A co-culture assay of embryonic zebrafish hearts to assess migration of epicardial cells \textit{in vitro}

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Abstract

\textbf{Background:} The vertebrate heart consists of three cell layers: the innermost endothelium, the contractile myocardium and the outermost epicardium. The epicardium is vital for heart development and function, and forms from epicardial progenitor cells (EPCs), which migrate to the myocardium during early development. Disruptions in EPC migration and epicardium formation result in a number of cardiac malformations, many of which resemble congenital heart diseases in humans. Hence, it is important to understand the mechanisms that influence EPC migration and spreading in the developing heart. \textit{In vitro} approaches heretofore have been limited to monolayer epicardial cell cultures, which may not fully capture the complex interactions that can occur between epicardial and myocardial cells \textit{in vivo}.

\textbf{Results:} Here we describe a novel \textit{in vitro} co-culture assay for assessing epicardial cell migration using embryonic zebrafish hearts. We isolated donor hearts from embryonic zebrafish carrying an epicardial-specific fluorescent reporter after epicardial cells were present on the heart. These were co-cultured with recipient hearts expressing a myocardial-specific fluorescent reporter, isolated prior to EPC migration. Using this method, we can clearly visualize the movement of epicardial cells from the donor heart onto the myocardium of the recipient heart. We demonstrate the utility of this method by showing that epicardial cell migration is significantly delayed or absent when myocardial cells lack contractility and when myocardial cells are deficient in \textit{tbx5} expression.

\textbf{Conclusions:} We present a method to assess the migration of epicardial cells in an \textit{in vitro} assay, wherein the migration of epicardial cells from a donor heart onto the myocardium of a recipient heart in co-culture is monitored and scored. The donor and recipient hearts can be independently manipulated, using either genetic tools or pharmacological agents. This allows flexibility in experimental design for determining the role that target genes/signaling pathways in specific cell types may have on epicardial cell migration.

\textbf{Keywords:} Epicardial cell, Epicardium, Proepicardium, Myocardial cell, Myocardium, Migration, Co-culture

Background

The heart is one of the first organs to form in vertebrate embryogenesis and during early development consists of three major cell layers: endocardium, myocardium, and epicardium. The endocardial and myocardial cells originate from populations of mesodermal cells that migrate from the midbrain-hindbrain boundary to form the linear heart tube [1, 2]. These cardiogenic mesoderm cells form the ventricle, atrium, outflow tract myocardium, and contribute to the cardiac conduction system [3]. The epicardium originates from a different population of cells, the proepicardium (PE), a transitory structure of progenitor cells arising from coelomic mesenchyme of the septum transversum [3]. Epicardial progenitor cells (EPCs) from the PE migrate onto the bare myocardium and envelop the heart, forming the epicardium. There are two known mechanisms of cell migration from the PE to the heart: 1) the release of free-floating EPC aggregates that land on the myocardium (e.g., mouse); 2) the formation of a tissue bridge between the PE and myocardium (e.g., chick). Both mechanisms of PE cell migration are observed in some species (e.g., zebrafish, axolotl) [4–6]. As the heart...
develops, a subset of epicardial cells undergo epithelial-to-
mesenchymal transition and invade the subepicardial
space. These mesenchymal cells, called epicardium-
derived cells (EPDCs), are important for normal heart
maturation and have been shown to differentiate into
interstitial cardiac fibroblasts, coronary vascular smooth
muscle cells, and adventitial fibroblasts. Though some-
what controversial, it is suggested that EPDCs also con-
tribute to the coronary endothelium, valve development,
myocardial cells, and Purkinje fiber differentiation [4, 7].

