardiomyocyte [8, 9]. Alterations to the normal cardiac/skeletal protein ratios in the heart have been linked to heart diseases with arrhythmogenic consequences [10]. Thus, an important contribution to the overall MSC regenerative therapy is to understand the temporal development of these two myogenic cell groups and find a method to separate or suppress the skeletal-like cells and further increase the number of cardiac-like cells. These data will improve our ability to identify cells from MSC cultures that are more likely to differentiate into a cardiac lineage and increase the yield of cardiogenic cells from MSC cultures by suppressing skeletal myogenic development.

2. MATERIALS AND METHODS

2.1 Animals

Mice were housed in the Biological Resources Laboratory at UIC (AAALAC accredited) and maintained in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, revised 1996). Experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at UIC.

2.2 Bone Marrow Mesenchymal Stem Cell (MSC) Harvest

Bone marrow was isolated from FVB.Cg-Tg(GFPu)5Nagy/J mice (Jackson Laboratory) as previously described [7, 11]. Briefly, tibia and femur bones were stripped of muscle and placed in ice cold PBS + 2% FBS. The epiphyseal ends were removed and the bones were

centrifuged at 4,000 x g for 1 minute in a microfuge tube. The bone marrow cells were suspended in ice cold PBS + 2% FBS, passed through a 70 μ m filter and counted with a hemocytometer.

Filtered bone marrow cells were suspended in PBS + 2% FBS + 0.1 g/L phenol red and enriched for lineage negative (Lin-) cells using the SpinSep system (Stem Cell Technologies). The cells were incubated with Murine Progenitor Enrichment Cocktail (anti-CD5, anti-CD45R, anti-CD11b, anti-Gr-1, anti-TER119, and anti-7/4; Stem Cell Technologies) on ice for 30 minutes and, after washing, incubated with dense particles on ice for 20 minutes. The cells were layered on density medium, centrifuged at 1200 x g for 10 minutes, and the layer of cells at the density medium/PBS interface was collected, washed and counted.

Enriched bone marrow cells were placed on tissue culture treated plates at a density of 0.1×10^6 cells/cm² in murine Mesencult media (Stem Cell Technologies) with 100 units/ml penicillin, 100 μ g/ml streptomycin, and 0.25 μ g/ml amphotericin B added. The media was changed after 48 hrs and adherent cells were maintained in culture with twice a week media changes. After 4 weeks the confluent cells were detached with trypsin and split 1:3. Lin⁻ MSC were characterized for surface antigens using flow cytometry.

2.3 MSC Differentiation Treatment with 2% and 5% BCS

Cells (P11–P13) were allowed to attach to the plate by culturing them for 24 hours in Eagle's MEM with 15% BCS. After the plating time, cells were treated with low serum media (Eagle's MEM, sigma M0643, with 2% BCS) for 72 h. After 72 h, the media was replaced every three days using either MEM with 5% or 2% BCS and 5 μ g/ml of Insulin and Transferrin, 5 ng/ml of sodium selenite (Sigma: ITS, I1884 prepared as recommended) for each media group. All cells were plated at a density of 1550 cells per cm². For immunohistochemistry measurements, MSC were plated over sterile 22mm glass cover slips placed in 10cm plates. For patch clamp experiments, cells were cultured in 35mm dishes at a density of 15×10^3 cells per dish.

2.4 MSC Differentiation Treatment with BMP4

Cells (P11–P13) were allowed to attach to the plate by culturing them for 24 hours in Eagle's MEM with 15% BCS. After the plating time, cells were treated with bone morphogenetic protein-4 (BMP4) media, composed of low serum media (Eagle's MEM, sigma M0643, with 2% BCS) and 25ng/ml of BMP4 (Human BMP4: H4916 Sigma-Aldrich) for 84 h. After 84 h, the media was replaced every three days with MEM with 5% BCS and 5 μ g/ml of Insulin and Transferrin, and 5 ng/ml of sodium selenite (Sigma: ITS, I1884 prepared as recommended)

2.5 Skeletal Satellite Cell Isolation and Differentiation

Primary myoblasts were isolated from limb muscles of neonatal mice (0–48hrs), as previously described [12]. Briefly, muscles were finely minced and incubated for a total of 40 min in Ca²⁺- and Mg²⁺-free rodent Ringer solution (in mM): 155 NaCl, 5 KCl, 10 HEPES, and 11 (2g/L) D-glucose containing 0.3% trypsin, 0.1% Collagenase, and 0.01% DNase. Large debris was removed by centrifugation and filtration. Cells (2.50×10^5) were plated onto 35-mm Falcon culture dishes containing 80% Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, 10% horse serum, and 10% calf serum. After 48 hrs, the plating media was replaced with DMEM plus 2% horse serum to promote cell differentiation. After an additional 72 hrs, the media was changed to DMEM plus 5% horse serum.

