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Embryonic stem cell-derived cardiomyocytes harbor a subpopulation of niche-forming Sca-1$^+$ progenitor cells

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Abstract The adult mammalian heart is known to contain a population of cardiac progenitor cells. It has not been unambiguously determined, however, whether these cells form as part of the developmental program of the heart or migrate there by way of the circulatory system. This study was done in order to determine the origin of this population of cells. A population of cardiomyocytes was established from mouse embryonic stem (ES) cells using a genetic selection technique. In order to determine whether cardiac progenitor cells exist within this ES cell-derived cardiomyocyte population, the cells were analyzed by fluorescence activated cell sorting (FACS) using an antibody directed against stem cell antigen-1 (Sca-1). We observed that approximately 4% of the cardiomyocyte population was composed of Sca-1$^+$ cells. When the Sca-1$^+$ cells were isolated by magnetic cell sorting and differentiated as cellular aggregates, contractions were observed in 100% of the aggregates. Gene expression studies using quantitative RT-PCR showed that these cells expressed terminally differentiated cardiac-specific genes. When three-dimensional cellular aggregates were formed from ES cell-derived cardiomyocytes co-cultured with adult HL-1 cardiomyocytes, the Sca-1$^+$ cells were found to "sort out" and form niches within the cell aggregates. Our data demonstrate that cardiac progenitor cells in the adult heart originate as part of the developmental program of the heart and that Sca-1$^+$ progenitor cells can provide an important in vitro model system to study the formation of cellular niches in the heart.

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Introduction

It is well established that the majority of cardiomyocytes permanently withdraw from the cell cycle shortly after birth [1–4]. This limited capability for regeneration leaves the heart with virtually no way to repair itself following myocardial damage, contributing to statistics that consistently identify cardiovascular disease as the leading cause of death worldwide [5].

Recent reports have described the existence of progenitor cells in the adult heart which have been shown to be able to mature into smooth muscle, cardiac muscle and endothelial cells [6–11]. In the heart, progenitor cells have been shown to reside within niches and have been identified by their expression of cell surface markers such as c-Kit or stem cell antigen-1 (Sca-1) [12–15]. It has not been unambiguously established, however, whether these progenitor cells originate in the heart, or may have migrated there by way of the circulatory system. One way to eliminate this ambiguity would be to demonstrate the presence of progenitor cells within a population of cardiomyocytes derived from embryonic stem (ES) cells.

We report here the isolation of a population of purified cardiomyocytes from mouse embryonic stem cells, using a modified version of an antibiotic selection technique first described by Field [16]. Within these cardiomyocytes, we have identified a Sca-1$^+$ subpopulation. Using a magnetic cell sorting system, we isolated Sca-1$^+$ cells from the ES cell-derived cardiomyocytes and demonstrate that when grown as three-dimensional aggregates, these cardiac

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progenitor cells differentiated into contracting cardiomyocytes. Gene expression analyses by qRT-PCR also showed that cardiomyocytes differentiated from the Sca-1^+ cells express genes that are normally detected only in adult terminally differentiated cardiac muscle cells. We demonstrate that the Sca-1^+ cell population in the adult heart originate as part of the developmental program of the heart. We further demonstrate that the Sca-1^+ cells will self-organize into a cellular niche when co-cultured with adult cardiomyocytes in three-dimensional cellular aggregates.

Materials and methods

Generation of ES cell-derived cardiomyocytes

A genetic selection technique [16] was used to isolate cardiomyocytes from ES cells. A 4.5 kb α-MHC promoter (a gift from Jeffrey Robbins [17]) driving the neomycin resistance gene was subcloned into the pcDNA3.1/Hygro vector (Invitrogen, Carlsbad, CA). Undifferentiated mouse J1 ES cells (a gift from Jaenisch [18]) were used as the cell source. Briefly, transfected ES cells were selected for 8 days with Hygromycin B (200 μl/ml) before being differentiated as embryoid bodies (EBs) in suspension culture [19]. After 5 days in suspension, the EBs were plated onto gelatin-coated tissue culture dishes. G418 selection was started after contracting areas were observed in the EBs. To ensure that the antibiotic would reach every cell in the multi-layered EBs, we added G418 (200 μg/ml) to our differentiating ES cells as monolayer cultures rather than as whole EBs.

