Title: Amniotic fluid-derived stem cells demonstrate limited cardiac differentiation following small molecule-based modulation of Wnt signaling pathway

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Abstract

Amniotic fluid-derived stem cells (AFSC) are a promising cell source for regenerative medicine and cardiac tissue engineering. However, a non-xenotropic differentiation protocol has not been established for cardiac differentiation of AFSC. We tested a small molecule-based modulation of Wnt signaling for directed cardiac differentiation of AFSC. Cells were treated with inhibitors of glycogen synthase kinase 3 and Wnt production and secretion in a time-dependent and sequential manner, as has been demonstrated successful for cardiac differentiation of embryonic and induced pluripotent stem cells. Cells were then analyzed for gene and protein expression of markers along the cardiac lineage at multiple days during the differentiation protocol. At the midpoint of the differentiation, an increase in the percentage of AFSC expressing Islet-1, a transcription factor found in cardiac progenitor cells, and Nkx-2.5, a cardiac transcription factor, was observed. After a 15-day differentiation, a subpopulation of AFSC upregulated protein expression of smooth muscle actin, myosin light chain-2, and troponin I, all indicative of progression down a cardiac lineage. AFSC at the end of the differentiation also demonstrated organization of connexin 43, a key component of gap junctions, to cell membranes. However, no organized sarcomeres or spontaneous contraction were observed. These results demonstrate that small molecule-based modulation of Wnt signaling alone is not sufficient to generate functional cardiomyocytes from AFSC, though an upregulation of genes and proteins common to cardiac lineage cells was observed.

Introduction

Heart related defects are the most common form of fatal birth defect [1], and birth defects are the leading cause of infant death in the United States [2]. The incidence of moderate and severe congenital heart defects (CHD) is approximately 6 per 1000 births [3], and these defects are likely to require expert cardiology care, including surgeries, which may involve the use of patches. Current patches available for surgical repair have several shortcomings, including being non-conductive and non-contractile. These shortcomings could be addressed by development of a tissue engineered cardiac patch, but an ideal cell source for cardiac tissue engineering remains an issue. While pluripotent stem cells, such as induced pluripotent stem cells (iPSC) and embryonic stem cells (ESC), have been demonstrated to successfully differentiate into functional cardiomyocytes [4,5], issues remain that prevent these cells from being used therapeutically, including potential teratoma formation following implantation.

Amniotic fluid-derived stem cells (AFSC) present a possible alternative cell source. Amniotic fluid has been reported to contain a population of broadly multipotent stem cells that have traits and express markers characteristic of both mesenchymal stem cells (MSC) and pluripotent stem cells. AFSC can differentiate across all three germ layers and maintain proliferation rates similar to pluripotent stem cells, but do not form teratomas when implanted [6]. AFSC represent a promising cell source for tissue engineering applications, including potential autologous application to congenital defect repair, because they have many of the advantages of pluripotent stem cells such as ESC and iPSC and of MSC but lack many of the disadvantages of these cell types.

AFSC have been investigated for a variety of cardiovascular applications, including *in vitro* cardiomyocyte differentiation, implantation after myocardial infarction, heart valve tissue engineering, and vascular therapies – see review in ref. [7]. Of particular interest is the cardiac potential of AFSC *in vitro*. Upon direct co-culture of AFSC with neonatal rat ventricular myocytes (NRVM), human cells positive for cardiac markers such as sarcomeric proteins have been observed [8–10]. However, when AFSC are co-cultured with NRVM in systems that do not allow for cell fusion, including on opposite sides of a porous membrane, in shared media dishes, and AFSC in conditioned medium from NRVM, it is observed that AFSC form functional gap junctions but do not express sarcomere proteins [11]. It has also been observed that treatment of AFSC with the demethylating agent 5-aza-2'-deoxycytidine for 24 hours at the beginning of a 10 day culture period results in phenotypic changes of the cells consistent with muscle cells and protein expression of cardiac troponin I (cTnI) and cardiac troponin T (cTNT), but no mature sarcomeres or spontaneous contraction [9].