2.6 Immunofluorescence Staining

Cells were stained for cardiac specific Troponin T (cTnT; Thermo Scientific MS-295-P0, 1:200), slow skeletal troponin I, ssTnI (TNNI1, Abcam ab85087, 1:350) or ventricular myosin light chain-2, MLC2V, (MYL2-C17, Santa Cruz SC34490, 1:300). The cells were fixed with 2% paraformaldehyde for 20 minutes. We used horse, donkey, and goat serum (5%) diluted in 0.1% Tween in PBS for blocking and permeabilization for 45 minutes. The primary antibody was diluted in PBS in the ratios described above and incubated over-night at 4°C. Biotinylated horse anti-mouse IgG (Vector Labs BA-2001) for 1 hr at RT, 1:250 and streptavidin Alexa Fluor 555 conjugate (Invitrogen: S21381, S21375, 1:1000) was used for fluorescent detection (15 min at room temp) of cTnT. Other secondary antibodies used were Cy5 goat anti-rabbit (Jackson: 111-496-144 1:750) for ssTnI, and donkey anti-goat (Santa Cruz SC2024, 1:400) for MLC2V. After washing, cells were mounted with DAPI (Vector Laboratories, Inc). As a negative control we used an isotype specific IgG1 antibody (R&D Systems: MAB 002, 1:200).

2.7 cDNA Sequencing and RT-qPCR

Total RNA was obtained from MSC, MSC-BMP4, and Sk-sat cultures in the differentiated media described earlier. Extractions for Sk-sat were obtained from two 35mm culture plates for each day (0–6). Extractions for MSC were obtained from two (d0, d3, d6) or one (d9, d12, d15, d20) 10 cm culture plates, and days 0,3,6,12,20 for MSC-BMP4. RNA extractions were performed using Qiagen RNeasy mini-kits with the additional step of DNase digestion. The RNA density for each sample was measured (Thermo Scientific: spectrophotometer NanoDrop 8000) and normalized to the lowest RNA density found in the sample group and reverse-transcribed to cDNA using an ImProm-II kit from Promega with a random primer. The total Real-Time quantitative Polymerase Chain Reaction (RT-qPCR) (Applied Biosystems 7500) volume was 20 μ l, composed of Fast SYBR® Green Master Mix, molecular grade water, 0.5 μ M each of forward and reverse gene specific primer, and 1 μ l of cDNA. All PCR analyses except 18s were run between 59–60°C. Annealing temperature and dissociation curves were obtained for all primer/cDNA mixes and for the primer without cDNA for control. The 18s primer was run at 55°C. We used three separate cell cultures for each condition and the same primer/cDNA mix was analyzed in triplicate in qPCR 96 well plates. All genes were referenced to the geometric mean of at least two control genes selected from YWHAZ, 18s, and HPRT1 [13]. The primer sequences are given in the supplemental Table 2.

2.8 FACS Analysis

Differentiated MSC and BMP4 treated cells were cultured for 3 and 20 days and detached from the plates with 0.05% trypsin. Each cell group and day 0 cells were fixed with 2% paraformaldehyde. Day 0 and day 3 cells were incubated with the Sca-1 antibody (Bioscience: PE-Cy5 Ly-6A/E 155981–8) and IgG (Bioscience: PE-Cy5 IgG2b 553990) for 1 hr on ice, washed and analyzed. Day 20 cells were permeabilized with ice cold Methanol for 1hr and washed. Thereafter, four groups of MSC and BMP4 day 20 cells were incubated with the Nkx2.5 antibody (Santa Cruz: sc14033, 1:70). Separate groups of MSC and BMP4 day 20 cells were used for controls and treated with IgG antibody (R&D Systems: MAB002, 1:200) overnight at 4°C. The next day cells were washed and treated with the Cy5-conjugated secondary goat anti-rabbit (Jackson: 111-496-144, 1:300) for 45 min. After washing, cells were analyzed by FACS.

2.9 Electrophysiological Measurements

Data acquisition and processing were performed with pCLAMP 8.0 software (Axon Instruments). Recording electrodes were pulled from borosilicate glass with resistances

between 2–3.0M Ω when filled with a solution containing in mM: 137 KCl, 5 Mg 2 Cl, 10 K 2 -EGTA and 10 Hepes with pH adjusted for 7.2 with KOH. The extracellular solution contained Tyrodes salt (Sigma T2145) with pH 7.4. The action potential measurements were done in current clamp mode applying a 5 ms current pulse in steps of 1 nA with a 2 s interval between pulses. The inward (sodium) and the outward (potassium) currents were measured in voltage clamp mode from a holding potential of –80mV, applying 160 ms voltage pulses in 10 mV steps, with a 10s interval between pulses.

2.9 Statistical Data Analysis

Data are presented as Mean $\pm$ SEM with the number (N) of experiments. Significant differences between groups were evaluated using One-way Analysis of Variance (ANOVA) with Tukey's test for multiple comparisons. For experiments where only two sets of data were compared, we used Student's t-test. A p-value of 0.05 was considered statistically significant.