Culture of HL-1 cardiomyocytes as cellular aggregates

HL-1 cardiomyocytes were maintained in Claycomb Medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum, L-glutamine (2 mM), and norepinephrine (0.1 mM) as previously described [20, 21]. Three-dimensional cellular aggregates were created by the formation of hanging drops [22]. Each hanging drop was composed of 500 cardiomyocytes in 25 μl differentiation medium, which consists of DMEM (high glucose), 0.1 mM MEM non-essential amino acids solution, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10% fetal bovine serum, and 50 μM β-mercaptoethanol. The hanging drops were transferred onto the uncoated bottom of an ultralow-adhesion tissue culture dish. The dish was covered, inverted, and placed into a humidified CO_2 incubator for 4 days, after which it was turned right side up, and 15 ml of differentiation medium was carefully added to the uniform-sized cellular aggregates. The clusters were then plated onto 0.1% gelatin-coated glass cover slips and maintained until they were analyzed by immunohistochemistry.

Creation of ES cell-derived cardiomyocyte aggregates and ES cell-derived cardiomyocyte/HL-1 aggregates

The hanging drop technique was used to create cellular aggregates from ES cell-derived cardiomyocytes (500 cells per 25 μl hanging drop of differentiation medium). Since HL-1 cells are maintained in supplemented Claycomb Medium while ES cell-derived cardiomyocytes are cultured in differentiation medium; preliminary studies were conducted to determine the optimal culture medium to use in growing ES cell-derived cardiomyocyte/HL-1 aggregates. ES cell-derived cardiomyocytes and HL-1 cells also proliferate at different rates, prompting us to carry out additional preliminary experiments to determine the number of each cell type to use in the creation of co-cultured aggregates. The optimal combination for each hanging drop was determined to be 125 ES cell-derived cardiomyocytes and 375 HL-1 cardiomyocytes per 25 μl differentiation medium.

Immunohistochemical staining of ES cell-derived cardiomyocytes

For immunohistochemical analyses, 1.5×10^5 ES cell-derived cardiomyocytes were plated on 0.1% gelatin-coated 15 mm glass coverslips in differentiation medium for 3 days before fixing in 4% paraformaldehyde (Thermo Fisher Scientific, Waltham, MA). Immunostaining was performed for 1 h at room temperature using cardiac troponin T, titin and sarcomeric myosin (MF-20) antibodies (all from University of Iowa Developmental Studies Hybridoma Bank, Iowa City, IA). Each sample was then incubated for 45 min at room temperature with the appropriate secondary antibody. Cells stained with cardiac troponin T and MF-20 were incubated with an Alexa fluor 568 goat anti-mouse IgG (H+L) antibody and those stained with titin were incubated with an Alexa fluor 568 goat anti-mouse IgM antibody (both from Invitrogen). All samples were counter-stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) to label cell nuclei. The samples were visualized with a Nikon MICROPHOT-SA microscope. A CCD camera was used to acquire images, which were analyzed with MetaMorph software (Molecular Devices, Sunnyvale, CA).

Immunohistochemical staining of cellular aggregates

Cellular aggregates were cultured on 0.1% gelatin-coated 15 mm glass coverslips in differentiation medium for 9 days before fixing in 4% paraformaldehyde. Immunostaining was

performed using both mouse monoclonal anti-SV40 large T antigen antibody (anti-T Ag; EMD Chemicals, Gibbstown, NJ) and anti-Sca-1 antibody (Becton, Dickinson Biosciences, Franklin Lakes, NJ) in 1% normal goat serum for 1 h at room temperature. Preparations were then incubated for 45 min at room temperature with Alexa fluor 568 anti-mouse and Alexa fluor 488 anti-rat secondary antibodies (both from Invitrogen). Nuclei were counterstained with DAPI. Confocal images were acquired using a Leica DM IRE2 inverted epifluorescent microscope (Leica, Heerbrugg, Switzerland).