Given the limited success of cardiac differentiation observed in AFSC using the above methods, we tested a method of defined small molecule-based modulation of the Wnt signaling pathway for cardiac differentiation of AFSC. Multiple studies show that modulation of the canonical Wnt signaling pathway by small molecules or protein growth factors leads to cardiac differentiation of ESC and iPSC into functional and beating cardiomyocytes [12–23]. These cardiac differentiation methods are based on the role of Wnt signaling in cardiac development. Inhibition of canonical Wnt signaling induces cardiomyogenesis [24].

To address the ability of AFSC to differentiate towards a cardiomyocyte lineage following directed differentiation using small molecule-based modulation of Wnt signaling, we cultured AFSC at varying densities on Matrigel-coated surfaces and added a glycogen synthase kinase 3 (GSK3) inhibitor and Wnt processing inhibitor sequentially in a specific time course. Expression of mesodermal transcription factor brachyury was assessed to select the appropriate GSK3 inhibitor concentration. Then expression of cardiac specific transcription factor and sarcomere genes and proteins were evaluated at time points during the differentiation process to evaluate success of the differentiation protocol. This differentiation method is based off an approach shown successful at generating beating cardiomyocytes from iPSC with high efficiency [23].

Materials and Methods

Isolation and culture of AFSC

Human amniotic fluid was obtained from second trimester pregnancies from patients undergoing planned amnioreduction for treatment of twin-twin transfusion syndrome. The research was carried out according to the World Medical Association Declaration of Helsinki, and informed consent was obtained from all subjects. The experimental protocol and informed consent form were approved by the Institutional Review Boards of Baylor College of Medicine and Rice University.

Amniotic fluid was centrifuged and cells were resuspended in a modified α -Minimum Essential Medium (α MEM): 63% α MEM (HyClone), 18% Chang Basal Medium (Irvine Scientific), 2% Chang C supplement (Irvine Scientific), 15% embryonic stem cell-tested fetal bovine serum (FBS; Gemini Bio-Products), 1% GlutaMAX

(Invitrogen) and antibiotics. Cells were plated at 2500 cells/cm² on standard plastic Petri dishes (BD Biosciences) and cultured at 37°C and 5% CO₂ in a humidified environment.

Cells were routinely passaged using 0.25% trypsin at approximately 70 to 80% confluence, approximately every 5-7 days. All differentiation experiments were performed on cells at passage 5.

Small molecule-based cardiac differentiation of AFSC

Differentiation was performed as has previously been published for iPSC [23]. On day -4 of the differentiation, AFSC were plated in 12-well cell culture plates coated with ESC-qualified Matrigel (Corning). For initial studies to determine optimal cell seeding density cells were plated at 50,000, 100,000, 150,000, and 200,000 cells per cm². Cells were maintained in modified α MEM, and the medium was changed every day for 3 days. Throughout the differentiation, cells were maintained with 2 ml of medium per well.

On day 0, medium was changed to RPMI1640 with GlutaMAX (Gibco) supplemented with B-27 supplement minus insulin (Gibco). A small molecule inhibitor of GSK3, CHIR99021 (Selleckchem), was tested at concentrations varying from 0 to 12 μ M. Exactly 24 hours later (day 1) the cell culture medium was changed to RPMI1640+B-27 minus insulin. Forty-eight hours after that (day 3), the second small molecule, Inhibitor of Wnt Processing and Secretion 2 (IWP2; R&D Systems, Minneapolis, MN), was added. At this media change, 1 ml from each well was transferred to a sterile secondary container and 1 ml of 10 μ M IWP2 in RPMI1640+B-27 minus insulin was added, for a final concentration of 5 μ M IWP2 as describe in Lian et al [23]. After aspiration of the second ml of media from the cell culture well, the combined

media was added back to the corresponding original well. After forty-eight hours in media containing 5 μ M IWP2, the media was changed to RPMI1640+B-27 minus insulin (day 5). On day 7 of the differentiation, media was changed to RPMI1640 with GlutaMAX supplemented with complete B-27 supplement. Cells were maintained in this media to day 15, with media changes performed every 2-3 days.

Flow cytometry

Monolayers of adherent cells were detached with Accutase (Sigma-Aldrich) or 0.25% trypsin (for intracellular staining only) and resuspended in phosphate buffered saline (PBS) with 1% FBS.