3 RESULTS

3.1 MSC and Sk-sat Protein Expression

To determine if differentiated MSC myotubes lead to cardiac or skeletal-like cells, we compared their temporal cTnT and ssTnI protein expression with those of differentiated Sk-sat cells. Our previous experience with Sk-sat cultures [15], revealed that Sk-sat differentiation is accelerated when they are cultured in low serum media. For example, when serum concentrations are decreased from 20% to 2%, Sk-sat start forming myotubes by culture day 3, and show early spontaneous beating by day 4, with increasing beating frequency and myotube length by day 6 (Supplemental movie 2). Thus, we examined Sk-sat differentiation starting from the day the media was changed to 2% serum (day 0) and followed their temporal gene expression up to day 6. It has been shown that Sk-sat calcium currents exhibit the greatest changes between day 2 to day 6 when placed in 2% serum, thereafter no significant changes were measured [15]. Therefore, we predicted that Sk-sat reached their differentiated stage when placed in 2% serum by culture day 6. Our protein expression studies showed that Sk-sat cells express cTnT early (day 2) during differentiation but by day 6 cTnT was mainly expressed at the periphery of the cell and ssTnI was expressed throughout the cell, as shown in Figure 1A. Similarly, differentiated MSC expressed cTnT early during differentiation (day 6) and by day 20 some multinucleated cells lost their cTnT and expressed it mainly at the periphery of the cell similar to Sk-sat cells. Other multi-nucleated cells expressed cTnT throughout the cytoplasm along with expression of ssTnI, Figure 1B. We also examined ventricular myosin light chain-2 (MLC2V) in both cell groups and noticed that its expression decreased by day 6 in Sk-sat cells, whereas some of the differentiated MSC expressed it throughout the cell by day 20, Figure 1C.

3.2 Cardiac and Skeletal Specific Gene Expression

Our initial observation of cTnT and ssTnI protein expression in MSC and Sk-sat cells led us to study the RNA expression of some of the cardiac- and skeletal-specific genes during low serum differentiation. For the cardiac pathway, we selected two early expressing genes, Nkx2.5 and GATA4 that are key regulators of cardiac development [16–18], and three late expressing genes cTnT, cTnI and MLC2. For the skeletal pathway, we selected MyoD for early gene expression evaluation [19], and myogenin (MyoG), skeletal-actin (Sk-Act), and ssTnI as late expressing genes. We evaluated the RNA in Sk-sat from day 0–6, with day 0 beginning after 48hrs in 20% serum. We obtained RNA from cells cultured at two serum concentrations. One group was kept at 2% serum and a second group was changed to 5% serum at day 3. All the genes were expressed as fold-change relative to the day at which there was a measurable RNA value and for which the first derivative of the dissociation

curve peaked at the corresponding amplicon melting temperature as shown in Table 1. Sk-sat did not exhibit significant expression of Nkx2.5 and had low levels of GATA4 (Figure 2). In addition, cardiac-specific gene expression, such as cTnT, cTnI and MLC2V were expressed from day 0 and peak at day 4, but significantly decreased by day 6 in the 2% serum cultures. This was especially the case for MLC2V. MyoD expression did not vary significantly from day 0 but ssTnI and Sk-Act increased significantly by days 3 and 4 respectively. In contrast, myogenin was consistently expressed from day 0–6 in the 2% serum, but significantly decreased by day 5 in the 5% serum media condition. ssTnI also had a significant decrease in expression in the 5% serum condition.

Collectively, these data suggest that cardiac-specific gene expression (cTnT, cTnI and MLC2V) decreased during Sk-sat differentiation, a result that is in agreement with our early observations for protein expression. In addition, the skeletal-specific genes (MyoG, ssTnI, Sk-Act) expression during Sk-sat differentiation was enhanced in the 2% compared to the 5% serum condition by day 5.

3.3 Cardiac and Skeletal Specific Gene Expression during MSC Differentiation

We also studied the same cardiac- and skeletal-specific gene expression in MSC from day 0–20 for two serum conditions 2% and 5%. Unlike Sk-sat, MSC express Nkx2.5 (Figure 3). During MSC differentiation Nkx2.5 and GATA4 expression levels remain unchanged from day 0 through day 20. In contrast, MyoD expression increased 4-fold by day 3; and in the 5% serum media, it significantly increased 6-fold by days 9 and 15 compared to 2% serum media condition. Data from Figure 3 also demonstrates that unlike the Sk-sat cells, RNA expression of the cardiac-specific genes cTnT, cTnI, and MLC2V increased to 35.7 ± 19 , 5.9 ± 2 , and 96.3 ± 32 fold respectively by day 15 compared to their reference day for the 5% serum. Similarly, the skeletal-specific genes Sk-Act, MyoG, and ssTnI significantly increased to 1258 ± 810 , 162 ± 90 , and 2506 ± 998 fold, respectively by day 15, relative to their reference day for 5% serum.

To understand the significance of cardiac and skeletal gene expression between the two cell types (Sk-sat and MSCs) we examined RNA expression of all the studied genes at day 0. Supplemental Table 1 shows the RNA expression at day 0 relative to the average RNA expression of cTnT in MSC at day 0. The RNA expression ratios were multiplied by 100 for clarity. At day 0, except for Nkx2.5 and GATA4, both skeletal and cardiac-specific genes were more highly expressed in Sk-sat cells compared to MSC. For example, cTnT, MyoD and Sk-Act were nearly 10-fold, 20-fold and 93-fold higher in Sk-sat cells than in MSC. In addition, cTnI and MLC2V were not detectable at day 0 in the MSC, but were expressed at low levels in the Sk-sat cells. During differentiation of MSC, cardiac expression continued to rise throughout the 20 days. cTnT and MLC2V for example increased nearly 6-fold more than in Sk-sat cells when compared to their highest expression (35.7 ± 19 vs. 5.2 ± 1.3) and (162 ± 67 vs. 25.2 ± 11) respectively.