Fluorescence activated cell sorting (FACS)

ES cell-derived cardiomyocytes were dissociated enzymatically with trypsin–EDTA and the resulting cells were resuspended in a buffer consisting of 0.5% BSA and 4 mM EDTA in PBS to prevent cell clumping. To further remove cell clumps, the cell suspensions were passed through a 30 μm nylon mesh filter (Miltenyi Biotec, Auburn, CA) before incubation with a Sca-1 antibody (Miltenyi Biotec) according to the manufacturer's protocol. The samples were then analyzed by flow cytometry using a FACS Calibur flow cytometer (BD Biosciences) to determine the percentage of Sca-1^+ cells within each cell sample.

Isolation of Sca-1^+ cells

Sca-1^+ cells were isolated from ES cell-derived cardiomyocytes using a Magnetic Cell Sorting System (Miltenyi Biotech), following the manufacturer's protocol, with slight modification. Briefly, ES cell-derived cardiomyocytes cultured as a monolayer were collected for separation. The cells were resuspended in Separation Buffer, which consisted of 0.5% BSA, 4 mM EDTA in PBS. The cells were passed through a pre-separation filter (Miltenyi Biotec) to remove cell clumps before being incubated with a Sca-1 antibody and magnetic microbeads according to the manufacturer's protocol. Separation was performed utilizing an AutoMACS Separator (Miltenyi Biotec) using the possel_d2 software program. To increase the purity of the Sca-1^+ fraction, these cells were processed through the magnetic sorting program twice. Sorted populations of cells were analyzed by FACS to determine the purity of the Sca-1^+ cells.

Differentiation of Sca-1^+ cells as cellular aggregates

To demonstrate the potential of the putative Sca-1 progenitor cells to differentiate into cardiomyocytes, Sca-1^+ cells were cultured for 9 days as cellular aggregates using the hanging drop technique (500 cells per 25 μl hanging drop of differentiation medium). Quantitative RT-PCR was performed to determine the expression levels of genes that code for cardiac-specific transcription factors and structural proteins. The amplified products were separated on 1.5% agarose gels, and images were captured and analyzed using the Quantity One imaging and analysis software from Bio-Rad.

Quantitative RT-PCR

Total RNA was isolated using the RNeasy RNA Isolation kit (Qiagen, Valencia, CA) according to the manufacturer's protocol, including the optional DNase digestion step. Five microgram of total RNA was reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Primers (Table 1) were designed using the Primer3 software (Whitehead Institute/MIT Center for Genome Research, Cambridge, MA) and purchased from Integrated DNA Technologies (Coraville, IA). For each primer, we conducted a BLASTN search against the GenBank database to confirm the specificity of each oligonucleotide.

PCR reactions were performed in a 25 μl reaction volume using the Cepheid Smart Cycler (Sunnyvale, CA). Each final reaction contained 1× SYBR Green Jump Start Taq Ready Mix (Sigma-Aldrich) and 25 ng cDNA. PCR reaction conditions included an initial stage of 2 min at 95°C, followed by 40 PCR cycles, each with a three-stage temperature cycle. Each three-stage cycle consisted of 95°C for 15 s, 56–60°C for 60 s depending on the primer set (Table 1) and 72°C for 60 s. PCR amplification was followed with melt curve analysis to ensure the purity of the products generated.