For cell surface proteins, cells were stained with fluorophor-conjugated antibodies in the dark at room temperature for 15 minutes then rinsed twice with PBS containing 1% FBS. Antibodies used were Alexa Fluor 647-conjugated anti-SSEA4, FITC-conjugated anti-Tra-1-60, PE-conjugated anti-CD117/c-kit, PE-conjugated anti-Islet-1, PE-conjugated anti-CD29, FITC-conjugated anti-CD44, APC-conjugated anti-CD90, PE-conjugated anti-CD105, PE-conjugated anti-CD140a, Alexa Fluor 647-conjugated anti-CD309, FITC-conjugated anti-CD31, APC-conjugated anti-CD45, PE-conjugated anti-HLA-ABC, and FITC-conjugated anti-HLA-DR. Antibodies and corresponding isotype controls were developed in mouse and purchased from BD Biosciences.

For analysis of intracellular proteins, suspended cells were fixed in 4% paraformaldehyde for 20 minutes at room temperature, washed with PBS, and resuspended in permeabilizing buffer (PBS with 0.5% Tween20, Sigma-Aldrich). Cells were then incubated with fluorophor-conjugated antibodies for 30 minutes at room

temperature and rinsed twice with PBS with 1% FBS. These antibodies included PE-conjugated anti-Sox2 (mouse IgG2a, R&D Systems), PE-conjugated anti-Islet-1 (BD Biosciences), and PE-conjugated anti-brachyury (goat IgG, R&D Systems).

Unconjugated primary antibodies were incubated with cells at 4°C overnight, rinsed, incubated with fluorophor-conjugated secondary antibodies for 30 minutes at room temperature, and rinsed again. Primary antibodies used were anti-Nkx-2.5 (rabbit IgG, Santa Cruz Biotechnology, Inc.), anti-myosin light chain 2 (MLC2, rabbit IgG, ProteinTech), anti-Troponin I (TnI, rabbit IgG, Santa Cruz Biotechnology, Inc), anti-smooth muscle actin (SMA, mouse IgG2a, Thermo Scientific), and anti-cardiac Troponin T (cTnT, mouse IgG1, Thermo Scientific). Secondary antibodies used were Alexa Fluor 488-conjugated goat anti-rabbit, Alexa Fluor 488-conjugated goat anti-mouse IgG1, Alexa Fluor 647-conjugated goat anti-mouse IgG2a, and Alexa Fluor 647-conjugated goat anti-rabbit. All secondary antibodies were purchased from Life Technologies. Controls of primary antibody specific isotype controls or secondary antibody only were performed.

Data collection was performed on a BD LSR II Flow cytometer with FACSDiva software (BD Biosciences). Data analysis was performed using FlowJo software (Tree Star, Inc.).

Quantitative reverse transcriptase – polymerase chain reaction (qRT-PCR)

Total RNA was extracted from AFSC at days 0, 5, and 15 of the differentiation protocol using the RNeasy kit according to manufacturer's protocols (Qiagen). cDNA

synthesis was performed using High Capacity cDNA Reverse Transcription Kit, according to manufacturer's protocol (Applied Biosystems).

Relative quantification of GATA4, NKX-2.5, TNNT2, and ACTN2 were performed using TaqMan Gene Expression Assays Hs00171403_m1, Hs00231763_m1, Hs00943911_m1, and Hs00153809_m1, respectively. Expression was normalized to GAPDH (TaqMan Gene Expression Assay Hs02758991_g1). Briefly, 1 µl cDNA was amplified in TaqMan Gene Expression Master Mix with 250 nM TaqMan probe in a 20 µl reaction using the Standard program for 60 cycles on an ABI ViiA 7 Real-Time PCR System. Data was analyzed using the comparative C_T method, with all samples normalized to GAPDH and then to a Day 0 sample group. All TaqMan reagents were from Applied Biosystems.

