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Article

Wnt/β-Catenin Stimulation and Laminins Support Cardiovascular Cell Progenitor Expansion from Human Fetal Cardiac Mesenchymal Stromal Cells

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SUMMARY

The intrinsic regenerative capacity of human fetal cardiac mesenchymal stromal cells (MSCs) has not been fully characterized. Here we demonstrate that we can expand cells with characteristics of cardiovascular progenitor cells from the MSC population of human fetal hearts. Cells cultured on cardiac muscle laminin (LN)-based substrata in combination with stimulation of the canonical Wnt/β-catenin pathway showed increased gene expression of ISL1, OCT4, KDR, and NKX2.5. The majority of cells stained positive for PDGFR-a, ISL1, and NKX2.5, and subpopulations also expressed the progenitor markers TBX18, KDR, c-KIT, and SSEA-1. Upon culture of the cardiac MSCs in differentiation media and on relevant LNs, portions of the cells differentiated into spontaneously beating cardiomyocytes, and endothelial and smooth muscle-like cells. Our protocol for large-scale culture of human fetal cardiac MSCs enables future exploration of the regenerative functions of these cells in the context of myocardial injury in vitro and in vivo.

INTRODUCTION

In the developing heart, lineage-tracing experiments in chicks and mice have revealed that the heart mainly develops from mesenchymal progenitors of the first and second heart fields (FHF and SHF, respectively) (Buckingham et al., 2005; Kelly et al., 2014). The cells of the FHF are defined by their co-expression of hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4), TBX5 as well as NKX2.5 (Buckingham et al., 2005; Spater et al., 2014). The mesenchymal progenitors of the SHF originate from the pharyngeal mesoderm and are characterized by their expression of the LIM-homeodomain transcription factor transcription factor Islet-1 (ISL1; Cai et al., 2003; Laugwitz et al., 2004). The progenitors of the SHF mainly contribute to the atria, the outflow tract (OFT), and the right ventricle, whereas the FHF progenitors are involved in the development of the left ventricle (Cai et al., 2003; Moretti et al., 2006; Genead et al., 2010; Laugwitz et al., 2004, 2005; Laugwitz et al., 2008; Lam et al., 2009; Yang et al., 2013). Apart from these two major cell sources of the FHF and SHF, the cardiogenic mesoderm also seems to contain a progenitor subpopulation, defined by its expression of TBX18, which contributes to the sinus venous myocardium (Mommersteeg et al., 2010; Christoffels et al., 2006), while cardiac neural crest cells seem to be involved in valve formation and parasympathetic innervation, as well as the septation of the OFT (Hildreth et al., 2008; Hutson and Kirby, 2007; Waldo et al., 2005).

Cardiac colony-forming unit fibroblasts with cardiovascular progenitor properties have also been identified in the fetal and adult hearts of mice and humans (Chong et al., 2011, 2013). These cells demonstrate characteristics of mesenchymal stromal cells (MSCs), express platelet-derived growth factor receptor-alpha (PDGFR-α), and give rise not only to epicardial cells but also to cells that occupy...
the interstitial, perivascular, and adventitial niches of the heart (Chong et al., 2011, 2013). MSCs in hearts of adult mice have also been shown to express higher levels of specific transcription factors representing the FHF and SHF than bone marrow MSCs (Pelekanos et al., 2012), which might indicate organ-specific characteristics of the MSCs persisting in the myocardium from the fetal stages to adulthood. These findings collectively support the idea that cardiac MSCs may be used as a source of cells with cardiac progenitor characteristics.

