Human Embryonic Non-haematopoietic SSEA-1+ Cells are Cardiac Progenitors Expressing Markers of Both the First and Second Heart Field

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Abstract

Background: Damage to the myocardium following a myocardial infarction can be severe and possibly non reversible leading to heart failure. However, recent evidence suggests that cardiomyocyte progenitor cells may be able to assist in healing the injured cells. The study purpose is to investigate the use of "Stage Specific Embryonic Antigen 1" (SSEA-1) surface marker to isolate cardiac progenitors from the human embryonic heart.

Materials and methods: The surface marker SSEA-1 was used to isolate cardiac progenitor cells from human embryonic hearts obtained from abortion material.

The fast and reliable Fluorescence Activated Cell Sorting (FACS) technique was used to sort out pure population of SSEA-1+ cells. Cells were then cultured and characterized for multipotent stem cells markers using qRT-PCR and flow cytometry. The cardiopoietic capacity of SSEA-1+ cells was evaluated using a 5-azacytidine differentiation protocol.

Results: Isolated SSEA-1+ cells expressed the pluripotent stem cell transcription factor (Oct4) as well as the multipotent cardiac progenitor cell transcription factors (Nkx2.5, Isl-1 and Tbx5) at the mRNA level, but they did not express the mature cardiomyocyte marker-troponin T (TnT). Furthermore, these cells were found to co-express the mesenchymal stem cells markers (CD105, CD166, CD73, CD59 and CD44), but not the hematopoietic markers (CD45, CD133 and CD34). The cultured SSEA-1+ cells were cardiopoietic and responded to 5-azacytidine treatment by differentiating into cardiomyocytes with high expression of TnT and actin as well demonstrating contractile filaments on transmission electron microscopy.

Conclusions: These data demonstrate that isolated SSEA-1+ from human fetal hearts using the FACS method might be used as a template for the generation of cardiac progenitor cells from other stem cells sources. This knowledge assists in the expansion of our knowledge on the regeneration of damaged myocardium.

Keywords: SSEA-1; Cardiac progenitors cells; Cardiomyocytes; Non-haematopoietic

Introduction

Ischemic heart disease and subsequent heart failure remain the leading cause of death in the Western World. Although post-myocardial infarction survival rate has improved with recent medical advances, reduced heart function attributed to irreversible loss of viable cardiomyocytes is still a major clinical problem. This loss of viable tissue results in the formation of scar tissue, and subsequently in left ventricular remodeling, and progression of congestive heart failure. Medical and interventional therapy can manage the initial stage of heart failure but treatment options for end-stage heart failure patients are limited. This has encouraged development of cardiomyocyte replacement therapies by cell transplantation [1-8] or promotion of endogenous regenerative processes [9].

Earlier clinical trials involving patients with ischemic heart disease and congestive heart failure where bone marrow mononuclear stem cells as well as mesenchymal stem cells were used to improve the left ventricular function, have demonstrated limited effect [3-5,10]. Although these trials are disappointing, an increasing body of evidence has indicated that progenitor cells present in the myocardium may have a better therapeutic potential to repair the myocardium. Blin et al. have focused on stem cells that expressed the surface marker Stage Specific Embryonic Antigen-1 (SSEA-1) which is derived from human

embryonic stem and iPS cells [11]. These cells have been shown to be multipotent and able to differentiate into cardiomyocytes, smooth muscle cells and endothelium. Another advantage using the SSEA-1 sorting strategy would be enrichment of cells that can give rise to both the right and left ventricle. So far, SSEA-1+ cells have only been characterized from human embryonic stem cells, iPS cells and primate pluripotent stem cells [11], but if these cells represent the same cell type present in the human embryonic heart has not been clarified.

In this study we isolate SSEA-1+ cells from the human embryonic hearts and demonstrate that these cells express pluripotency markers

as well as early cardiomyocyte markers and mesenchymal stem cells phenotypic markers. After *in vitro* differentiation, the SSEA-1+ cells differentiate into cardiomyocytes expressing TnT. This study supports that the surface marker SSEA-1 can be used for enrichment of cardiac progenitors that can be used for cardiomyoplasty.

Materials and Methods

Cell culture

Three human embryonic hearts (9-11 gestational weeks) were obtained after voluntary abortion. Approval for these studies was obtained from the Karolinska University Hospital´s institutional review board. Informed consent was obtained from the volunteers in accordance with the Declaration of Helsinki.