Immunostaining

Immunostaining was performed to observe the presence and localization of proteins in the differentiated AFSC populations. At day 14 of the differentiation protocol, cells were lifted with 0.25% trypsin and replated in RPMI 1640 medium with 20% FBS on 1% gelatin coated dishes at 100,000 cells per well in 12-well plates. Two days later the media was changed to RPMI 1640 with complete B27 supplement, and three days after that the cell cultures were washed with PBS and fixed with 4% paraformaldehyde (Alfa Aesar) for 20 minutes at 4°C. Cells were washed with PBS and permeabilized with 0.5% Triton X100 (Sigma-Aldrich). Cells were again washed with PBS, blocked with 1% bovine serum albumin (BSA; CalBioChem) for 1 hour at 25°C, and then stained overnight at 4°C with primary antibodies against: connexin 43/GJA1 (rabbit polyclonal;

Abcam), SSEA-4 (mouse monoclonal; Santa Cruz Biotechnology), smooth muscle actin (Thermo Scientific), troponin I (Santa Cruz Biotechnology), and myosin light chain 2 (ProteinTech). Cells were again rinsed with 1% BSA in PBS then stained with Alexa Fluor 488-conjugated goat secondary antibodies (Life Technologies) diluted to 1:1000 for 30 minutes at room temperature. Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) with Vecta Shield (Vector). Undifferentiated AFSC and differentiated AFSC incubated with secondary antibodies alone served as negative controls, while NRVM served as positive controls. Images were obtained using a DMI 6000B (Leica Microsystems) fluorescence microscope.

Statistical Analysis

Results are presented as means $\pm$ standard deviations. Sample numbers are presented in their respective figure legends. ANOVA with Tukey's post-hoc tests or Student t-tests were used to test for significant differences, as appropriate. Significance was determined as $p < 0.05$. Statistics were performed using R statistical software.

Results

Characterization of AFSC

Flow cytometry on undifferentiated AFSC cultured through passage 5, showed the cells were strongly positive for the pluripotent stem cell marker SSEA4 (68%) but negative for pluripotent stem cell marker Tra-1-60. Subpopulations of cells expressed the transcription factors Sox2 (12%) and brachyury (17%). Sox2 is a transcription factor characteristic of pluripotent stem cells while brachyury is indicative of a mesodermal

lineage. Unfractionated AFSC at passage 5 were negative for c-kit/CD117 and the transcription factor Islet-1, a marker for cardiac progenitors. C-kit is also known as stem cell growth factor receptor and is expressed on the surface of hematopoietic stem cells and other cell types. AFSC were negative for CD309, also known as vascular endothelial growth factor receptor-2 (VEGFR2) or kinase insert domain receptor (KDR), and 20% of cells were positive for CD140a, or platelet-derived growth factor receptor alpha (PDGFR α). VEGFR2 and PDGFR α are co-expressed on cells of the cardiac mesoderm and on embryonic or induced pluripotent stem cells undergoing cardiac differentiation through Activin A and BMP4 treatment [25]. AFSC were positive for MSC markers CD29, CD44, CD90, and CD105. The stem cells were positive for the immunological marker HLA-ABC and negative for the immunological marker HLA-DR. AFSC were also negative for protein expression of the hematopoietic differentiation marker CD45 and the endothelial marker PECAM-1/CD31 (Figure 1).

Figure 1 – Analysis of protein expression in undifferentiated AFSC.

AFSC expanded out to passage 5 underwent flow cytometry analysis for protein expression of surface markers and transcription factors. Isotype controls are in grey. Expression of each marker or isotype control was analyzed on 100,000 cells.

Effect of cell density and CHIR99021 concentration

To determine the optimal cell seeding density and CHIR99021 concentration, brachyury protein expression at day 0 and day 1 was analyzed by flow cytometry. CHIR99021 is the small molecule inhibitor of GSK3. Cells were plated at 50,000, 100,000, 150,000, and 200,000 cells per cm² in Matrigel-coated 12-well plates. At day 0,

CHIR99021 concentrations of 0, 4, 8, and 12 μM in RPMI1640 medium with B-27 supplement without insulin were added to the cell cultures.

According to statistical analysis by two-way ANOVA, cell density did not have a significant effect on protein expression of brachyury ($p=0.12$). CHIR99021 concentration did have a significant effect of brachyury expression ($p<0.001$). Cells cultured in medium containing 8 μM CHIR99021 had significantly higher brachyury expression than those at day 0 or cultured in 0 μM CHIR99021 or 12 μM CHIR99021, according to Tukey's post-hoc testing. Cells cultured in medium containing 4 μM CHIR99021 had significantly higher brachyury expression than those cultured in medium containing 0 μM CHIR99021 or 12 μM CHIR99021 (Figure 2).