At day 0 the skeletal-specific gene Sk-Act was nearly 93-fold higher in Sk-sat than in MSC, but by day 15, MSC on average increased its expression to nearly 131-fold relative to the highest expression (day 4) in Sk-sat cells, (1258 ± 810 vs. 9.6 ± 6.4). Similarly, ssTnI and MyoG were expressed in Sk-sat cells at day 0, but were nearly absent in MSC. At day 3 and day 15, MSC on average had nearly a 134-fold and 95-fold change compared to the maximum Sk-sat fold change (2506 ± 998 vs. 18.6 ± 10) and (162 ± 90 vs. 1.7 ± 0.9) for ssTnI and MyoG respectively.

These RNA data demonstrate that during myogenic differentiation some MSC had an increased expression of cardiac-specific genes and proteins as if following the cardiogenic pathway, a result that is in agreement with our observations for cTnT, MLC2V and ssTnI

protein expression. Similarly, other cells increased their expression of the skeletal-specific proteins to reach the level expressed by the Sk-sat cells, indicating a predominant skeletal genotype.

3.4 Cardiac and Skeletal Specific Gene Expression in BMP4 treated MSC

Our MSC RNA and protein expression profiles demonstrate dual induction of cardiac- and skeletal-like pathways under low serum differentiating media. This led us to investigate whether we could further increase the cardiac-specific gene and protein expression and suppress the skeletal-specific expression with the addition of the growth factor protein, BMP4. Other investigators [20, 21] demonstrated cardiac-like induction in embryonic and induced pluripotent stem cells when BMP4 and Activin were added to the media. Thus, we modified the differentiation protocol to include the addition of BMP4 during the 2% serum differentiation period (days 0–3) and followed the 5% serum differentiation media protocol. We selected this protocol based on our qPCR findings, noticing that we obtained significantly higher expressions of cTnT, cTnI and MLC2V with 5% serum condition over 2% serum by day 15 (Figure 3). The BMP4 application time and protocol were selected to be similar to protocols previously reported [20, 21].

Figure 4 shows a comparison of the expression of both cardiac- and skeletal-specific genes in MSC with 5% serum compared to BMP4 treated MSC (MSC-BMP4) for days 0–20. Days 9 and 15 were omitted in MSC-BMP4 because examination at this culture time in the previous experiments did not yield any additionally relevant data. MSC-BMP4 treated cells had a significant increase in the early cardiac and skeletal markers Nkx2.5, GATA4, and MyoD by day 3. These changes were nearly 14-, 5-, and 2.5-fold compared to MSC by day 20. Similarly there was a significant increase of nearly 6.5-fold cTnI expression at day 12 compared to the untreated MSC. Cardiac TnT and MLC2V expressions were significantly decreased at days 3–6 in the BMP4 treated, but reached comparable levels in the MSC by day 20. There was a delay in the expression of the skeletal gene MyoG with low detectable levels by day 6 as opposed to day 3 in the untreated MSC but it reached similar expression levels by day 20. Suppression in the other skeletal-specific genes Sk-Act, and ssTnI was detected, but their expression increased at day 12 and by day 20 they reached higher levels than those seen in the untreated MSC.

3.5 Temporal Protein Expression in BMP4 treated MSC

From our RNA studies we concluded that BMP4 significantly influenced the RNA expression of the cardiac specific markers. The greatest changes were observed for Nkx2.5, GATA4 and cTnI compared to the untreated MSC. To further evaluate whether BMP4 treatment similarly influenced protein expression, we examined Nkx2.5 expression in MSC and MSC-BMP4 using FACS analysis. Our FACS data demonstrated a positive bimodal distribution of Nkx2.5 expression compared to the isotype control (Figure 5A). Sample gating revealed that the first (left) peak represented cells with smaller forward and side scatter compared to cells in the second (right) peak (data not shown), indicating the existence of two cell populations. BMP4 treated MSC had 11.08 ± 3.2 % (Mean $\pm$ SEM) greater Nkx2.5 expression in the right-most peak compared to untreated 5% MSC by day 20. We have previously demonstrated that Lin⁻ MSC in culture express high levels of the stem cell surface protein Sca-1 [11]. To verify that MSC were functionally differentiating, we used FACS analysis to evaluate the expression of Sca-1 in MSC at day 3 and compared it with day 0. There was a decrease in the mean distribution of Sca-1 for both BMP4 treated and low serum treated MSC by day 3 compared to day 0, indicating a loss of “stemness” for both cell culture conditions by day 3 (Figure 5B). The calculated values for a count of 15,000 cells in each group were 324.6 ± 3.3 , 272.5 ± 4.3 , 174.8 ± 3.2 (Mean $\pm$ SEM) for day 0, MSC-day 3 and BMP4-day 3 respectively (Figure 5B). Immunocytochemical analysis

of the cells treated with BMP4 also revealed a shift in the temporal expression pattern of cTnT from days 6–15 (Figure 5C–D), compared to low serum treated MSC at days 6–20 (Figure 1B). BMP-4 suppressed early cTnT in MSC but induced high levels of cTnT expression by day 15 (Figure 5C–D). In contrast, cTnT expression was clearly visible at day 6 in rounded MSC under low serum alone and was further increased by day 20 in the elongated cells.