Congenital heart disease, which affects between 0.4-
5% of live births, is often due to defective cardiac
morphogenesis involving problems with cardiac progeni-
tor cells [3]. There has been increasing interest on epi-
cardium formation and importance of the epicardium in
subsequent heart development [7]. Ablation of the PE
inhibits epicardium formation, causing an array of
cardiac malformations that resemble malformations
observed in human congenital heart disease. For
example, chicks lacking an epicardium developed thin
compact myocardia similar to human left ventricular
non-compaction cardiomyopathy [8]. Aberrant crosstalk
between the epicardial layer and underlying myocardial
and endocardial cells has been implicated in several con-
genital diseases, such as hypoplastic left heart syndrome
and endocardial fibroelastosis [3, 7].

In vivo approaches to studying PE migration and epi-
cardium formation often include microsurgery to ablate
the PE or the use of physical barriers to block migration.
Manipulation of specific genes involved in signaling or
cell adhesion has also been used to assess involvement
in PE formation [5, 8–10]. However, the genes of inter-
est, including Wt1, Tbx18, Tcf21, are expressed during
development in other organs besides the heart: the use
of mutants or morpholino oligonucleotide (MO) knock-
down produces effects wherever the target gene is nor-
mally expressed. This creates a concern that the results
have been influenced by altered gene expression not spe-
cific to heart cells [7, 9, 11].

Common in vitro approaches involve excising the PE
or heart segment and monitoring effects on EPC migra-
tion in culture [9, 12, 13]. One advantage of this ap-
proach is the ease with which signaling factors can be
added to the culture media to assess effects on migration
[9]. In addition, it avoids the problem of off-target effects
in gene manipulation experiments. However, most of
these studies have focused on the effects of factors in
the culture medium, rather than on the cell-cell interac-
tions between epicardial cells and myocardial cells,
which have been shown to play an important role in
heart development in vivo [6, 8, 14].

Here we describe an in vitro assay to assess and quan-
tify the migration of epicardial cells from a donor heart
onto the bare myocardium of a recipient heart. In this
assay, the important cell-cell interactions between differ-
cent cell types remain intact. Because the technique cul-
tures multiple cell types, differentiated cell phenotypes
are better preserved [15], allowing the hearts to remain
healthy in culture for several days. This permits for
lengthy observations, not possible with most in vitro
approaches. In this method, the source of migrating epicar-
dial cells is different from the source of target myocardial
cells, making it possible to manipulate either or both types
of cells independently.

We use embryonic zebrafish as the source of hearts.
This is advantageous for several reasons: zebrafish pro-
duce large numbers of offspring, embryonic hearts can
be efficiently isolated, externally fertilized eggs allow for
gene manipulation with MO or CRISPR-Cas9, and there
is clear observation of effects during early development.
Additionally, a variety of transgenic lines are readily
available [1].

In this report, we demonstrate key features of this
assay by assessing the ability of epicardial cells from a
donor heart (marked with tcf21:DsRed2) to migrate onto
the myocardial surface of a recipient heart (marked with
cmlc2:EGFP). We show the normal course of migration,
and how migration was inhibited when the recipient
hearts were extracted from embryos injected with MOs
against silent heart (sih), and tbx5. Sih morphants lack a
heartbeat [16]. Tbx5, which is expressed in multiple tis-
ues in the heart, has been implicated in EPC migration
in vivo [12]. Because this approach maintains the mul-
tiple cell types of the in vivo setting, yet allows for ma-
nipulation of individual cell types, this assay can be used
to identify not only genes important in epicardial forma-
tion, but also where they function.

Methods

Zebrafish

Embryos were obtained from adult zebrafish (Danio
rerio) housed and maintained according to guidelines
described in Westerfield (2000) [17]. Embryos were har-
vested at 84 h post fertilization (hpf) for obtaining
“donor hearts”. These hearts were obtained from the
transgenic line tcf21:DsRed2 [Tg(tcf21:DsRed2)pd37],
which marks epicardial cells with a red fluorescent pro-
tein. The “recipient hearts” were collected from 60 hpf
embryos from the transgenic line cmlc2:EGFP
[Tg(cmlc2:EGFP)], which marks myocardial cells with a
green fluorescent protein (Fig. 1a).