For all further experiments cells were plated at 200,000 cells per cm^2 and a concentration of 8 μM CHIR99021 was used. This cell seeding density is recommended for iPSC undergoing directed cardiac differentiation using small molecules [23].

Figure 2 – Effect of cell seeding density and CHIR99021 concentration on brachyury expression.

Cells were plated on Matrigel-coated tissue culture plates at 50,000, 100,000, 150,000, and 200,000 cells per cm². At day 0 cells were treated with 0-12 μ M CHIR99021 for 24 hours and then analyzed for percentage of cells expressing brachyury using flow cytometry. The percentage of cells expressing brachyury was not significantly effected by cell seeding density, but CHIR99021 concentration did have a significant effect. Graph is mean $\pm$ standard deviation with n=3. * indicates bars are significantly different from each other with $p<0.05$; ** indicates bars are significantly different from each other with $p<0.01$. *** indicates bars are significantly different from each other with $p<0.001$. Statistical significance was determine by two-way ANOVA with posthoc tests.

Cardiac gene expression

Significant changes in gene expression were observed during the differentiation period (Figure 3). Relative GATA4 gene expression was significantly higher in day 15 samples than undifferentiated AFSC or day 0 samples (undifferentiated AFSC: 0.818 ± 0.563 ; day 0: 1.15 ± 0.51 ; day 5: 3.25 ± 1.03 ; day 15: 6.83 ± 4.05). Relative NKX-2.5 gene expression was significantly higher in day 0 and day 5 samples, compared to day 15 samples (undifferentiated AFSC: 0.701 ± 0.175 ; day 0: 0.951 ± 0.244 ; day 5: 0.787 ± 0.346 ; day 15: 0.278 ± 0.211). GATA4 and NKX-2.5 are both cardiac transcription factors. Relative ACTN2 gene expression was significantly higher in day 5 samples compared to all other samples (undifferentiated AFSC: 0.0847 ± 0.0117 ; day 0: 1.20 ± 0.17 ; day 5: 5.80 ± 1.66 ; day 15: 0.684 ± 0.410). ACTN2 is the gene that encodes α -actinin 2, the specific isoform expressed in skeletal and cardiac muscle. Relative TNNT2 gene expression was significantly higher in both day 5 and day 15 samples compared to undifferentiated AFSC and day 0 samples (undifferentiated AFSC: 1.90 ± 0.66 ; day 0: 1.70 ± 0.44 ; day 5: 7.56 ± 0.44 ; day 15: 5.87 ± 1.01). TNNT2 is the gene that encodes cTnT.

Figure 3 – Gene expression in AFSC following differentiation.

Relative gene expression was measured using qRT-PCR. (a) GATA4 gene expression was increased in cells at day 15 of the differentiation compared to undifferentiated AFSC and day 0 cells. (b) NKX-2.5 gene expression was decreased in cells at day 15 of the differentiation compared to cells from days 0 and 5. (c) ACTN2 gene expression was significantly increased in cells at day 5 compared to all other groups. (d) TNNT2 gene expression was increased in both day 5 and 15 groups compared to the undifferentiated AFSC and Day 0 group. N=6 for all groups. Error bars are standard deviation. * indicates bars are significantly different from each other with $p < 0.05$. # indicates that bar is significantly different from all other bars with $p < 0.05$.