Results

Immunohistochemical staining of ES cell-derived cardiomyocytes

Using a genetic selection technique, we isolated a population of cardiomyocytes from mouse ES cells. When cultured as cellular aggregates, these cardiomyocytes differentiate into contracting cardiac muscle cells expressing cardiac muscle-specific sarcomeric proteins (Figs. 1, 3). These ES cell-derived cardiomyocytes were examined with antibodies directed against cardiac troponin T, titin, and sarcomeric myosin heavy chain (MF-20) (Fig. 1). We observed the cells to be both mononucleated and binucleated and to exhibit strong immunoreactivity against all three sarcomeric antibodies (Fig. 1). Occasionally, however, we observed cells that did not express these sarcomeric proteins (arrows in Fig. 1a, c). This observation led us to investigate whether these cells possibly represented a

Table 1 PCR primer pair sequences, accession numbers, annealing temperatures, and PCR product sizes of cardiac-specific genes

Primer	Accession number	Annealing temperature (°C)	Product size
GATA4	U85046	58	161
5′-AGAAGGCAGAGAGTGTGTCAAT-3′			
5′-GCCGTTCATCTTGTGATAGAG-3′			
MEF2C	L13171	60	151
5′-CCCCTTCGAGATACCCACAA-3			
5′-CCATTGGACTCACCAGACCTTC-3′			
Myocardin	AF384055	58	124
5′-TCCAACTGAATTCCATGACC-3′			
5′-GGCTTGGAGAATGTGCATAG-3′			
Nkx2.5	NM 008700.2	58	141
5′-TTAGGAGAAGGGCGATGACT-3′			
5′-AGGTCCGAGACACCAGGCTA-3′			
a-Cardiac actin	M15501	58	104
5′-TCTGAGATGTCTCTCTCTTAGCCTAC-3′			
5′-CGTACAATGACTGATGAGAGATG-3′			
a-MHC	NM_010856.2	56	291
5′-GAAGATGCACGACGAGGAAT-3′			
5′-CGAACGTTTATGTTTATTGTGGA-3′			
Cardiac troponin I	NM_009406.2	58	225
5′-TCAGTTGAAGACTCTGATGCTG-3′			
5′-ATGTTCTTGGTGACTTTTGCTT-3′			
MLC2a	NM_022879.1	58	150
5′-CTCGGGAGGGTAAGTGTTCC-3′			
5′-CATGCGGAAGGCACTCAG-3′			
MLC2v	NM 010861.2	59	499
5′-GCCAAGAAGCGGATAGAAGG-3′			
5′-CTGTGGTTCAGGGCTCAGTC-3′			

Forward and reverse cardiac-specific primer pairs, designed using Primer3 software, are listed along with their corresponding annealing temperatures. *GATA-4* cardiac transcription factor GATA-4, *MEF2C* myocyte-specific enhancer factor 2, *Nkx2.5* NK2 transcription factor related, locus 5, *α-MHC* cardiac muscle myosin heavy chain, *MLC2a* cardiac myosin light chain 2a, *MLC2v* cardiac myosin light chain 2v

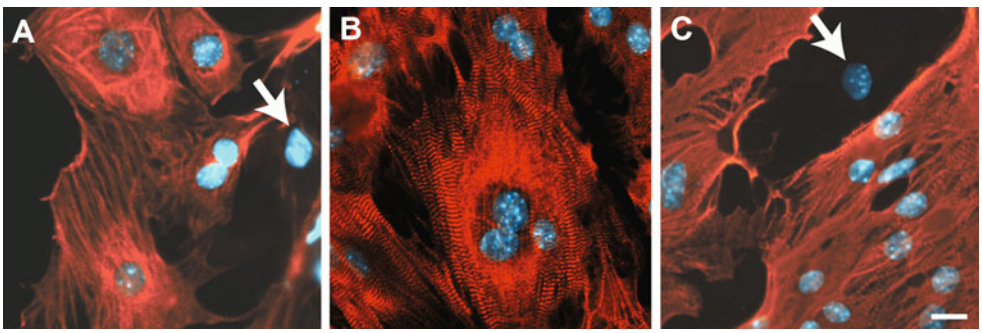

Fig. 1 Immunohistochemical analysis of ES cell-derived cardiomyocytes. ES cell-derived cardiomyocytes were analyzed for expression of **a** cardiac troponin T, **b** titin, and **c** sarcomeric myosin (MF-20). The *arrows* in (**a**) and (**c**) point to cells that are not immunoreactive to cardiac troponin T and MF-20, respectively. Bar, 10 μm

subpopulation of cardiac progenitor cells that exist within the ES cell-derived cardiomyocyte population.