Isolation of the cells was performed as described in earlier work by Smits et al. and Elsheikh et al. [12,13] Briefly, hearts were dissociated into single cell suspension by several rounds of digestion in collagenase enzyme. The cells were then frozen using the STEM-CELLBANKER freezing medium (Nippon Zenyaku Kogyo Co., Ltd. Japan).

Anti-SSEA-1 antibodies were used to enrich the SSEA-1+ population from the frozen cell by fluorescence-activated cell sorting. Frozen cells were washed twice with Cell wash and recovery solution (CELLOTION, Nippon Zenyaku Kogyo Co., Ltd. Japan) and filtered on 70 μm filters. Then cells were distributed into two tubes. Twenty microliters of FITC conjugated anti-SSEA-1 (Becton and Dickinson, San Jose, CA, USA) was added to one tube and incubated for 30 minutes on ice. For the viable cell isolation, SYTOX® Blue staining was also used. The other tube was left unstained. Positive sorting gates were set according to unstained controls and performed using FACSAria (Becton and Dickinson). The SSEA-1 isolated cells were plated into 0.1% gelatin coated flask in M199 (Gibco, invitrogen, Carlsbad, CA, USA)/EBM-2 (3:1) medium supplemented with EGM-2 Single Quots (vascular endothelial growth factor, basic fibroblast growth factor, epithelial growth factor and insulin-like growth factor, hydrocortisone, fetal bovine serum, ascorbic acid, heparin, gentamicin) (Clonetics, Lonza, Basel Switzerland).

To induce cell differentiation, we used 5-azacytidine, the most common *in vitro* differentiation model [14-16]. Cells were treated with 5 μM 5-azacytidine for the initial 72 h in a differentiation medium (Iscove's modified Dulbecco's medium/Hams F12 (1:1) (Gibco)) which was supplemented with L-glutamine (Gibco), 2% horse serum, nonessential amino acids, insulin-transferrin-selenium supplement, and 10-4 M ascorbic acid (Sigma) . After treatment, the medium was changed every 3 days. To determine the morphological changes in SSEA-1+ cells induced by 5-azacytidine treatment, phase-contrast photography was performed.

Phenotyping of SSEA-1+ cells

The SSEA-1+ cells were characterized for their mesenchymal stromal cell, endothelial progenitor cell and hematopoietic stem cell profiles by flow cytometry. The panels included fluorochrome conjugated mAbs against the following surface antigens: CD166 (PE), CD90 (PE-Cy5), CD73 (PE-Cy7), CD59 (APC), CD44 (APC-Cy7), CD14 (V500), and dump cannel (Pacific Blue/V450, CD31, CD45, CD34, CD11b and HLA-DR), CD105 (APC), C-Kit (APC) purchased from BD Biosciences, San Jose, CA, USA; CD133(PE) from Miltenyi Biotec GmbH, BergischGladbach, Germany; KDR (FITC)from R&D Systems, Minneapolis, MN, USA. Data were analyzed with FACSDiva (BD), and FlowJo.

Gene expression

Total RNA was isolated from SSEA-1+ cells by using the Pico Pure RNA extraction kit. cDNA was synthesized with the High Capacity cDNA Reverse Transcription kit, both from Applied Biosystems, according to the manufacturer's instructions. During the RNA extraction process the samples were treated with DNase (Qiagen) to remove contaminating genomic DNA.

Real-time quantification of cDNA was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). TaqMan Gene Expression Assays for human GATA4, Nkx2.5, Tbx5, Troponin T (TnnT2), Islet 1 (Isl-1) and GAPDH (Applied Biosystems) were used for quantification of gene expression. PCR reactions were performed in duplicates in 96-well optical plates. In this study we used the comparative ΔCt method to calculate the ratio between the gene of interest and the endogenous control GAPDH in the same sample.