The silent heart (sih; cardiac troponin T2, tntt2) and
tbx5 (T-box 5) MOs were obtained from Gene Tools
(Philomath, OR). The Gene Tools standard control mor-
pholino (control MO) was used as a control. The MO
sequences were: sih, 5’ CATGTTTGTCTCTGATGAC
ACGA 3’ [16]; tbx5, 5’ GAAAGTGTCTCTACTGTCC
GCCAT 3’ [18]; control MO, 5’ CCTCTTACCTC
AGTTACAATTTATA 3’. A 2 nM MO solution was prepared with either the sih, tbx5, or control MO, and microinjected into cm1c2:EGFP eggs in the 1-2 cell stage. Microinjections were done as previously described [19]. Eggs were collected into a petri dish with autoclave-sterilized egg water (60 μg/ml Instant Ocean Sea Salts with 0.2 ppm methylene blue). Embryos were screened for MO incorporation at 48 hpf, and only MO positive embryos were used. Clean water changes were made daily.

All procedures involving zebrafish were approved by the Animal Care and Use Committee of the University of Wisconsin-Madison and adhered to the National Institute of Health’s “Guide for the Care and Use of Laboratory Animals.”

Culture media and plate preparation
Culture medium consisted of Leibovitz’s L-15 medium (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies) and 4x penicillin/streptomycin.
(Fisher Scientific). The culture medium was filtered through a disposable sterile filter unit, and stored at 4 °C prior to use. Conical-bottom 60-well plates with lids (Electron Microscopy Sciences) were pre-plated with a mix made from Matrigel basement membrane matrix mix (Corning), with a protein concentration diluted to 4 mg/mL in 1X DMEM (Corning) and supplemented with 4x penicillin/streptomycin. The matrigel mix was prepared ahead of time in a sterile hood and stored at -20 °C. On the culture start day, the matrigel mix was thawed and placed onto a culture dish following the manufacturer-recommended thin gel coating method. Each well required approximately 10 µL of matrigel mix to coat. After the culture dish was set (37 °C, 30 min) it was stored in the cell culture incubator (28 °C, 5 % CO₂) until time for placement of hearts into culture.

**Isolation of embryonic hearts and placement into culture**

Hearts were extracted from embryos according to methods adapted from Burns and MacRae [20]. Briefly, 50 to 80 embryos were lightly anesthetized with tricine (MS 222, Sigma-Aldrich) and placed into a 1.7 mL microcentrifuge tube. Excess water was drawn off and 1 mL of culture medium was added. The microcentrifuge tube with embryos was placed beneath a 5 mL syringe fitted with a 19-gauge needle, adjusted such that the beveled end of the needle aligned with the 0.25 mL mark on the microcentrifuge tube. The syringe was gently pumped up and down, bringing culture media and embryos into and out of the syringe, in a rhythm guided by beats on a metronome. Hydrodynamic shear forces remove the hearts from the bodies. These forces are proportional to the rate of flow through the needle, and inversely proportional to the needle diameter, thus the rate of syringe pumping is critical. All contents from the syringe and microcentrifuge tube were then quantitatively emptied from the syringe with washes and filtered through a 105 micron nylon mesh (Component Supply) to separate the bodies and other large debris from the hearts in the filtrate. If necessary, the media was filtered again with a 37 micron nylon mesh (Component Supply) to retain the hearts on the filter and remove smaller debris. Hearts in medium were placed in a Petri dish following the manufacturer-recommended thin gel coating method. Each well required approximately 10 µL of matrigel mix to coat. After the culture dish was set (37 °C, 30 min) it was stored in the cell culture incubator (28 °C, 5 % CO₂) until time for placement of hearts into culture.

Culture medium was refreshed on a daily basis by removing up to 5 µL of old medium and adding 5 to 8 µL of fresh culture medium (Fig. 1c). The culture dish was carefully monitored for signs of contamination and any questionable samples were removed and not used for analysis. As previously mentioned, the cell culture incubator was maintained at 28 °C with 5 % CO₂.