Cardiac protein expression

Transcription factors

Cells were analyzed by flow cytometry to assess protein expression of cardiac transcription factors, following the time course for analysis demonstrated previously for iPSC and ESC [22,23]. Islet-1 was analyzed on samples from day 0 and day 8 of the

cardiac differentiation. The percentage of cells expressing Islet-1 significantly increased in the day 8 samples ($p = 0.02$; day 0: $1.41 \pm 0.25\%$; day 8: $10.38 \pm 3.85\%$; Figure 4-a). Nkx-2.5 was analyzed for samples from days 0, 5, and 8 of the cardiac differentiation. Samples from days 5 and 8 contained a significantly larger percentage of cells expressing Nkx-2.5 compared to the samples from day 0, but no significant difference was observed between samples from days 5 and 8 (day 0: $2.75 \pm 1.82\%$; day 5: $24.6 \pm 12.5\%$; day 8: $35.1 \pm 13.4\%$; Figure 4-c). All samples stained for Nkx-2.5 were shifted to the right (higher fluorescence intensity) on the histogram of analyzed cells compared to the secondary only controls, which had a relative fluorescence intensity less than 10^3 . Therefore gating was done based on a day 0 sample. Representative histograms of each day and the gate used are provided in Figure 4-d.

Figure 4 – Cardiac transcription factor proteins in AFSC following differentiation. Following small molecule-based cardiac differentiation for 0, 5, or 8 days, cells were analyzed by flow cytometry for expression of transcription factor proteins. (a) Islet-1 was expressed in a larger percentage of cells at day 8 than at day 0 ($n = 4$ for both groups). (b)

Representative histograms and gate for Islet-1 analysis. (c) Nkx2.5 was strongly expressed in more cells in day 5 and 8 samples, compared to day 0 samples. No significant difference was observed between days 5 and 8. (d) Representative histograms and gates for Nkx2.5 analysis. Bar graphs are means $\pm$ SD. * indicates bars are significantly different from each other with $p<0.05$. N=4 for Islet-1 analysis and n=7 for day 0 and 8 for days 5 and 8 analysis of Nkx2.5 protein expression.

Sarcomeric proteins

Cells were also analyzed by flow cytometry to assess the percentage of cells expressing sarcomeric proteins at days 0 and 15 of the cardiac differentiation (Figure 5). Day 15 samples stained for MLC2, TnI, and SMA showed a bimodal distribution, with a larger population at the lower fluorescence intensity, corresponding to lower amounts of the protein of interest per cell. Though the portion of cells positive for the protein of interest was smaller than the negative portion, cells at day 15 still had a significantly higher percentage of cells expressing MLC2, TnI, and SMA compared to day 0 samples (MLC2: $2.22\pm0.43\%$ vs. $21.4\pm4.7\%$; TnI: $1.46\pm0.78\%$ vs. $15.3\pm3.3\%$; SMA: $1.07\pm0.10\%$ vs. $11.1\pm2.1\%$). While the percentage of cells positive for cTnT was also statistically significantly lower at day 0 compared to day 15 ($0.163\pm0.462\%$ vs. $3.22\pm0.57\%$), the percentage of cells expressing cTnT was still less than 5% at day 15. Additionally, there was not a bimodal distribution observed, as observed in the other markers, so even the positive cells were not strongly positive. A small percentage of cells were double positive for SMA and cTnT, an evaluation used to determine the success of this protocol in pluripotent stem cells [23]. The difference between day 0 and day 15 was statistically significant ($0.0433\pm0.0148\%$ vs. $2.04\pm0.41\%$). The gating was based off of the day 0 samples, which were shifted to higher fluorescence intensities than the isotype or secondary antibody alone controls, indicating some low level expression of the proteins

of interest. All isotype and secondary antibody only controls had relative fluorescence intensities less than 10^3 . Representative histograms and plots, including gating, can be seen in Figure 5-f-j.

Figure 5 – Sarcomeric protein expression in AFSC following differentiation.

Following small molecule-based cardiac differentiation for 0 or 15 days, cells were analyzed by flow cytometry to determine the percentage of cells expressing the sarcomere proteins of interest. (a) MLC2, (b) TnI, and (c) SMA analysis indicated a significant increase in expression of the proteins of interest. Representative histograms (f-h) exhibit a bimodal distribution in cells differentiated for 15 days. (d) cTnT was expressed in significantly more cells at day 15 compared to day 0, but no bimodal

distribution was observed (i). There was also a small percentage of cells that were double positive for cTnT and SMA (e, j). Bar graphs are means $\pm$ SD. * indicates bars are significantly different from each other with $p < 0.05$. N=4 for day 0 and n=6 for day 8.