Transmission Electron Microscopy (TEM)

SSEA-1+ cells were grown on membrane filters on 24-well plates. After fixation in 2% glutaraldehyde, the membranes were cut free, fixed for 1 hour at 4°C in a buffer containing 0.15 mol/L sodium cacodylate, 1% osmium tetraoxide, and 3 mmol/L $CaCl_2$, pH 7.4. Subsequently, the wells were rinsed briefly, in 0.15 mol/L sodium cacodylate buffer, dehydrated in ethanol as described above, and imbedded in Spurr resin (Agar Scientific Ltd., Essex, UK). The sections were contrasted with uranyl acetate followed by lead citrate, and examined at 80 kV in a Leo 906 (Oberkochen, Germany) transmission electron microscope.

Cytosolic Ca^{2+} imaging

To investigate the effect of IGF-1 on intracellular Ca^{2+} dynamics, SSEA-1+ cells were incubated with the Ca^{2+}-sensitive dye Fluo-4 and time-lapse Ca^{2+} imaging was performed.

Cells were loaded with the Ca^{2+}-sensitive fluorescence indicator Fluo-4/AM (5 μM, Molecular-Probes) in cell culture medium at 37°C for 30 min. Dynamic Ca^{2+} imaging was conducted at 37°C in a heat-controlled chamber (Warner Instruments) with a cooled back-illuminated EMCCD camera Cascade II:512 (Photometrics) mounted on an up-right microscope Axiovert 100 M (Carl Zeiss) equipped with a LCI Plan-Neofluar 25×/0.8NA water immersion lens (Carl Zeiss). Excitation at 480 nm took place using a Lambda LS xenon-arc lamp (Sutter Instrument) equipped with a Lambda 10-3 filter-wheel (Sutter Instrument) and a SmartShutter (Sutter Instrument). Emission wavelengths were detected at 510 nm, and the sampling frequency was set to 0.5 Hz. MetaFluor software (Molecular Devices) was used to control all devices and to analyze the acquired images. The experiments were performed in Krebs-Ringer's buffer containing 119.0 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 20.0 mM Hepes (pH 7.4). IGF-1 was bath-applied at a final concentration of 10 nM.

Results

Cardiac SSEA-1+ cells expressing pluripotency genes as well as early cardiomyocyte transcription factors

After the SSEA-1+ cells were sorted by flow cytometry from frozen human fetal hearts (n=3), a yield of 1 to 2% SSEA-1+ cells per heart was generated. The cultured cells were spindle-shaped and demonstrated high growth potential (Figure 1A). The SSEA-1 expression was kept unaltered between each passage and after five passages 70% of the cells

still expressed SSEA-1 (n=3) (Figure 1B). The SSEA-1+ cells expressed markers of pluripotency (Oct4) as well as early cardiomyocyte progenitors (Isl-1, Nkx2.5, Tbx5 and GATA4) but not markers representative of mature cardiomyocytes (TnT). The cardiac SSEA-1+ cells appeared to originate from the cardiac mesenchymal stem cell characterized by expression of CD105, CD166, CD73, CD59 and CD44 while at the same time being negative for the hematopoietic stem cell markers CD45, CD34 and CD133 (Figure 2 and Table 1).

Figure 1: (A) Phase contrast images of SSEA-1+ Cells. (B) Expression of SSEA-1 in isolated cells at different passages. (C and D) RT-PCR analysis of cardiomyocyte-specific genes in RNA isolated from SSEA-1+ cells. Results were calculated as the ratio between the gene of interest and the endogenous control GAPDH in the same sample n=3.
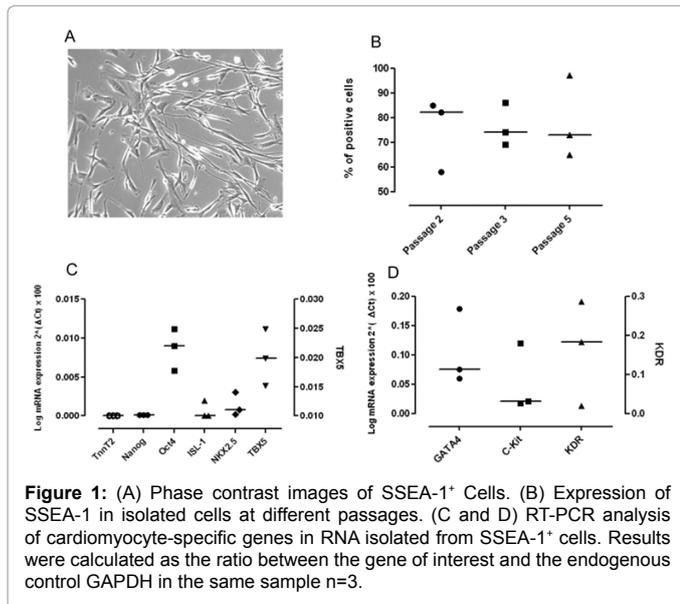
Ca2+