**Assay imaging and scoring**

Beginning on the day after hearts were placed in culture, images of each well were obtained daily (Day 1 through Day 7 in culture) using an Olympus DP72 camera mounted on an Olympus SZX16 epifluorescence stereo microscope with cellSens software (Fig. 1c). Images were processed using Adobe Photoshop (Adobe). The migration of epicardial cells (red) from the donor heart onto the myocardium of a recipient heart (green) was scored by an experimenter blinded to sample identity (Fig. 1d). Scoring was based on a scale from 0 to 7: 0 = no migration of epicardial cells was observed during the duration of culture; 1 = epicardial cells were only observed on the recipient myocardium on Day 7; 2 = migration of epicardial cells began on Day 6 and continued to expand coverage of the recipient myocardium through Day 7; 3 = migration began on Day 5, etc.; 4 = migration began on Day 4, etc.; 5 = migration began on Day 3, etc.; 6 = migration began on Day 2, etc.; 7 = migration began on Day 1, etc.
Assay analysis
Each culture dish well containing one donor and one recipient heart that remained in contact throughout the seven days in culture was considered n = 1 for statistics. In order to assess whether data for each treatment group could be pooled from two experimental replicates, two-way analysis of variance was conducted to confirm that variation from different experimental days did not have an effect. Since both the experimental day and interaction variables were not significant (p < 0.05) for both sih MO and tbx5 MO groups and their respective controls, replicate data sets were pooled. The pooled sample size for the control vs. sih MO group was n = 13 to 14, and the pooled sample size for control vs. tbx5 MO group was n = 14 to 19. Student’s t-test was used to compare the pooled migration scores of control MO recipient hearts with respective sih MO or tbx5 MO recipient hearts. F-test was used to check homoscedasticity of data and significance was set at p < 0.05. All statistical analysis was conducted with GraphPad Prism statistics software (GraphPad Software).

Immunohistochemistry
On Day 7, the donor/recipient hearts from each well were collected from the culture dish and processed for confocal imaging using a method from Plavicki et al. [6] (Fig. 1e). Primary antibody rabbit anti-DsRed2 (Ana-Spec) was used in a 1:200 dilution in PBT (0.3 % Triton X-100 in phosphate buffered saline) buffer. Secondary antibody, anti-rabbit Alexa 568 antibody (Invitrogen), was used in a 1:100 dilution in PBT buffer. Confocal images were collected with an Olympus Fluoview FV1000 microscope. Brightest point projections were made using Olympus Fluoview software (Olympus) and images were processed in Adobe Photoshop (Adobe).

Results
To verify that migration from a donor heart to a recipient heart can be assessed in vitro, we collected normal donor hearts from tcf21:DsRed2 embryos and placed them in culture with control MO recipient hearts from cm1lc2:EGFP embryos. In zebrafish, EPCs begin migrating to the ventricle between 60-72 hpf. By 96 hpf, epicardial cells cover most of the ventricle, and, by 120 hpf, also cover most of the atrium. Hence, donor hearts were collected at 84 hpf, a time point at which epicardial cells were present and actively spreading on the ventricle. In contrast, recipient hearts were collected at 60 hpf before EPCs began migrating to the ventricle in order to prevent recipient-epicardial cells from confounding our results. To confirm that recipient hearts extracted at 60 hpf lacked epicardial cells, we examined hearts from embryos with both a red epicardial marker, tcf21:DsRed2, and green myocardial marker, cm1lc2:EGFP. Hearts extracted from cm1lc2:EGFP; tcf21:DsRed2 embryos at 60 hpf lacked epicardial cells on the ventricle and atrium (n = 10). Neither tcf21+ cells nor DAPI-stained cells with the flattened epicardial cell phenotype were observed on the myocardia of these hearts (Additional file 1: Figure S1A). In addition, we confirmed that recipient hearts did not contain cells that were capable of independently differentiating into epicardial cells after 7 days in the presented culture conditions. Hearts from 60 hpf cm1lc2:EGFP; tcf21:DsRed2 embryos that were individually maintained in culture for 7 days did not have any tcf21+ cells or DAPI-stained cells with the epicardial phenotype present on their myocardia (n = 8, Additional file 1: Figure S1B).