Taken together, the FACS analysis and immunocytochemical findings are consistent with our gene expression data. BMP-4 induced early developmental cardiac protein (e.g., Nkx2.5) with a concomitant loss of “stemness” in MSC and delayed expression of the late cardiac specific protein, cTnT. Previous studies examining embryonic cardiogenic responses reported similar early increases of Nkx2.5 with suppression of the late expressing genes cTnT, MLC2V and cTnI [6]. It is noteworthy that by day 15, most of the observable BMP-4 treated MSC expressed cTnT and were mononuclear, with no observable multinucleated and elongated cells exhibiting cTnT expression.

3.6 Action Potentials and Currents in Sk-sat, MSC, and BMP4 treated MSC

We previously reported spontaneous calcium cycling in differentiated MSC [7]. Thus we questioned whether these cells exhibited action potentials and if treatment with BMP4 could have an effect on their electrophysiological properties. MSC and MSC treated cells with BMP4 were patch clamped and compared to Sk-sat. Representative action potential measurements are shown for Sk-sat, MSC, and BMP4 treated MSC (Figure 6A–C). BMP4 treated MSC had an average action potential peak value of 98.1 ± 13.5 mV, a 30% decrease from the 141.3 ± 5.7 mV peak value of untreated MSC and both MSC groups displayed significantly lower action potential peaks than Sk-sat (Figure 6D). MSC-BMP4 action potential amplitudes are comparable to those reported for ventricular and primary myocardial-like cells [8]. The action potential average duration at 50% amplitude (APD₅₀), and at 90% amplitude (APD₉₀) are illustrated in Figure 6 E and F, respectively. Mean MSC time durations were significantly longer than those of Sk-sat, and MSC exhibited a bimodal distribution of cells having short and longer time durations. BMP4 treatment led to a significant increase of the average time duration in the action potentials to 25.8 ± 4.2 ms for APD₅₀ and 54.4 ± 7.6 ms for APD₉₀, nearly a 1.7 fold increase compared to untreated MSC. These significant increases in action potential time durations suggest that the electrical activity of BMP-4 treated MSC became more distinct from the short time durations exhibited by the Sk-sat cells and resembled action potential durations reported in embryonic stem cell-derived cardiocytes and fetal cardiomyocytes [8, 9].

We also measured the membrane capacitance and the currents in the same patched cells. Representative current measurements are shown in Figures 7 A, B, and C for Sk-sat, MSC, and MSC-BMP4 respectively. Results of the average membrane capacitance revealed a significant reduction in the capacitance value for MSC after BMP4 treatment (Figure 7 D). A lower capacitance value correlates with a smaller size cell, as is consistent with our immunohistochemistry data. There was also a significant difference in the average inward peak currents between MSC and Sk-sat cells. The average values of the maximum inward currents were -27 ± 3 (n=23), -14 ± 4 (n=16), and -8 ± 2 (n=12) pA/pF for Sk-sat, MSC, and MSC-BMP4 respectively. The outward currents, measured at 100ms had an average value of 15.5 ± 2 (n=23), 4.2 ± 2 (n=16), and 1.7 ± 1 (n=12) pA/pF for Sk-sat, MSC and MSC-BMP4 respectively. Sk-sat outward currents were significantly greater than MSC. We observed that within the MSC, there were a subset of cells that exhibited large outward currents and others in which these currents were very small or close to zero at 100ms. After BMP4 treatment, MSC outward current decreased to nearly 1/3 compared to the untreated MSC. In addition to the average current reductions there was a nearly 50% reduction in the standard deviation measurements, indicating the presence of a more homogeneous

population with many more mononuclear cells whose outward currents remained low during the voltage clamp measurement.

Overall, these patch clamp measurements show that during myogenic differentiation MSC exhibit action potentials and currents between days 14–20 whose average values lie between Sk-sat cells and cardiomyocytes. After BMP4 treatment, MSC-BMP4 exhibit a significant increase in their action potential duration, deviating farther away from the typical short duration characteristic of Sk-sat cells. Furthermore, MSC-BMP4 had a significant decrease in the average membrane capacitance value compared to the untreated cells, revealing a possible shift from electrically active long skeletal-like myotubes to electrically active mononuclear cardiac-like cells.

4 DISCUSSION

The current study is the first, to our knowledge, that demonstrates the ability to modulate skeletal myogenesis in bone marrow-derived mesenchymal stem cell cultures as a means of producing a greater population of cardiogenic MSCs with concomitant changes in action potential duration.

MSC transplantation following ischemic insult in the heart is an effective therapy for improving cardiac function. It has also been well established that bone marrow mesenchymal stem cells are heterogeneous in nature and manipulation of cell cultures induces differentiation into multiple cell lineages. Multipotency is a hallmark of stem cells and is critical for effective regenerative therapy in a variety of organs/tissues. But the multipotent phenotype also complicates the use of these cells because specific cardiac muscle lineage development is required for repair of the ischemic heart tissue and a large number of non-cardiac cell lineages are induced in culture [22]. Previously published work suggests that custom tailored cocktails of growth factors that include proteins from the transforming growth factor beta (TGF-beta) family may be necessary to guide early muscle specific gene expression leading to myotube formation and sarcomeric protein assembly in mesenchymal stem cells [23]. One caveat of this approach is the presence of MSCs that also express skeletal muscle specific markers and progress to multi-nucleated myotubes more characteristic of a skeletal muscle cell phenotype

Previously published work addressed the dual myogenic nature of MSCs, where MSCs differentiate into skeletal-like and cardiac-like cells in the same culture [14]. However, the enrichment of specific cells necessary for transplantation or a more in depth assessment of early gene expression in MSCs is lacking and would advance our understanding of how MSCs differentiate into specific lineages in culture.