We have explored the possibility of using the cardiac MSCs of the human fetal heart for expansion of cells with characteristics similar to those of mesenchymal progenitors that contribute to mammalian cardiogenesis. In this study cardiac MSCs were reproducibly recovered from the adherent cell fraction from first trimester human fetal hearts by using defined culturing conditions, including the use of specific laminin (LN) isoforms, in combination with canonical Wnt/β-catenin stimulation. The MSCs expressed ISL1, PDGFR-α, and NKX2.5, where subpopulations also expressed the stemness and potential cardiovascular progenitor markers: kinase insert domain protein receptor (KDR), the human orthologue of FLK1; c-KIT, the receptor for stem cell factor (Beltrami et al., 2003; Bearzi et al., 2007, 2009), stage-specific embryonic antigen 1 (SSEA-1) (Blin et al., 2010), and TBX18 (Christoffers et al., 2006; Mommersteeg et al., 2010). The fetal cardiac MSCs were multipotent and could be differentiated into elongated, striated, and spontaneously beating cardiomyocytes, endothelial cells, and smooth muscle cell-like cells, also when using defined laminins as substrata. This implies that the unique culturing conditions of human fetal cardiac MSCs using a combination of canonical Wnt/β-catenin stimulation and LN-based matrices facilitate the in vitro propagation of cells with cardiac progenitor characteristics.

**RESULTS**

**Enrichment and Expansion of Fetal Cardiac MSCs**

In order to develop a culture protocol for successful propagation of cardiac MSCs, the adherent cell fraction from whole human fetal hearts (gestational weeks 6–10) was prepared as previously described by Laugwitz et al. (2005). This cell fraction contains a mixture of different cell types, including the cardiac MSCs. To activate the transcriptional network known to be involved in embryonic cardiogenesis (Klaus et al., 2007), we focused on the canonical Wnt/β-catenin signaling pathway, which has previously been shown to be effective in expansion of SHF progenitors derived from embryonic stem cells from mice and humans (Qyang et al., 2007; Kwon et al., 2007; Moretti et al., 2006; Bu et al., 2009) (Figure 1A).

Based on a strategy previously introduced by Qyang et al. (2007), we used a Wnt3a-containing cell culture medium (Wnt3a 100 ng/ml, see Experimental Procedures) to stimulate the canonical Wnt/β-catenin pathway. When cells of the adherent cell fraction of human fetal hearts were expanded on plastic, they multiplied rapidly (Figure 1B) but showed a low expression of the cardiac progenitor markers ISL1, KDR, and NKX2.5, as well as the pluripotency marker OCT4 (Figure 1C).

In order to improve the culturing conditions, the adherent cells were cultured on dishes pre-coated with the Engelbreth-Holm Swarm mouse tumor-derived extracellular matrix extract, Geltrex (GibcoBRL). This extract, which contains basement membrane proteins such as LN-111, has been widely used for culturing stem cells and other cell types (Xu et al., 2001; Ludwig et al., 2006). When the adherent cells were cultured on Geltrex in combination with Wnt3a-containing medium, the cells divided exponentially and were passaged every other day. At 2 weeks, the adherent cells had expanded more than 1,000-fold (Figure 1B) and demonstrated activation of transcription factors involved in defining cardiac progenitors and multipotent stem cells (Figure 1C). Although cells expanded on Geltrex, in combination with the presence of Wnt3a, demonstrated increased gene-expression levels of the pluripotency marker OCT4 in comparison with the other culturing conditions, this expression was $10^3$ times lower than that found in pluripotent human embryonic stem cells (Figure S1).

As expected for activation of the canonical Wnt/β-catenin pathway, when using Wnt3a-containing medium in combination with Geltrex, the cultured cells showed an increased phosphorylation of S1490-Lrp6 and Dvl3 as well as increased levels of active (dephosphorylated) β-catenin compared with the cells of the initial adherent cell fraction (Figure 1D). These data suggest that both the Wnt3a-containing medium and Geltrex may provide important signals for expansion of cells from the adherent cell fraction of fetal human hearts, with transcriptional signatures akin to those of cardiac progenitors (Moretti et al., 2006).

Exclusion of either Wnt3a or Geltrex from the culture protocol negatively affected proliferation of the cells in the initial fraction (Figure 1B) and thereby preserved survival of the limited numbers of remaining endothelial cells and cardiomyocytes among the adherent cells. This was also reflected in a relatively high mRNA expression of troponin T (TNNT2) under these culture conditions (Figure 1C).