In control, unstimulated conditions, undifferentiated SSEA-1+ cells exhibited stable Ca^{2+} levels, without any evidence of spontaneous Ca^{2+} activity either in the presence or absence of extracellular Ca^{2+} (Figures 3A and 3B, respectively). Notably, when the cells were exposed to IGF-1 (10 nM) in the presence of extracellular Ca^{2+}, a clear intracellular Ca^{2+} response was observed within 5-15 minutes after IGF-1 addition (Figures 3C and 3D). This response was characterized as Ca^{2+} oscillations. Spectral analysis of the IGF-1 induced Ca^{2+} oscillations demonstrated an average periodicity of 7.0 ± 1.1 mins and a mean frequency of 2.40 ± 0.37 mHz (Figure 3E). Interestingly, when the cells were challenged with the same concentration of IGF-1 in the absence of external Ca^{2+}, the oscillatory Ca^{2+} response was not observed (Figure 3F), suggesting that IGF-1 activates extracellular Ca^{2+} influxes in human SSEA-1+ cells.

In vitro differentiation of cardiac SSEA-1+ Cells

As shown in Figure 4, after three weeks of the differentiation protocol SSEA-1+ cells show an increase in cell size with no further proliferation, while cells grown in the differentiation medium (without 5-azacytidine) showed cobblestone-like morphology with a slight increase in cell size and proliferation . In contrast, cells in the growth medium showed spindled shaped morphology and became confluent.

After examing the gene expression of cardiac transcription factors and cardiac structural proteins in SSEA-1+ cells, we found that the cardiac structural proteins, Troponin T, was highly induced after differentiation regardless of the addition of 5-azacytidine (Figure 5). Yet, this protocol did not affect the expression of multipotent cardiac progenitor cells transcription factors (Figure 5).