Scoring for epicardial cell migration was assessed by the increasing overlap between red epicardial signal (tcf21+) and green myocardial signal (cm1lc2+) over time (Fig. 2a-c). In control experiments, epicardial cell migration was observed in 12 of 13 samples (controls for sih MO cohort) and 17 of 19 samples (controls for tbx5 MO cohort). In general, epicardial cells from control samples showed clear signs of migration onto recipient myocardia between Day 4 and 5 (Figs. 2a, and 3). This was reflected in the scoring: average migration scores for control samples were 3.615 (SEM ±0.385) for the sih MO cohort, and 3.737 (SEM ±0.445) for the tbx5 MO cohort (Fig. 3). In contrast, no migration was observed at all in 5 of 14 samples in the sih MO group. If migration occurred it was minimal and significantly delayed, beginning in most cases between Day 6 and 7 (Fig. 2b). This was reflected in a significantly lower average migration score of 1.786 (SEM ±0.435) (Fig. 3). Similarly, there was no migration in 6 of 14 samples in the tbx5 MO group. Again, in those cases in which migration occurred the area of overlap was small and migration was significantly delayed, beginning in most cases on Day 6, with an average migration score of 2.000 (SEM ±0.584) (Fig. 3).

While migration can be readily observed with normal fluorescence microscopy, it can be difficult to determine whether the merged signal is due to true overlap of spreading epicardial cells in contact with the underlying myocardia; a merged signal can also result when the two tissues are simply positioned above and below each other but not in actual contact. Thus, migration was confirmed using confocal microscopy to examine samples on Day 7 for the presence of tcf21+ epicardial cells on top of, and associated closely with, the myocardial cells of recipient hearts (Fig. 2d-f). Donor epicardial cells (red) were observed covering recipient myocardial cells (green) in control samples (yellow arrows, Fig. 2d). In contrast, many recipient hearts from the sih MO and tbx5 MO groups did not have any observable donor epicardial cells covering the labeled myocardial cells (Fig. 2e-f).
Discussion

The heartbeat is halted in sih morphants [16]. Our results show that epicardial cell migration is significantly delayed or inhibited when the myocardial cells do not contract. This supports our previous results, which showed that pharmacological inhibition of heartbeat inhibited migration of PE cells and spreading of epicardial cells over the myocardium, both in vitro and in vivo [6]. A similar co-culture assay comparing migration of epicardial cells onto normal and sih MO recipient hearts was also presented in those experiments [6]. We have developed the assay further to increase precision: instead of mixing a large number of donor and recipient hearts in a 24-well culture dish, we have refined the method into using only one donor and one recipient heart per well in a culture dish. This reduces the number of hearts needed per experiment, and reduces the risk of widespread contamination across many hearts. This also addresses potential concerns that neighboring hearts could influence migration, for example, by locally increasing the concentration of a secreted paracrine factor.