The cells were analyzed by flow cytometry using an MSC antibody panel to confirm that the adherent cells expanded for 2 weeks on Geltrex in Wnt3a-containing medium were derived from the fetal cardiac MSCs (Pittenger
and Martin, 2004). With a purity of >85%, the cultured adherent cells expressed MSC markers CD105, CD90, CD73, and HLA-1, while being negative for HLA-DR and the hematopoietic lineage markers CD14, CD11, CD80, CD83, CD34, CD45, and endothelial marker CD31 (Figure S2). The cultured cells were also able to differentiate in vitro into the three mesenchymal lineages, osteoblasts, chondrocytes, and adipocytes (Figure 2), and thus fulfilled the International Society of Cellular Therapy criteria for being MSCs (Horwitz et al., 2005). Cryopreservation had no influence on the expression of the above-mentioned surface markers or the ability to differentiate into the three mesenchymal lineages.

Characterization of Cultured Cardiac MSCs

The gene-expression profile of the cardiac MSCs was analyzed by microarray over time of culture, and we focused on the core group of transcription factors known to be important in regulation of cardiogenesis and especially the SHF development in mice (Black, 2007). The gene-expression profile of the human fetal cardiac MSCs showed only minor fluctuations over time in culture (from day 15 to day 48), including the expression of mesodermal markers TBX6, TBX1/Brachyury, and TBX18, together with the key transcription factors involved in the transcriptional network of the SHF (ISL1, GATA4, NXX2.5, FOXH1, FGF8), the FHF (TBX5, NXX2.5), as well as other markers related to cardiovascular progenitors like PDGFR-α and the stemness markers KDR, SSEA-1, and KIT (Figure 3A). The activated ISL1-GATA4 pathway in the cultured cardiac MSCs correlated well with the mRNA levels of cardiogenic bone morphogenetic proteins (Cohen et al., 2007; Marvin et al., 2001) and the concomitant expression of the early cardiomyocyte markers NXX2.5 and mesoderm posterior 1 (MESPI). However, the cultured cardiac MSCs also express mature cardiomyocyte markers such as myosin heavy chain (MYH) 6 and 7, the endothelial marker VCAM1, as well as the smooth muscle markers MYH11 and myocardin (MYOCID).

Another important finding is that after 3 weeks in culture (eight passages), the majority of the cardiac MSCs stained positive for ISL1 (>90%) and also for NXX2.5 (>80%) (Figure 3B). Cardiac MSCs staining positive for the mesodermal progenitor marker TBX18 were also sparsely identified (Figure 3B).
majority of the cardiac MSCs expressed PDGFR- 
with low variability between cultures. From 8- to 9-week human fetal hearts identified subpopula-
tion (Figure 3C) analyses of the cultured cardiac MSCs derived
were differentiated into the three mesenchymal lineages: bone (alkaline phosphatase, ALP), cartilage (Aggrecan), and adipose
tissue (oil red 0, ORO). See also Figure S2. Scale bars represent
100 μm.

Flow cytometry (Figure 3D) and immunofluorescence
(Figure 3C) analyses of the cultured cardiac MSCs derived
from 8- to 9-week human fetal hearts identified subpopulations
of cells that express the cell stemness markers KDR, c-KIT, or SSEA-1, with low variability between cultures. Moreover, immunofluorescence staining showed that the majority of the cardiac MSCs expressed PDGFR-α.

Accordingly, the gene-expression profiles in combination
with the immunohistochemical and flow cytometry
analyses demonstrate that the culture protocol utilized in
this study reproducibly allows for large-scale expansion of
fetal cardiac MSCs while retaining cardiovascular progenitor phenotypes, with low gene-expression variability during
the course of culture.