Protein organization

Immunostaining was performed following the directed differentiation regime to observe the protein organization within cells (Figure 6). Cells were replated prior to immunostaining because cell death occurred during the differentiation protocol and an increased cell density was desired for staining. This increased density was especially relevant to connexin 43 staining, as the protein would not localize to the membranes of cells that were not in contact with other cells. This replating step was following the model previously published for this differentiation protocol in pluripotent stem cells [23]. Cells were cultured for 5 days after being replated to allow time for organization damaged or changed during the trypsinization to be restored. TnI and MLC2 immunostaining revealed a subpopulation of cells that were positively stained for the proteins of interest by with no organization into sarcomeres. SMA immunostaining revealed a strongly stained subpopulation of cells, with most of the positively stained cells exhibiting organization of the actin into filaments. Connexin 43 immunostaining of the differentiated cells revealed connexin 43 preferentially located between cells. While some undifferentiated AFSC displayed connexin 43 expression, no organization to the cell membrane was observed. Controls of secondary antibodies only contained no staining.

Figure 6 – Protein organization in AFSC following differentiation.

Immunostaining for TnI in (a) undifferentiated AFSC, (b) differentiated AFSC, and (c) NRVM. Sarcomere structures are present in NRVM while differentiated AFSC show a population of cells stained for TnI but without any sarcomere organization. Similarly, immunostaining for MLC2 in (d) undifferentiated AFSC, (e) differentiated AFSC, and (f) NRVM show a large portion of NRVM positive stained and with more organization than the AFSC following the differentiation regime. Undifferentiated AFSC are negative for both TnI and MLC2. Immunostaining for SMA shows weak and unorganized staining in (g) undifferentiated AFSC with more filament like organization in (h) differentiated AFSC and a larger proportion of strongly positive cells in (i) NRVM cultures. (j) Undifferentiated AFSC stain for connexin 43 but with no organization while (k) differentiated AFSC and (l) NRVM exhibit localization of connexin43 to cell membranes. Scale bars are 100 μ m.

Discussion

AFSC provide a promising cell source for tissue engineering applications to repair of congenital defects because they can be used autologously after defects are identified *in utero*. Amniotic fluid is routinely collected following detection of a congenital defect with minimal risk [26,27]. AFSC are also well-suited for tissue engineering and regenerative medicine because of their broad differentiation potential and high proliferation rate [6].

Several previous studies have investigated injection of AFSC after a myocardial infarction in rodent models, with positive functional success similar to MSC injection (reviewed in [7]). For a full-thickness, functional patch, as needed to restructure the heart for repair of a congenital defect, mature differentiated AFSC would be required.

This study supports previous studies that indicate AFSC can be induced to express cardiac specific genes and proteins, but lack organized sarcomere structures and spontaneous contraction [8,11]. The chemically defined method studied here provides higher potential to examine the specific roles of the involved factors, compared to co-culture systems dependent on a primary cardiac cell source.

A subpopulation of AFSC expresses brachyury prior to any differentiation protocol, indicating that there is a portion of cells that may already be directed towards a mesoendodermal lineage. This subpopulation warrants further study. If an isolation method were determined to preferentially select these cells, it may be possible to skip the first step of the differentiation and instead proceed directly to second small molecule for inhibition of Wnt processing and signaling.

The undifferentiated AFSC expressed brachyury in 17% of cells while the day 0 cultures in the differentiation experiments expressed brachyury on only approximately 10% of cells. The differences between these two culture conditions are the culture surface (untreated plastic vs. Matrigel) and the cell density, which is 25 to 100 times higher in the differentiation experiments than in expansion culture of AFSC. These changes in culture conditions affected the percentage of cells expressing brachyury. This effect may have been caused by increased proliferation of the brachyury negative cells compared to that of brachyury positive cells under the differentiation set-up conditions.

Results of this study indicated that AFSC can be induced to increase gene expression of GATA4; ACTN2, the skeletal and cardiac muscle form of α -actinin; and TNNT2, the cardiac specific troponin T gene. NKX-2.5 gene expression was decreased on day 15 compared to days 0 and 5, which supports the idea that small molecule modulation of Wnt signaling is not enough to fully commit AFSC to a cardiac lineage. However, based on the data presented here it is not known if Nkx2.5 protein expression does ultimately decrease significantly, corresponding to gene expression. Since ACTN2 gene expression was also decreased at day 15 compared to day 5, it may be possible that the cells were de-differentiating or, alternatively, a population that did not differentiate may have continued to proliferate and dominate the culture.