Fig. 2 Migration of epicardial cells from donor hearts onto control, sih, or tbx5 MO recipient hearts. a-c Fluorescence images taken on Days 1, 3, 5, and 7 in culture show progression of epicardial cell migration. Red (tcf21:DsRed2) shows epicardial cells from the donor, green (cmic2:EGFP) shows recipient myocardial cells. a Migration of donor epicardial cells onto a control MO recipient heart is apparent by Day 5 in culture. The merged red-on-green signal, appearing yellow, is significantly noticeable by Day 7. There does not appear to be any significant migration of donor epicardial cells onto either the sih MO recipient heart (b) or the tbx5 MO heart (c) throughout the 7 days in culture. d-f Confocal microscopy images of donor/recipient heart samples after 7 days in culture. d-f Confocal microscopy verifies the presence of donor epicardial cells that have migrated onto the control MO recipient myocardium (yellow arrows). In contrast, there does not seem be any donor epicardial cells on the sih MO recipient heart (e) or the tbx5 MO recipient heart (f), which is consistent with the epifluorescence images. In this figure, single hearts were followed throughout the 7-day time course in panels a-c, and were then collected to produce the confocal images shown in panels d-f. Scale bars in all images represent 100 μm.
Although \( \text{tbx5} \) is expressed in several cardiac tissues, many studies have focused on investigating the role of \( \text{tbx5} \) expression in the PE [12]. In zebrafish, \( \text{tbx5a} \) is required for PE specification, a process that also involves BMP (bone morphogenetic protein) signals [21]. In the chick, \( \text{tbx5} \) expression is regulated in concert with initiation and cessation of cell migration. Either reducing or increasing \( \text{tbx5} \) expression in PE explants could inhibit EPC migration, \textit{in vivo} and \textit{in vitro} [12]. The investigators assessed \textit{in vitro} migration as the ability of an epicardial monolayer of cells to spread out (migrate) in a cell culture dish. However, this approach does not provide for the crosstalk that may occur with myocardial cells, which also express \( \text{tbx5} \) [12]. Here, we show that inhibiting expression of \( \text{tbx5} \) in the myocardium alone is sufficient to substantially affect epicardial cell migration. Our results add to our overall understanding of \( \text{tbx5} \) in epicardium development.

![Fig. 3 Migration scores for sih and tbx5 MO recipient hearts are significantly lower than respective controls. Each sample was scored for migration of epicardial cells as described in the Methods. Bar graphs show average migration score for each group, error bars represent standard error of the mean (SEM), asterisk indicates that the treatment group is significantly different from its respective control (Student’s t-test, \( p < 0.05 \)).](image)

It is important to recognize that in the presented assay the age of the recipient myocardial cells is different than that of the migrating donor epicardial cells, an interaction that does not happen in natural circumstances. It was necessary to use younger recipient myocardial cells in this assay so that recipient hearts lacked epicardial cells, which could influence the migration of donor epicardial cells and confound the interpretation of results. In chick models, it is possible to remove the source of epicardial cells by blocking or ablating the PE using microsurgery techniques [8, 10]. However this is difficult to replicate in zebrafish larvae. The size of the zebrafish PE is considerably smaller, making physical manipulations logistically challenging. Furthermore, multiple PEs form over multiple days of development and contribute to the zebrafish epicardium [6]. Therefore, a single ablation event cannot remove the PE.

Another possible approach is to genetically ablate epicardial cells from the recipient heart \textit{in vivo} before extraction and placement into culture with a same-age donor heart. For example, bacterial nitroreductase can be expressed in epicardial tissues using the \( \text{tcf21} \) promoter to convert nontoxic metronidazole into a cytotoxin in \( \text{tcf21}^+ \) cells [22]. However, complete ablation of epicardial cells is difficult, especially given the regenerative capacity of the zebrafish heart, as surviving epicardial cells are capable of repopulating the epicardium [22, 23]. Given these challenges, we felt that using the 60 hpf recipient heart was appropriate for the intended scope of this assay.