Development of a Chemically Defined LN-Based Cell
Culture System
We have demonstrated that a combination of a basement
membrane (LN)-containing culture matrix (Geltrex) and
molecules that stimulate the canonical Wnt/β-catenin
signaling pathway enables propagation of cardiac MSCs
with cardiovascular progenitor characteristics. To obtain a
more defined matrix while maintaining successful propa-
gation of cardiac MSCs, we explored the possibility of utilizing chemically defined LN-based matrices for expansion and differentiation of these cells. Cultured pluripotent stem cells and pluripotent cells of the inner cell mass of the blastocyst express LN-511 and LN-521 (Rodin et al., 2010; Domogatskaya et al., 2012). Since these LNs have previ-
ously been demonstrated to support clonal expansion of
cultured embryonic stem cells (Rodin et al., 2014) and
the LN subunits α5 and γ1 are also abundantly expressed in human fetal hearts (Figure 4A), this suggests that LN-511 and LN-521 may be suitable for derivation and expansion of cardiac MSCs. LN-211, on the other hand, might be linked to cardiomyocyte development and/or function, as it has been shown to be defective in congenital muscular dystrophic patients with cardiomyopathy (Fins-
ter et al., 2010), and α2 chains were also expressed in the fetal heart (Figure 4A).

The human fetal cardiac MSCs were cultured on human recombinant LN-511 and LN-521 using the same Wnt-con-
taining medium as described above. MSCs cultured on LN-
511 and LN-521 displayed a tendency toward increased
mRNA levels of ISL1 in comparison with cells cultured on Geltrex. At the same time, TNNT2 levels remained low
(Figure 4B) and the expansion potential of the cells was un-
affected. In order to explore the capacity of LN-211 to sup-
port cardiomyocyte differentiation, cardiac MSCs derived
and expanded on LN-521 were cultured on LN-211 in a me-
dium devoid of Wnt3a. After 2 weeks, the gene expression of TNNT2 had increased 150 times, concomitantly with a significant downregulation of KDR (Figure 4C). This indicates that LN-211 has the potential to stimulate cardiac commitment of cardiac MSCs at the gene-expression level. In order to study the signaling pathways involved, we blocked cell-laminin interactions with antibodies against α-dystroglycan (α-DG) and β1-integrin, which are important laminin receptors (Domogatskaya et al., 2012). This caused a substantial reduction of TNNT2 expression (Figure 4D), which suggests that LN-211 interacts to a large extent with the cardiac MSCs through these cellular receptors.

Differentiation into Cardiomyocytes, Smooth Muscle
Cells, and Endothelial Cells
After 2 weeks of culture, the fetal cardiac MSCs expressed
cardiovascular progenitor markers, supporting their potential
to differentiate into the different cell types of the heart.
In order to initiate cardiomyocyte differentiation, we
used a protocol previously developed for differentiation
of pluripotent stem cells (Lian et al., 2012, 2013). Expanded
cardiac MSCs from fetal hearts of 6, 8, and 9 weeks’ gesta-
tion were seeded on Matrigel and canonical Wnt signaling
was blocked using a defined serum-free medium to induce
cardiomyocyte differentiation. A portion of the cultured
cardiac MSCs derived from the 9-week heart differentiated
into spontaneously beating, troponin T+ (TnT+) striated
cardiomyocytes (Figures 5A and 5B and Movie S1) within
3 weeks, with no residual cardiomyocytes present before
initiation of differentiation (Figure 5C). The cells derived from 6- to 8-week human fetal hearts did not differentiate into TnT+ cardiomyocytes, despite similar expression of ISL1, NKX2.5, PDGFR-α, SSEA-1, KDR, and c-KIT before
initiation of differentiation.