Further analysis by electron microscopy confirmed the cardiac

Figure 2: Immunophenotype of SSEA-1+ cells. SSEA-1+ cells express CD166, CD73, CD59 and CD44 while at the same time not express haemotopoietic markers (CD14, CD45 and CD34).
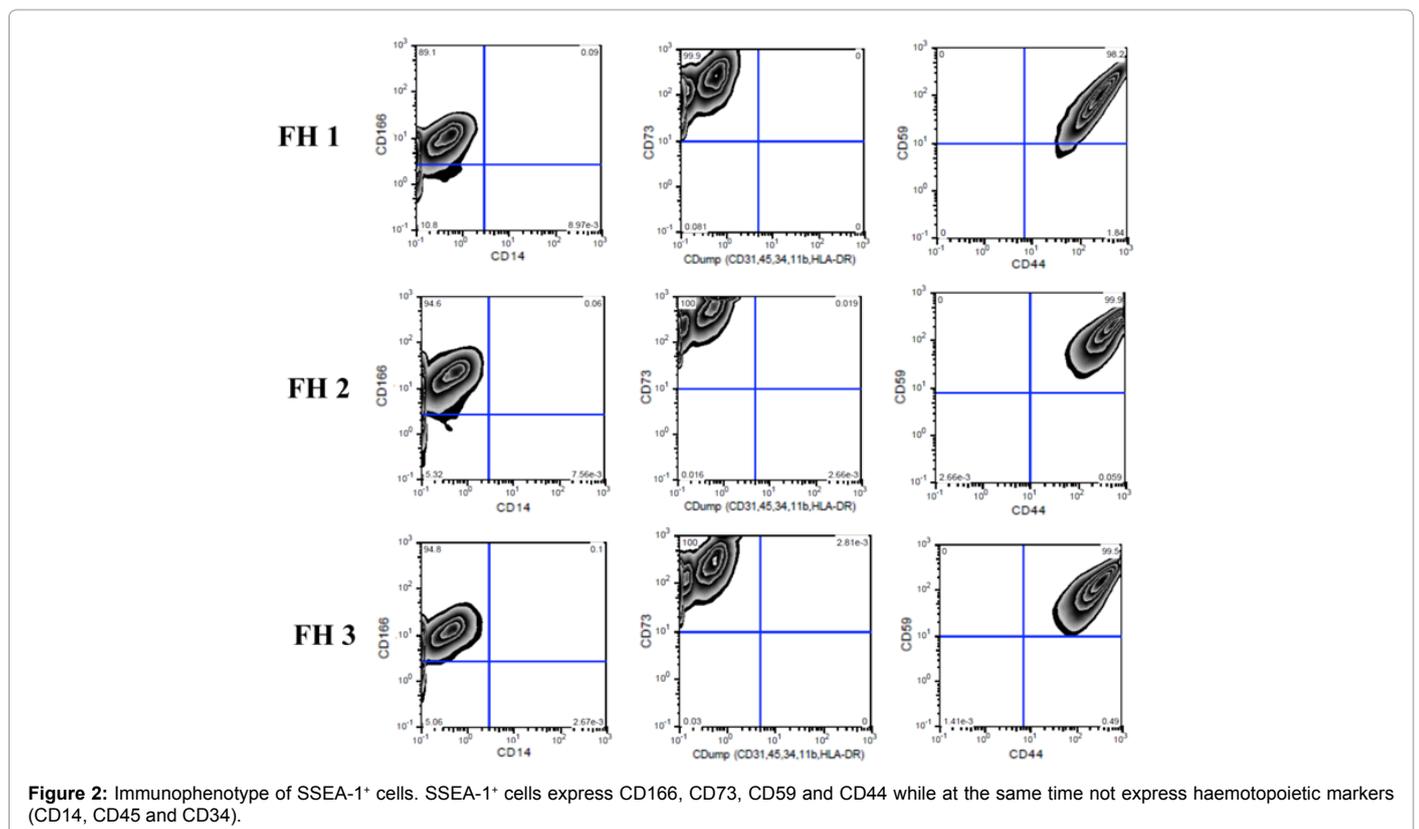
morphology of the differentiated SSEA-1+ cells (Figures 6A and 6B). Differentiated SSEA-1+ cells showed large areas of actin organized along the cell membrane as functional unit. There were no sarcomeres or a clear increase in mitochondrial density. These cells, thus, have been "pushed" towards contractile cells. While SSEA-1+ cells in growth medium showed a few scattered areas of actin with microtubuli and thus fibroblast like and no indication to be differentiated to mature cardiac cells (Figures 6C and 6D).

Discussion

In this study, we for the first time show that human embryonic SSEA-1+ cells represent early cardiac progenitors, which in vitro can be

Surface Marker	% of positive cells		
	FH 1	FH 2	FH 3
CD105	99	93	98
CD133-1	< 1	< 1	< 1
CD133-2	< 1	< 1	< 1
KDR	2	< 1	< 1
C-Kit	2	< 1	27

Table 1: Phenotype of SSEA-1+ cells.

differentiated into cardiomyocytes expressing TnT. Other studies have found that SSEA-1+ cells isolated from human embryonic stem cells and iPS cells were multipotent but not whether they could be used to isolate cardiac progenitors from the human embryonic heart [11].

In addition, these cells appear to represent a novel cell population of mesenchymal stem cells since they co-express CD105, CD166, CD73, CD59 and CD44. This hypothesis is supported by Anjos-Afonso and Bonnet [17] who proposed that SSEA-1+ cells are the most primitive mesenchymal subset described so far. They showed that SSEA-1+ mesenchymal cells gave rise to SSEA-1- mesenchymal cells, whereas the reverse could not be observed. Also, these SSEA-1+ cells have a much higher capacity to differentiate than their negative counterparts, not only to several mesenchymal cell types but also to unconventional cell types such as astrocyte-, endothelial-, and hepatocyte-like cells in vitro, and capable of differentiating into different mesenchymal cell types in vivo [17]. Furthermore, our findings support that human embryonic SSEA-1+ cells are cardiogenic and might be a template for generation of cardiac stem cells for cardiomyoplasty and are distinct from hematopoietic stem cells (based on CD45-, CD34-) and endothelial progenitor cells (based on CD45-, CD34-, CD133-, KDR-).