To determine whether the undifferentiated cells we identified in our ES cell-derived cardiomyocyte population might possibly represent progenitor cells, we analyzed them by FACS for the presence of Sca-1 (Fig. 2) and observed that about 4% of the cells expressed Sca-1.

Differentiation of Sca-1-positive cells into cardiomyocytes

To demonstrate that the Sca-1$^+$ cells are cardiac progenitor cells, they were isolated using a magnetic cell sorting system and cultured for 9 days as cellular aggregates. Gene expression patterns of cardiac-specific transcription factors

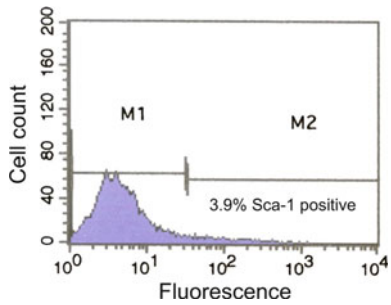

Fig. 2 Sca-1^+ cells are present within the ES cell-derived cardiomyocyte population. ES cell-derived cardiomyocytes labeled with FITC-conjugated anti-Sca-1 antibody were analyzed by FACS. This one-parameter histogram shows that about 4% of the ES cell-derived cardiomyocytes are Sca-1-positive. The area under the M1 portion of the graph shows Sca-1^- cells while the area under M2 shows Sca-1^+ cells

and structural genes were determined by RT-PCR. The expression level of each cardiac gene in cellular aggregates formed from Sca-1^+ cells was compared to the level in freshly isolated Sca-1^+ cells (Fig. 3). Following differentiation, a decrease was observed in the levels of the Sca-1 transcripts (Fig. 3). This was accompanied by an increase in genes associated with the adult terminally differentiated cardiomyocyte, such as GATA4, MEF2c, myocardin, Nkx2.5, α-cardiac actin, α-myosin heavy chain (MHC), myosin light chain (MLC) 2a, MLC-2v, and cardiac troponin T. Our demonstration that Sca-1^+ cells can be differentiated into contracting cardiomyocytes that express cardiac-specific genes indicates that these Sca-1^+ cells are cardiac progenitor cells.

Three-dimensional aggregates formed from the co-culture of ES cell-derived cardiomyocytes mixed with HL-1 cardiomyocytes

The presence of stem cell niches has been documented in the adult mouse heart [11, 32]. Since we had developed an adult cardiomyocyte (HL-1) cell line in our laboratory [20, 21], we utilized these cells to study niche formation in vitro. HL-1 cells are an immortalized adult cardiomyocyte cell line isolated from a transgenic mouse heart in which the expression of the Simian virus 40 large T antigen is controlled by the atrial natriuretic promoter [20, 21]. These spontaneously contracting cardiomyocytes have been extensively characterized and have been shown to have an adult cardiomyocyte phenotype by electron microscopy, immunohistochemical analysis, RT-PCR analysis, and electrophysiology [20, 21]. In this study we utilize HL-1 cells to provide an adult cardiomyocyte microenvironment that we thought would be necessary to provide for in vivo niche formation if it were to occur. In an attempt to simulate an in vitro environment similar to an in vivo adult