As shown in Figure 4C, we observed prominently
increased levels of TNNT2 in the fetal cardiac MSCs upon
culture on LN-211. Thus we replaced Matrigel, which is not chemically defined and has a batch-to-batch vari-
ability, with LN-211 and used the same cardiomyocyte dif-
ferentiation protocol for the MSCs as described above (Lian et al., 2012, 2013). The cells differentiated on LN-211 in comparison with Matrigel showed equally increased levels
of TNNT2 (Figure 5E) and also similar numbers of TnT+ cells after differentiation (Figure 5F). Thus LN-211 proved to be just as effective as Matrigel for supporting cardiomyocyte differentiation of cardiac MSCs derived from the 9-week heart.

Endothelial cell differentiation was induced in cardiac MSCs by using a medium containing vascular endothelial growth factor (VEGF). The substrates used were gelatin or LNs present in the basal membranes of blood vessels (LN-521 and LN-411) (Hallmann et al., 2005; Simon-Assmann et al., 2011; Stenzel et al., 2011; Domogatskaya et al., 2012). After 3 weeks in culture, a small proportion of the cardiac MSCs expressed the endothelial marker CD31 (Figure 6, left panel) regardless of the substrate used, showing that the chemically defined laminin combination LN-521/LN-411 can support differentiation toward endothelial cells (Figure 6, left panel).

The fetal cardiac MSCs expressed α-SMA prior to differentiation (Figure 6, right panel), which has already been described for other PDGFR-α+ cells (Chong et al., 2013). After smooth muscle cell differentiation of the cardiac MSCs, the α-SMA staining intensity did not seem to increase but rather polarized into podosome-like structures (Figure 6, right panel), which can be found in vascular smooth muscle cells upon stimulation with exogenous PDGF-BB and TGF-β (Murphy and Courtneidge, 2011). Our results demonstrate that human fetal cardiac MSCs are multipotent, where different LN compositions can support their lineage specification.

**DISCUSSION**

Herein, we describe that human fetal cardiac MSCs with cardiac progenitor characteristics can be propagated by using a combination of a supplemented culture medium, which stimulates the canonical Wnt/β-catenin pathway and specific laminin isoforms as cell culture substrata, without using any genetic manipulation or feeder cells. During culture, the fetal cardiac MSCs stably expressed early cardiovascular markers and expanded rapidly without changing their phenotype during the culturing process. Excluding either Wnt3a or LN-based matrix from the culture protocol affected either cell growth or the gene expression of cardiac progenitor markers.

The origin of the different subpopulations of cardiac MSCs has not been examined in this study. It has previously been reported that interstitial cells of both the atria and ventricles, display a strong expression of PDGFR-α (Chong et al., 2011, 2013). Chong et al. (2013) showed
that PDGFR-α+ cells isolated from human fetal hearts of the second trimester were multipotent and could be differentiated into smooth muscle as well as endothelial cells, but they did not differentiate into cardiomyocytes. In the present study, the majority of the cultured cardiac MSCs stained positive for PDGFR-α. Whether these cells possibly originate from interstitial cells of the atria and ventricles remains to be elucidated, but they displayed a similar capacity to differentiate into endothelial cells and also cells with polarized α-SMA expression akin to that of mature smooth muscle cells. In contrast to the PDGFR-α+ cells previously derived from second trimester hearts (Chong et al., 2013), the fetal cardiac MSCs derived from a 9-week human heart in the present study could be differentiated into spontaneously beating, striated TnT+ cardiomyocytes, although it was only a small proportion of the cardiac MSCs that expressed c-KIT, we do not know if these cells are similar to the previously described cardiac progenitors in the adult heart (Urbanek et al., 2003; Beltrami et al., 2003; Bearzi et al., 2007, 2009).

A small population of the fetal cardiac MSCs also expresses SSEA-1, which has been reported to represent an early marker of cardiovascular progenitors derived from pluripotent stem cells (Blin et al., 2010). The existence of a corresponding progenitor in the human fetal heart has yet to be identified, and it remains to be further investigated whether the SSEA-1-expressing cells identified within the cardiac MSC population specifically have the potential to develop into the different cell types of the heart. Some of the cultured cardiac MSCs also stained positive for the mesodermal progenitor marker TBX18 (Mommersteeg et al., 2010; Christoffels et al., 2006); future studies need to be performed in order to address whether some of the PDGFR-α+ and TBX18+ cells potentially share a developmental origin.