Our temporal gene and protein expression studies revealed that Sk-sat exhibited early expression of cardiac-specific genes such as cTnT and MLC2V but after six days in culture, these cardiac-specific genes were significantly attenuated and the protein expression was limited to the periphery of the cell. Similarly, a group of differentiated MSC formed skeletal-like myotubes with cTnT expression at the periphery of the cell. Despite these similarities, MSC temporal gene expression demonstrated a sustained increase in the expression of cardiac-specific genes such as cTnT, cTnI, and MLC2V, as well as skeletal-specific MyoG, ssTnI and Sk-Act throughout the differentiation period.

In parallel with the differences in temporal gene expression, we observed that the action potentials in Sk-sat cells were short in duration and had a high overshoot while the action potentials in MSC were longer and had lower amplitudes. The differences in action potentials can be explained by the differences in ionic currents recorded from each cell type. Sk-sat cells had larger inward and outward currents compared to MSC, resulting in a large

overshoot and a short duration of the action potential. The smaller currents in MSC resulted in longer durations and lower amplitudes of the action potential.

A number of investigators [20, 21] have shown cardiac-like induction in embryonic stem cells when BMP4 and Activin are added to the media. More recently Behfar *et al.*, [23] reported improvements in MSC therapy in chronic myocardial infarction when hMSC were pre-treated with a cocktail of growth factors (including BMP4) prior to transplantation. In addition, Mohanty *et al.*, incubated hMSCs with TGF beta1 and demonstrated increased expression of GATA4 and Nkx2.5 compared to untreated MSCs [24]. Several investigators have reported that GATA4 overexpression promotes induction of early cardiac genes, brain natriuretic peptide, Islet-1, and alpha-sarcomeric actinin [25]. Similarly, Xiang *et al.*, [26] showed that when skeletal muscle-derived stem cells are transduced with a lentivirus encoding for Wnt11 and treated with BMP4, it led to MLC2a protein expression. Thus, we further hypothesized that BMP4 treatment would alter early gene expression leading to preferential expression of genes associated with a cardiogenic lineage. We compared here the early temporal expression of skeletal- and cardiac-specific genes in cultured MSC and Sk-sat when induced into myogenic differentiation and demonstrated that BMP4 treatment modulated the MSC gene and protein profile favoring cardiogenesis over skeletal myogenesis. BMP4 significantly increased the early expression of Nkx2.5 and GATA4, delayed the expression of MyoG, and significantly increased the expression of cTnI in treated compared to untreated MSCs. Most noteworthy, BMP4 also elicited changes in the functional characteristics of MSCs that were associated with the changes in gene expression. BMP4 treatment resulted in a shift further away from the skeletal-like short action potential durations towards longer action potential durations characteristic of a cardiac cell [8, 9]. The prolongation of the action potential was the result of a reduction of the outward repolarizing current.