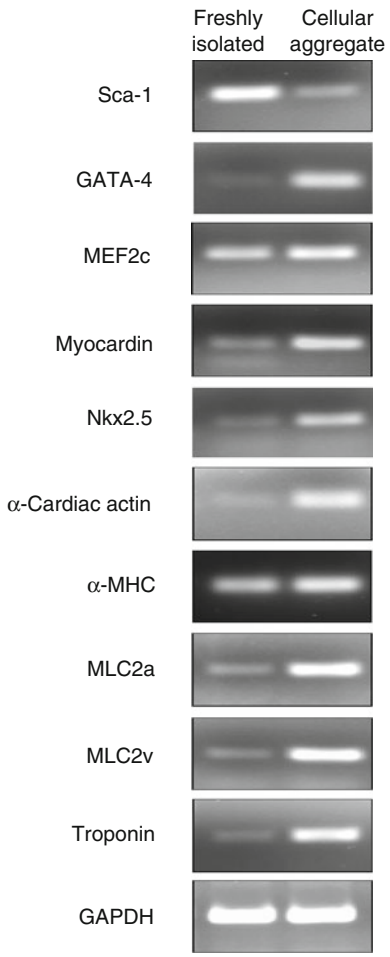

Fig. 3 Analysis of gene expression in cellular aggregates formed from Sca-1^+ cells. RT-PCR was performed on freshly isolated Sca-1^+ cells and on cellular aggregates formed from Sca-1^+ cells grown for 9 days. PCR products were separated by agarose electrophoresis and imaged using Quantity One imaging software

cardiac muscle niche, we created three-dimensional aggregates using HL-1 cardiomyocytes co-cultured with ES cell-derived cardiomyocytes.

We took advantage of the differential expression of SV40 large T antigen in HL-1 cardiomyocytes and the expression of Sca-1 in ES cell-derived cardiomyocytes to localize the distribution of these respective cells within the cellular aggregates. We first demonstrate in Fig. 4a–c that cellular aggregates of HL-1 cells express SV40 large T antigen but not Sca-1 and that cellular aggregates formed from ES cell-derived cardiomyocytes express Sca-1 but not SV40 large T antigen (Fig. 4d–f). Within the cellular aggregate formed from ES cell-derived cardiomyocytes, Sca-1^+ cells are not localized within a distinct niche-like region but instead are scattered throughout the cellular aggregate (arrows in Fig. 4f).

To determine the distribution and localization of Sca-1^+ cells within cellular aggregates formed by co-culturing ES

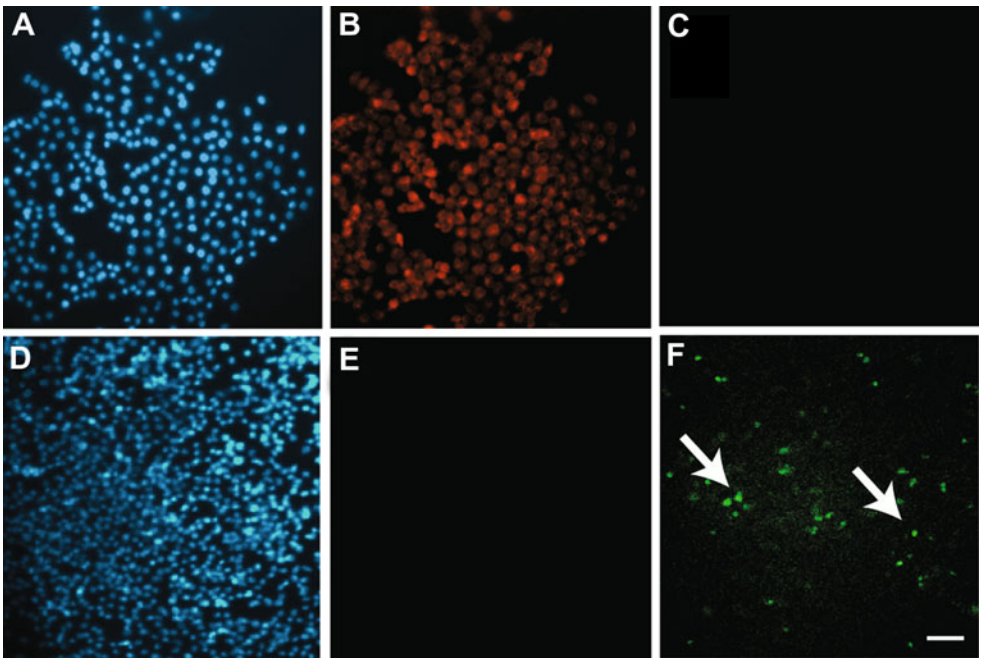

Fig. 4 Immunohistochemical analysis of the expression of SV40 large T antigen and Sca-1 in three-dimensional aggregates of HL-1 cells and ES cell-derived cardiomyocytes. Three-dimensional aggregates of HL-1 cardiomyocytes (**a**–**c**) and ES cell-derived cardiomyocytes (**d**–**f**) are stained with antibodies directed against SV40 large T Antigen (**b** and **e**) as well as Sca-1 (**c** and **f**), and counter-stained with the nuclear DAPI stain (**a** and **d**). Bar, 60 μm