In iPSC and ESC, it is expected that the directed differentiation protocol tested here will lead to contracting cardiomyocytes at approximately day 12 of the differentiation [23]. In AFSC, no spontaneous contraction was observed at any point in the 15-day differentiation culture. Additionally, previous studies found that more than 90% of pluripotent cells will express Nkx-2.5 and Islet-1 at day 8 of the differentiation

[23]. Cells that express Islet-1 may be cardiovascular progenitor cells, in which case expression of Islet-1 would be expected to decrease after an initial peak, as the cells become more fully differentiated. From AFSC we only observed approximately 10% of cells expressing Islet-1 and less than 40% of cells expressing Nkx-2.5 at the same time point. These results indicated that a much smaller fraction of AFSC were directed towards a cardiovascular progenitor lineage that seen for iPSC or ESC under the same differentiation protocol. However, it may be possible that at the same time point the cells are at a different point in the differentiation process, therefore having lower expression of these transiently expressed proteins.

A fraction of AFSC can be induced to increase protein expression of TnT, SMA, and MLC2 following small molecule-based modulation of canonical Wnt signaling. However, the larger portion of cells were negative for sarcomere proteins. And despite the observed increases in the percentage of cells expressing large amounts of sarcomere proteins TnT and MLC2, no organized sarcomere structures or spontaneous contraction were observed.

Following the attempted differentiation protocol, connexin 43 preferentially located to the cell membranes between cells. This occurrence has been observed following other cardiac differentiation attempts in AFSC [9–11]. Connexin 43, also known as gap junction alpha-1 protein, is a dominant connexin expressed in cardiovascular tissue and is a key component to gap junctions [28]. Gap junctions are necessary for action potential transfer between cells within the heart [29]. One of the shortcomings of current patch materials for congenital heart defect repair is that they are non-conductive, which leads to increased risk of arrhythmias and sudden cardiac death

[30]. Therefore, the preferential location of connexin 43 to the cell membranes, indicating possible formation of gap junctions, indicates that even in this limited differentiated state AFSC may be able to form electrical connections with neighboring cardiac cells.

However, connexin 43 is expressed in many other cell types beyond cardiomyocytes, including cardiac fibroblasts, so expression of connexin 43 alone is not enough to indicate successful cardiac differentiation. And similarly, connection with neighboring cells via gap junctions will not be sufficient to avoid cardiac arrhythmias if the cells are not able to propagate the signal at the same speed as the surrounding cells, but it is an important step in the process.

While a limited cardiac phenotype has been obtained from AFSC following the small molecule-based modulation of Wnt signaling, the cells are immature and not functional. This method of differentiation was chosen based on the impressive results observed in ESC and iPSC [20, 22, 23], but it requires further investigation and refinement to be useful for AFSC in cardiac tissue engineering applications. The goal for using AFSC for treatment of congenital heart defects would be to produce a fully functional tissue engineered patch for implantation, therefore these cells that may be predifferentiated are not sufficient for that purpose. Possible areas for further investigation include addition of demethylation agents, such as 5-aza-2'-deoxycytidine, or biophysical cues. Differentiation of ESC on substrates mimicking cardiac stiffness has been shown to improve the yield of cTnT+ cardiomyocytes compared to both more and less stiff surfaces [31]. Independent application of a demethylating agent to AFSC has been shown to lead to a limited cardiac phenotype [9]. By combining a demethylating

agent, substrate stiffness control, and the directed differentiation protocol tested here it may be possible to obtain a more mature cardiac phenotype from AFSC.

Conclusion

AFSC are a promising cell source for regenerative medicine, particularly for application to congenital heart defect repair. This study represents the first attempt in AFSC to use directed differentiation towards a cardiac lineage by small molecule-based modulation of WNT signaling and demonstrates that the method is not sufficient to generate functional cardiomyocytes from AFSC. Additional signals in the form of mechanical or electrical stimulation may enhance the cardiac differentiation.

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