Our gene expression results suggest that BMP4 treatment may alter the early myogenic precursor cells in differentiated MSC, increasing the expression of the early cardiac-specific genes Nkx2.5, GATA4 and the early skeletal-specific MyoD compared to untreated MSC. In addition, there was a significant and early increase of the cardiac-specific gene cTnI, suggesting that many of the cells reached a more cardiac-like condition differing from the Sk-sat cells in which expression of cTnI decreased with differentiation. The delayed expression of the skeletal-specific gene program by BMP4 in MSC cultures suggests that this growth factor may play a role in attenuating skeletal myogenesis while enhancing cardiogenesis. Furthermore, the addition of other growth factors particularly those in the TGF beta superfamily may further delay skeletal myogenesis in the MSC cultures and enhance the potential for enrichment of cardiogenic MSCs. We have previously demonstrated that enrichment of MSCs in culture by the $\alpha 2\delta 1$ subunit of the dihydropyridine receptor led to a greater population of cTnT⁺ cells displaying calcium transients than cells that were not enriched [7]. In this study, we show that some of those cTnT⁺ cells are following the skeletal-like pathway, and that the use of BMP4, elicits a dual response in the differentiated MSCs that favors cardiogenesis and attenuates skeletal myogenesis. Changes in the expression of cardiac specific genes of MSCs incubated with BMP4 occur in parallel with electrophysiologic properties of the cell and are established very early in treated cell cultures. Taken together, these data contribute to our understanding of cardiogenic induction in isolated MSCs and demonstrate that BMP4 alone is sufficient to alter both skeletal and cardiac lineage development simultaneously in heterogeneous MSC cultures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Phinney DG, Prockop DJ. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair--current views. *Stem Cells*. 2007; 25(11):2896–902. [PubMed: 17901396]
2. Pittenger MF, Martin BJ. Mesenchymal stem cells and their potential as cardiac therapeutics. *Circ Res*. 2004; 95(1):9–20. [PubMed: 15242981]
3. Quevedo HC, et al. Allogeneic mesenchymal stem cells restore cardiac function in chronic ischemic cardiomyopathy via trilineage differentiating capacity. *Proc Natl Acad Sci U S A*. 2009; 106(33):14022–7. [PubMed: 19666564]
4. Merritt EK, et al. Repair of traumatic skeletal muscle injury with bone-marrow-derived mesenchymal stem cells seeded on extracellular matrix. *Tissue Eng Part A*. 2010; 16(9):2871–81. [PubMed: 20412030]
5. Hare JM, Chaparro SV. Cardiac regeneration and stem cell therapy. *Curr Opin Organ Transplant*. 2008; 13(5):536–42. [PubMed: 19060539]
6. Muller-Ehmsen J, et al. Cellular cardiomyoplasty--a novel approach to treat heart disease. *Congest Heart Fail*. 2002; 8(4):220–7. [PubMed: 12147946]
7. Grajales L, et al. Delayed enrichment of mesenchymal cells promotes cardiac lineage and calcium transient development. *J Mol Cell Cardiol*. 2010; 48(4):735–45. [PubMed: 20060001]
8. Yamanaka S, et al. Enhanced proliferation of monolayer cultures of embryonic stem (ES) cell-derived cardiomyocytes following acute loss of retinoblastoma. *PLoS One*. 2008; 3(12):e3896. [PubMed: 19066628]
9. Liu A, et al. Functional characterization of inward rectifier potassium ion channel in murine fetal ventricular cardiomyocytes. *Cell Physiol Biochem*. 2010; 26(3):413–20. [PubMed: 20798526]
10. Gepstein L, et al. In vivo assessment of the electrophysiological integration and arrhythmogenic risk of myocardial cell transplantation strategies. *Stem Cells*. 2010; 28(12):2151–61. [PubMed: 20960511]
11. Boomsma RA, Swaminathan PD, Geenen DL. Intravenously injected mesenchymal stem cells home to viable myocardium after coronary occlusion and preserve systolic function without altering infarct size. *Int J Cardiol*. 2007; 122(1):17–28. [PubMed: 17187879]
12. Alden KJ, Garcia J. Differential effect of gabapentin on neuronal and muscle calcium currents. *J Pharmacol Exp Ther*. 2001; 297(2):727–35. [PubMed: 11303064]
13. Vandesompele J, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*. 2002; 3(7):RESEARCH0034. [PubMed: 12184808]
14. Shiota M, et al. Isolation and characterization of bone marrow-derived mesenchymal progenitor cells with myogenic and neuronal properties. *Exp Cell Res*. 2007; 313(5):1008–23. [PubMed: 17289022]
15. Nabhani T, Shah T, Garcia J. Skeletal muscle cells express different isoforms of the calcium channel alpha2/delta subunit. *Cell Biochem Biophys*. 2005; 42(1):13–20. [PubMed: 15673925]
16. Yamagishi H, et al. The combinatorial activities of Nkx2.5 and dHAND are essential for cardiac ventricle formation. *Dev Biol*. 2001; 239(2):190–203. [PubMed: 11784028]
17. Vincentz JW, et al. Cooperative interaction of Nkx2.5 and Mef2c transcription factors during heart development. *Dev Dyn*. 2008; 237(12):3809–19. [PubMed: 19035347]
18. Pikkarainen S, et al. GATA transcription factors in the developing and adult heart. *Cardiovasc Res*. 2004; 63(2):196–207. [PubMed: 15249177]

19. Tapscott SJ. The circuitry of a master switch: Myod and the regulation of skeletal muscle gene transcription. *Development*. 2005; 132(12):2685–95. [PubMed: 15930108]
20. Laflamme MA, et al. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nat Biotechnol*. 2007; 25(9):1015–24. [PubMed: 17721512]
21. Yang L, et al. Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. *Nature*. 2008; 453(7194):524–8. [PubMed: 18432194]
22. Rosca AM, Burlacu A. Effect of 5-azacytidine: evidence for alteration of the multipotent ability of mesenchymal stem cells. *Stem Cells Dev*. 2011; 20(7):1213–21. [PubMed: 21067364]
23. Behfar A, et al. Guided cardiopoiesis enhances therapeutic benefit of bone marrow human mesenchymal stem cells in chronic myocardial infarction. *J Am Coll Cardiol*. 2010; 56(9):721–34. [PubMed: 20723802]
24. Mohanty S, et al. TGFbeta1 contributes to cardiomyogenic-like differentiation of human bone marrow mesenchymal stem cells. *Int J Cardiol*. 2011
25. Li H, et al. GATA-4 promotes myocardial transdifferentiation of mesenchymal stromal cells via up-regulating IGFBP-4. *Cytotherapy*. 2011; 13(9):1057–65. [PubMed: 21846294]
26. Xiang G, et al. Lentivirus-mediated Wnt11 gene transfer enhances Cardiomyogenic differentiation of skeletal muscle-derived stem cells. *Mol Ther*. 2011; 19(4):790–6. [PubMed: 21304494]

Highlights

- BMP4 suppresses skeletal myogenic expression in mesenchymal stem cells
- Mesenchymal stem cells exhibit longer action potential durations than skeletal satellite cells
- Nkx2.5 protein expression is increased and Sca-1 levels are decreased in mesenchymal stem cells exposed to BMP4

Figure 1.

ssTnI (green) and cTnT (red) temporal protein expression: **A.** differentiated Sk-sat cells in 5% media express cTnT early (day 2) during differentiation but by day 6 cTnT is mainly expressed in the periphery and ssTnI is expressed throughout the cell; **B.** differentiated MSC in 5% media show expression of cTnT at day 6 and day 20; however by day 20, some multinucleated cells express cTnT mainly at the periphery whereas other mononuclear cells express cTnT everywhere in the cell, both showing expression of ssTnI. **C.** MLC2V protein expression is significantly reduced in Sk-sat cells by day 6, whereas some MSC express it throughout the cell by day 20.