cell-derived cardiomyocytes with HL-1 cells for 9 days, the aggregates were analyzed by immunohistochemistry with antibodies directed against both SV40 T antigen and Sca-1. In every cellular aggregate examined ($n = 30$), we found that the Sca-1^+ cells had "sorted out" and were organized within mainly one or two localized compact regions (niches) within the aggregate, although we did observe a few Sca-1^+ cells scattered throughout the aggregate. An image of a representative aggregate is shown in Fig. 5. The presence of HL-1 cardiomyocytes within this cellular aggregate is demonstrated by staining with an antibody directed against SV40 large T antigen and is shown in red. An arrow points to the location of a Sca-1-positive region within this same aggregate.

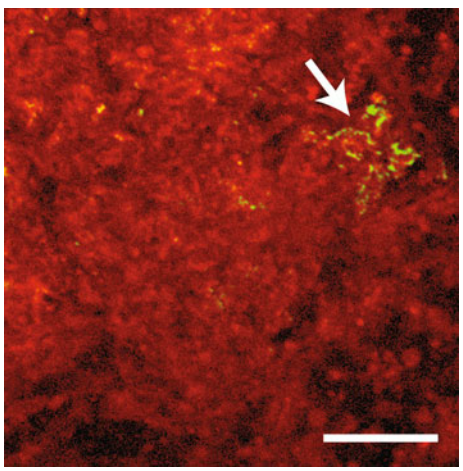

Fig. 5 Niche formation demonstrated by immunohistochemical analysis of an aggregate formed by co-culturing HL-1 cardiomyocytes with ES cell-derived cardiomyocytes. The cellular aggregate was cultured for 9 days. Staining with large T antigen is shown in red while an *arrow* points to the Sca-1^+ region within this merged confocal image. Bar, 100 μm

Discussion

Adult progenitor cells have been identified in hematopoietic [23, 24], neural [25, 26], epithelial [27, 28], intestinal [29, 30], and cardiac [11, 31–38] tissues, among others. Progenitor cells in the heart have been shown to be positive for various stem cell markers, including Sca-1 [11, 14, 31–38], and to reside in specialized microenvironments known as niches. These niches provide a microenvironment composed of an extracellular matrix in which progenitor cells are maintained in an undifferentiated state [39–43]. These cells are thought to function in the turnover of cardiomyocytes under normal conditions, regeneration in diseased states or adaptation to increased load [12–15]. Sca-1^+ cells have been isolated from adult mouse hearts and differentiated in vitro [33]. Under these conditions, differentiated Sca-1^+ cells contracted spontaneously and were shown by immunohistochemistry to express cardiac proteins such as troponin I and cardiac sarcomeric actin

and by RT-PCR analysis to express cardiac-specific genes [33]. We observed a similar pattern of differentiation for our Sca-1^+ cells which were isolated from ES cells (Fig. 3).

In this study, we show that when co-cultured with adult cardiomyocytes, the Sca-1^+ cells self-organized within the cellular aggregate, eventually residing in a distinct, localized niche-like region, demonstrating that Sca-1^+ cardiac progenitor cells can form a niche when present in an adult cardiomyocyte microenvironment. These data indicate that this in vitro differentiation model system can be a good investigational tool to study how stem cell niches organize in the heart.

In conclusion, our studies demonstrate that ES cell-derived cardiomyocytes harbor a subpopulation of Sca-1^+ progenitor cells. Further, we have established that these cardiomyocyte progenitor cells actually do originate as part of the differentiation program of the cardiac muscle cell during heart development and can form a putative niche-like region in a co-culture in vitro system.

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