Interestingly, the use of specific LN molecules supported lineage conversion of the cultured cardiac MSCs into cardiomyocytes and endothelial cells. By using LN-211 alone, the main component of the basement membrane surrounding adult cardiac and skeletal muscle fibers in situ (Domogatskaya et al., 2012), the cardiac MSCs could be differentiated into the cardiomyocyte lineage, with a 150 times increase of TNNt2 expression and a concomitant up-regulation of NKX2.5. In addition, culture on LN-521/411, which are present in the basal membranes of vessels
Hallmann et al., 2005; Simon-Assmann et al., 2011; Stemzel et al., 2011; Domogatskaya et al., 2012), supported differentiation toward endothelial cells expressing CD31.

In conclusion, we have developed a protocol for isolation and large-scale expansion of human fetal cardiac MSCs that exhibit properties of cardiac progenitor cells. These cells can be used as a tool to investigate the regenerative capacities of human fetal cardiac MSCs in a variety of contexts related to chronic and acute myocardial injury, such as hypoxia, fibrosis, and inflammation.

EXPERIMENTAL PROCEDURES

Derivation and Expansion of Human Fetal Cardiac MSCs
The fetal hearts were obtained from legal terminations of pregnancy after the donor’s informed consent and ethics approval from the Regional Ethics Board in Stockholm.

The fetal hearts were pre-digested overnight at 4°C in a 0.5 mg/ml Trypsin solution in Hank’s balanced salt solution (HBSS). The MSC fraction was prepared according to a modified version of the

Figure 5. Cardiomyocyte Differentiation Potential of Human Fetal Cardiac MSCs
(A) Upon exposure to cardiomyocyte differentiation medium, portions of the cardiac MSCs differentiated into elongated, striated TnT+ (green) cardiomyocytes. Scale bar represents 50 μm.
(B) A zoom-in of the box in (A) demonstrating the striations in the cardiomyocytes.
(C) All cardiac MSCs stained negative for TnT prior to initiation of the differentiation protocol. Scale bar represents 50 μm.
(D) Primary cardiomyocytes isolated from a human fetal heart were used as a positive control. Nuclei are stained with DAPI (blue). Scale bar represents 50 μm.
(E) Normalized mRNA levels of TNNT2 before and after differentiation on Matrigel and LN-211.
(F) Numbers of TnT+ cells of human fetal cardiac MSCs differentiated on plastic, LN-211, and Matrigel. Numbers of positive cells are normalized against the total number of nuclei in the same well multiplied by a factor of 100,000. Data are presented as means ± SD of three independent experiments performed in triplicate. *p < 0.05.

Figure 6. Differentiation into Endothelial and Smooth Muscle Cells
The cardiac MSCs underwent differentiation into endothelial cells (left panel) expressing CD31 (red) and smooth muscle cells (right panel) expressing α-SMA (green). There was no CD31 expression before differentiation, whereas the majority of the cardiac MSCs expressed α-SMA in abundance before initiation of differentiation. The intensity of the α-SMA staining did not increase as a result of differentiation but rather appeared in a more polarized fashion similar to what can be found in contractile, adult vascular smooth muscle cells exposed to PDGF-BB and TGF-β1. Human aortic endothelial cells (HAEC) and smooth muscle cells from a human carotid artery (HCtSMC) were positive controls for endothelial cells and smooth muscle cells, respectively. Nuclei are stained blue with DAPI. Scale bars represent 50 μm.
Fetal Cardiac MSCs

Laminin-Based Derivation and Expansion of Human Fetal Cardiac MSCs

To generate chemically defined matrices for derivation and expansion of human fetal cardiac MSCs (gestational weeks 6–8, n = 3), the cells were grown in wells coated with LN-511, 521, or 211, using the same culture medium as described above. The human recombinant laminins were generated as previously described (Kortesmaa et al., 2000; Doi et al., 2002; Rodin et al., 2014) or purchased from BioLamina AB (batches 80,051, 80,050, and 80,042, respectively).