Figure 2.

Sk-sat cells temporal cardiac (Gata-4, cTnT, cTnI, MLC2V) and skeletal (MyoG, Sk-Actin, ssTnI) specific gene expression in differentiating media with two serum levels, 2% and 5%. The fold change was calculated relative to the day listed on Table 1. NKx2.5 levels were insignificant compared to the other gene levels, and its expression was not recognizable throughout the differentiation period. Average values represent Mean + SEM and significant differences between each paired data is represented by lines, $p < 0.05$. The number of independent cell cultures analyzed were 3 and each gene was analyzed 3 times in the qPCR plate, $n = 9$. All genes were referenced to the geometric mean of at least two control genes selected between YWHAZ, 18s, and HPRT1.

Figure 3.

MSC temporal cardiac (Nkx2.5, GATA-4, cTnT, cTnI, MLC2V) and skeletal (MyoD, MyoG, Sk-Actin, ssTnI) specific gene expression in differentiating media with two serum levels, 2% and 5%. The fold change was calculated relative to the day listed on Table 1. Average values represent Mean + SEM significant differences between each paired data is represented by lines, $p < 0.05$. Points represented by (*) are significantly different to all other data points, $p < 0.05$. The number of independent cell cultures analyzed were 3 and each gene was analyzed 3 times in the qPCR plate, $n = 9$. All genes were referenced to the geometric mean of at least two control genes selected from YWHAZ, 18s, and HPRT1.

Figure 4.

MSC temporal cardiac (Nkx2.5, GATA-4, cTnT, cTnI, MLC2V) and skeletal (MyoG, Sk-Actin, ssTnI) specific gene expression in differentiating media with BMP4 treatment compared to MSC in 5% serum. Average values represent Mean + SEM significant differences between each paired data is represented by lines, $p < 0.05$. Points represented by (*) are significantly different to all other data points, $p < 0.05$. Points represented by (#) are significantly different to all other points except to those with (#), $p < 0.5$. The number of independent cell cultures analyzed was 3 and each gene was analyzed 3 times in the qPCR plate, $n = 9$. All genes were referenced to the geometric mean of at least two control genes selected among YWHAZ, 18s, and HPRT1.

Figure 5.

A–B. Protein expression using FACS analysis: **A.** FACS results show a positive bimodal distribution of the Nkx2.5 expression compared to the IgG expression. BMP4 treated MSC had $11.08 \pm 3.2\%$ (Mean $\pm$ SEM) greater Nkx2.5 expression on the right positive peak compared to untreated 5% MSC by day 20, $n=4$, and $p<0.05$. **B.** FACS results for the temporal expression of Sca-1 show that there is a decrease in the mean distribution of the stem cell marker during differentiation for both BMP4 treated and untreated MSC by day 3 compared to day 0. **C–D.** Temporal ssTnI and cTnT protein expression in BMP4 treated MSC: **C.** MSC-BMP4 cells show sparse areas of light cTnT (red) expression and diffuse ssTnI (green) at day 6, compared to untreated 5% MSC where some cells exhibited cTnT expression throughout the cell (Figure 1). **D.** At day 15 many mononuclear cells were seen with cTnT expression throughout the cell and no multinucleated cells or myotubes exhibited cTnT or ssTnI expression around the periphery as in the untreated 5% MSC culture (Figure 1).

Figure 6.

Electrophysiological patch clamp measurements: **A.** Representative action potential for Sk-sat, **B.** MSC, and **C.** MSC-BMP4 treated cells. **D.** Action potential average peak value in mV, **E.** Action potential average duration at 50% amplitude (APD_{50}). **F.** Action potential average duration at 90% amplitude (APD_{90}). Average values represent Mean + SEM and significant differences (*) are $p < 0.05$. The number of cells analyzed were $n=25$ for Sk-sat between days 4–6, $n=16$ for MSC between days 14–20, and $n=12$ for MSC-BMP4 between days 14–20.

Figure 7.

A, B, and **C** show representative measured currents for Sk-sat, MSC, and MSC-BMP4 respectively. **D.** Membrane capacitance average values. **E.** Inward peak current. The average peak value was measured at the maximum negative peak occurring during the first 20 ms. **F.** Outward maximum current. The average value measured at the 100 ms point. Average values represent Mean + SEM and significant differences (*) are $p < 0.05$. The number of cells analyzed were $n=25$ for Sk-sat between days 4–6, $n=16$ for MSC between days 14–20, and $n=12$ for MSC-BMP4 between days 14–20.

Table 1

Reference day at which there was a measurable RNA value and for which the first derivative of the dissociation curve peaked at the corresponding amplicon melting temperature. This table was used to calculate the fold changes in Figures 2, 3 and 4.

RNA	Sk-sat reference day	MSC reference day	MSC+BMP4 reference day
NKx2.5	Not detected	D0	D0
GATA-4	D2	D0	D0
MyoD	D0	D0	D0
cTnT	D0	D0	D0
cTnI	D0	D3	D3
MLC2V	D0	D6	D3
Sk-Act	D0	D0	D0
MyoG	D0	D3	D6
ssTnI	D0	D3	D3