It is desirable to use genetic tools and techniques to study developmental processes such as epicardium formation \textit{in vivo}, however, these genetic approaches rely on the availability of a cell-specific marker to drive expression of a recombinase, transcriptional factor, or other activating enzyme in a discrete expression pattern [24, 25]. While there are well-documented examples of myocardial-specific markers (e.g., \( \text{cmlc2} \)), there are no known PE- or EPC-specific markers that are not expressed in other tissues during development. Most common markers of the epicardial lineage, \( \text{Wt1, Tbx18, Tcf21} \), are expressed in other tissues, including the liver, kidney, pectoral fin mesenchyme, developing palate, and pharyngeal arches [26–29]. Furthermore, the PE and epicardium are composed of heterogeneous populations of cells [30], for example, there are both \( \text{tcf21}^+ \) and \( \text{tcf21}^- \) cells in the zebrafish epicardium [6]. Therefore, a truly precise genetic approach would require targeted modifications using intersectional epicardial markers (e.g., use a dual recombinase approach to target gene expression in cells that are both \( \text{tcf21}^+ \) and \( \text{tcf21}^- \)). Designing and establishing such highly specific transgenic lines would take considerable time and effort. Hence, \textit{in vitro} approaches, such as the one presented here, are desirable as comparatively faster and less logistically complex.
alternatives. The presented assay can aid in identifying candidate genes involved in EPC migration and provide insight into the tissue-specific role of these genes, while using readily available genetic tools.

Conclusions
In conclusion, we have developed an assay that can assess epicardial cell migration in vitro by co-culture of a donor and recipient heart. Our assay uses whole hearts in culture, allowing for important cell-to-cell interactions between the epicardial and myocardial cells. Given that donor and recipient hearts come from different individuals, these cells can be uniquely manipulated in order to determine how effects in each cell type can influence EPC/epicardial cell migration. In addition to genetic manipulation, signaling factors, blocking antibodies, or pharmacologic agents can be readily added to the hearts before or after placement in culture media. Using our assay, we demonstrated that epicardial cell migration was inhibited when myocardial cells lacked contractility (sih MO). In addition, we demonstrated that lack of tbx5 expression in myocardial cells alone was sufficient to inhibit epicardial cell migration.

Additional file

Additional file 1: Figure S1. Hearts extracted at 60 hpf lack epicardial cells. (A and B) Confocal micrographs of cmic2:EGFP; tcf21:DsRed2 hearts extracted at 60 hpf. Images show brightest point projections from confocal z-series. (A) cmic2:EGFP; tcf21:DsRed2 hearts before being placed into culture (Day 0). (B), cmic2:EGFP; tcf21:DsRed2 hearts after 7 days in culture (Day 7). There were no epicardial cells (red) observed on the heart myocardia (green) at Day 0 or Day 7. In addition, there were no observed tcf21+ cells with the stereotypically flattened phenotype of epicardial cells present on top of the myocardium (blue, DAPI nuclear staining). Scale bars in all images represent 50 μm. (PNG 965 kb)

Abbreviations
bpm: Beats per minute; BMP: Bone morphogenetic protein; cmic2: Cardiac myosin light chain 2; DAPI: 4',6-diamidino-2-phenylindole; DMEM: Dulbecco’s modified Eagle’s medium; DsRed2: variant of Discosoma sp. red fluorescent protein; EGFP: Enhanced green fluorescent protein; EPC: Epicardial progenitor cell; EPD: Epicardium-derived cell; hpf: Hours post fertilization; MO: Morpholino oligonucleotide; PBT: 0.3 % Triton X-100 in phosphate buffered saline; PE: Proepicardium; SEM: Standard error of the mean; sih: Silent heart; T-box 18; tcf21: Transcription factor 21; tnnt2: Cardiac troponin T2; WT1: Wilms tumor 1.

Competing interests
The authors declare no competing interests.

Authors’ contributions
MSY, JP, and XL were responsible for the planning and execution of laboratory work, collection and processing of data, and preparation of the manuscript. WH and REP directed research. WH and REP provided supervision, intellectual input and funding. All authors participated in the interpretation of results and approve of the submitted manuscript.

Acknowledgements
This research was funded by the National Institute for Environmental Health Sciences grants (T32 ES007015 and (R01 ES012716) to WH. and REP. The authors wish to thank Dr. K. Lanham, Dr. T. Baker, Dr. F. Burns, J. Gawdzik, and J. Gilbertson for advice and general support in conducting this research.

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Received: 7 July 2015 Accepted: 22 December 2015
Published online: 29 December 2015

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