Differentiation of Cardiac MSCs

For cardiomyocyte differentiation in vitro we used a modified protocol developed for human pluripotent stem cells (Lian et al., 2012, 2013), where the cardiac MSCs were cultured on Matrigel, LN-211 or plastic. Cardiac MSCs derived from fetal hearts of different ages (gestational week 6, 8, and 9, n = 3) were used. After 3 weeks, cells were fixed in 4% phosphate-buffered formalin and stained with a mouse monoclonal antibody against human troponin T (TnT) (ab8295, clone 1C11, Abcam) and visualized with an Alexa Fluor 488-conjugated rabbit anti-mouse secondary antibody (A11059, Thermo Fisher Scientific). RNA extraction and qRT-PCR for TNNT2 and GAPDH were performed as described in Supplemental Experimental Procedures. For imaging of calcium signaling dynamics, cells were labeled with the Ca2+-sensitive fluorescence indicator Fluo-4/AM (10 μM; Molecular Probes, Thermo Fisher Scientific) according to the manufacturers’ protocol.

For differentiation toward vascular smooth muscle cells, the cardiac MSCs were seeded on gelatin-coated plastic, and cultured for 18 days in a defined medium supplemented with PDGF-BB (10 ng/ml) and TGF-β1 (2 ng/ml) (PeproTech), according to a modified protocol previously described by Cheung et al. (2014). Cells were passaged 1:4 once per week into new gelatin-coated wells. Subsequently, the cells were fixed and stained with a mouse anti-human α-smooth muscle actin primary antibody (A2547, clone 1A4; Sigma-Aldrich) and visualized by a rabbit anti-mouse Alexa Fluor 488-conjugated secondary antibody (Thermo Fisher Scientific). The experiment was performed in duplicate using cells from four different hearts of gestational age 8 and 9 weeks.

For endothelial differentiation, cardiac MSCs were seeded in culture wells pre-coated overnight with a 1:4 mixture of LN-521/411 (5 μg/cm²) or gelatin (0.1%) and cultured for 21 days in EGM-2 medium (Lonza) with VEGF (50 ng/ml) from R&D Systems (Liu et al., 2007). Subsequently, the cells were fixed and stained with a monoclonal mouse anti-human CD31 antibody (M0823, clone JC70A). Stained cells were visualized using an Alexa Fluor 568-conjugated goat anti-mouse secondary antibody (Thermo Fisher Scientific). The experiment was performed in duplicate using cells from five different hearts of gestational age 8 and 9 weeks.

Statistics

Real-time qRT-PCR data are presented as means ± SD. Student’s t test (two-tailed, two samples, unequal variances) was used for statistical analysis. For analysis of TnT+ cells after differentiation, the Kruskal-Wallis test with Bonferroni correction was used. p < 0.05 was considered to indicate statistical significance.

Full details on the methods used are presented in the Supplemental Experimental Procedures.

ACCESSION NUMBERS

Microarray data was deposited in the Gene Expression Omnibus (GEO) repository at the National Center for Biotechnology Information (NCBI) (GEO: GSE78047).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.02.014.

AUTHOR CONTRIBUTIONS

K.H.G. conceived and designed the experiments together with A.M.B., S.R., I.B., O.E.S., C.O., C.S., M.L., and E.W., I.B., M.L., E.W., S.R., C.O., U.F., C.I., R.G., A.S., and C.G. performed the experiments and analyzed the data. C.S., E.A., K.L.B., C.I.E.S., M.W., E.S., P.U., E.A., K.T. and M.C. contributed reagents, materials and
analysis tools. K.H.G. wrote the paper with O.E.S., C.O., S.R., A.M.B., and M.C.

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