Figure 3: IGF-1 induces intracellular Ca2+ oscillations in SSEA-1+ cardiac stem cells. Human SSEA-1+ cells were loaded with Fluo-4 and dynamic Ca2+ measurements were performed using fluorescence Ca2+ imaging. (A) Representative basal Ca2+ levels in SSEA-1+ cells maintained in Ca2+-containing recording medium (n=28, from 3 independent cultures). (B) Representative basal Ca2+ level in SSEA-1+ cells maintained in Ca2+-free recording medium (n=32, from 3 independent culture). (C) Sequence of pseudocolor fluorescence images of a Fluo-4-preloaded SSEA-1+ cell stimulated in the presence of extracellular Ca2+ with IGF-1 10 nM as indicated. Representative Ca2+ oscillations are presented. Time and fluorescence intensity scales as shown. (D) Relative Ca2+ levels in SSEA-1+ cells stimulated with IGF-1 10 nM in a Ca2+-containing recording medium (n=36, from 3 independent cultures). (E) Spectral analysis of Ca2+ oscillations induced by IGF-1 in SSEA-1+ cells. The analysis was performed into a previously described spectral analysis software tool implemented in MATLAB, to characterize the temporal properties of Ca2+ oscillations. Average frequency obtained was 2.40 ± 0.37 mHz, which represent a periodicity 7.0 ± 1.1 minutes. (n=16, from 3 independent cultures). (F) Relative Ca2+ levels in SSEA-1+ cells stimulated with IGF-1 10 nM in a Ca2+-free recording medium (n=25, from 3 independent cultures).

Our derived SSEA-1+ cells expressed a number of cardiomyocyte-specific genes including Isl-1, Nkx2.5, Tbx5 and GATA4 as well as the pluripotent stem cell transcription factor (Oct4). They also expressed Ca channels, which were activated by IGF-1. The activation of the IP3-pathway was important for expansion of cardiac progenitor cells and for their differentiation [18]. These findings further support our hypothesis that SSEA-1+ cells are early cardiac progenitor cells.

In this study we also demonstrated that differentiated SSEA-1+ cells continue to express Isl-1, Nkx2.5, Tbx5 and GATA4. These results indicate that the stage of differentiation of the SSEA-1+ cell is between cardiomyocyte progenitor and differentiated cardiomyocytes.
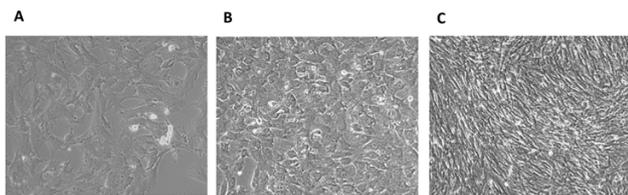
Figure 4: Phase-contrast photographs of SSEA-1+ cells with/without 5-azacytidine treatment after three weeks. (A) 5-azacytidine treated SSEA-1+ cells show an increase in size with no further proliferation. (B). Cells grow in differentiation medium only show cobblestone-like morphology with a slight increase in cell size and proliferation. (C) In the growth medium cells show spindle shape morphology and become confluent.

Furthermore, we found a similar result in the differentiation protocol with/without addition of 5-azacytidine, and this could be due to the low serum concentration in the culture medium (2%), which directly modulate serum response factor, a key cardiac transcription factor [19,20].

The fact that the SSEA-1+ cells can be easily be FACS sorted without changing their cardiopoietic characteristics is important. This sorting may allow for the development of a Good manufacturing practice (GMP) approved protocol that would be able to generate a large enough number of cells for cardiomyoplasty. Furthermore, the identification of the SSEA-1+ population that contains cardiovascular progenitors provides a unique opportunity to help investigators better understand cardiac embryogenesis, mechanisms that regulate the onset of human cardiac development as well as control specification to the cardiac lineage. This information will allow investigators to develop treatments for heart failure that develops as a result of damaged myocardium from a myocardial infarction or from an idiopathic cause.

In summary, we demonstrate that the early human embryonic heart is a source of optimal heart progenitor cells and that the SSEA-1+ cells obtained from the embryonic heart are cardiopoietic. Since SSEA-1 is a surface marker, these cells can easily be FACS sorted without changing their cardiopoietic characteristics and can serve as a template for generating progenitor cells from other sources so that in the future these cells can be used as a therapeutic alternative treatment for heart failure. Further research is needed to substantiate our findings.
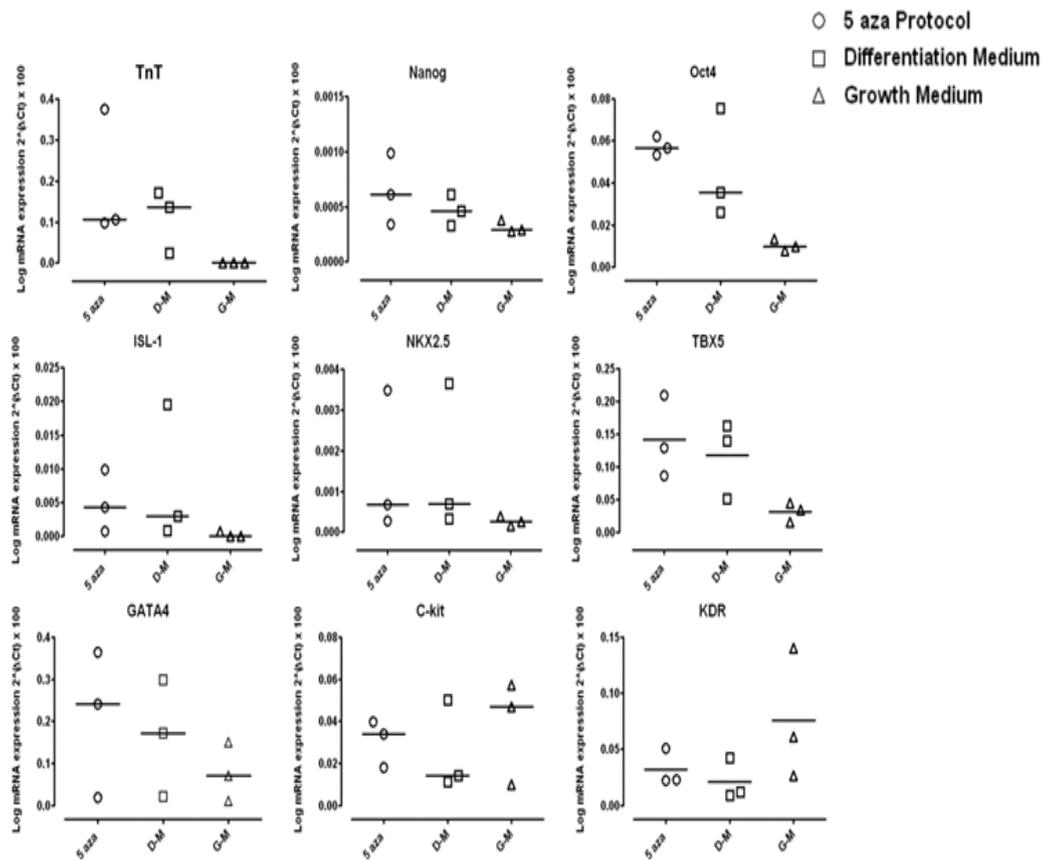
Figure 5: RT-PCR analysis of cardiomyocyte-specific genes in RNA isolated from SSEA-1+ cells with/without 5-azacytidine treatment after three weeks. Results were calculated as the ratio between the gene of interest and the endogenous control GAPDH in the same sample. n=3.
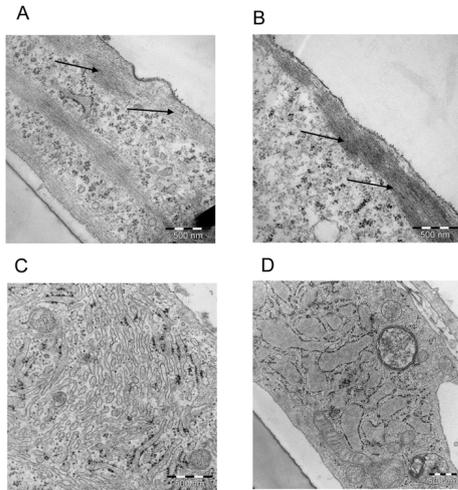
Figure 6: Representative transmission electron micrograph of SSEA-1⁺ cells after differentiation protocol. (A and B) Differentiated SSEA-1⁺ cells revealed large areas of actin organized along the cell membrane as functional unit (arrows). (C and D) SSEA-1⁺ cells in growth medium.

Acknowledgements

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