Subpopulation of Proepicardial Cells Is Derived From the Somatic Mesoderm in the Chick Embryo

Jan Schlueter, Thomas Brand

Rationale: The proepicardium (PE) is a transient structure forming at the venous pole of the heart and gives rise to the epicardium, fibroblasts, and smooth muscle cells. The embryological origin of the PE is presently unclear. Asymmetrical formation of the PE on the right inflow tract is a conserved feature of many vertebrate embryos, and in the chicken is under the control of fibroblast growth factor 8 and snail homolog 1.

Objective: To gain further insight into the process of asymmetrical PE formation, we studied the role of TWIST1 during PE formation in the chick embryo.

Methods and Results: TWIST1 is asymmetrically expressed on the right side in the somatic mesoderm under the control of snail homolog 1. Fate mapping experiments revealed a contribution of the somatic mesoderm to the PE. After colonization of the heart, this cell lineage gives rise to the epicardium, smooth muscle cells, and potentially fibroblast. Suppression of TWIST1 function in the right coelomic cavity caused a severe disruption of the villous protrusions of the PE and Wilms tumor 1 and transcription factor 21 expression. Rescue with the corresponding mouse cDNA normalized gene expression and PE morphology. Forced expression of TWIST1 on the left side induced ectopic expression domains of Wilms tumor 1 and transcription factor 21.

Conclusions: A significant proportion of the PE has its origin outside of the currently proposed domain in the splanchnic layer of the lateral plate mesoderm. The phenotype in embryos subjected to TWIST1 loss- or gain-of-function suggests an important contribution of somatic mesoderm to the mesothelial cell layer of the PE. (Circ Res. 2013;113:1128-1137.)

Key Words: developmental biology ■ epicardium ■ epithelium ■ fate mapping ■ genes, developmental ■ heart development ■ mesoderm ■ pericardium

The proepicardium (PE) is an agglomerate of progenitor cells at the venous pole of the heart and has been described in many vertebrate species ranging from lampreys to humans.1–5 The significance of this cell cluster lies in the formation of the epicardium, which covers the myocardial surface and the delivery of vasculogenic cells and fibroblasts to the developing heart.6,7 Recent studies that describe novel proepicardial marker genes gave support to the fact that the PE is a heterogeneous cell population.8–11 This is in accordance with earlier observations derived from quail-chick chimeras, which show that PE cells are the main source for the epicardium, coronary vasculature, and fibroblasts.12 Moreover, cultured PE cells are able to differentiate into cardiac myocytes,13,14 suggesting either a potential to differentiate into diverse cell types or alternatively that the PE harbors several progenitor populations. It is thought that cells forming the PE have their origin in the periphery of the heart fields15,16; however, at present, it cannot be ruled out that PE cells have different embryological origins.

Another aspect of PE development, which has been described in several vertebrates, is an asymmetrical development on the right side of the cardiac inflow tract.1–5 Visceral organs display left-right asymmetry, which is under the control of the highly conserved NODAL/paired-like homeodomain transcription factor 2 (PITX2) pathway.17,18 This pathway acts in the left lateral plate mesoderm (LPM) and is induced during gastrulation. The initial events that lead to asymmetrical NODAL expression are possibly diverse and involve directed nodal flow,19,20 asymmetrical cell migration guided by an asymmetrical ion flux,21,22 and intracellular tubulin.23 There is only limited knowledge about the processes that translate asymmetrical gene expression into asymmetrical organogenesis. Recent insight from studying gut formation, for example, suggests that asymmetries in both extracellular matrix and cell adhesion molecules establish the direction of gut looping.24,25
In this study, we sought to gain insight into the mechanism of asymmetrical PE formation. We have previously shown that asymmetry of PE formation in the chick embryo is not determined by the left-sided NODAL/PITX2 pathway but is under the control of a right-sided fibroblast growth factor 8 (FGF8)/snail homolog 1 (SNAI1) pathway.26 However, it was unclear as to how this early right-sided pathway is capable of affecting PE formation in the cardiac inflow tract, given that the morphogenesis of most structures of the venous pole are under the control of the cardiac inflow tract, given that the morphogenesis of most sided pathway is capable of affecting PE formation in the metrical organogenesis.

We now identified NODAL/PITX2 structures of the venous pole are under the control of the cardiac inflow tract, given that the morphogenesis of most sided pathway is capable of affecting PE formation in the metrical organogenesis.

**Results**

**TWIST1 is Asymmetrically Expressed in the LPM**

TWIST1 is a basic helix-loop-helix transcription factor and has been described in many studies to be regulated by SNAI1 and to promote epithelial-mesenchymal transition and cell invasion.37,38 To search for target genes of the FGF8/SNAI1 pathway, which regulate right-sided proepicardial development,26 we analyzed TWIST1 expression before and during PE formation. At HH stage 10,29 we found TWIST1 to be asymmetrically expressed in the LPM. Expression was more pronounced on the right side in a domain close to the venous pole of the heart (Figure 1A and 1E). Moreover, TWIST1 expression was confined to the somatic mesoderm, whereas the splanchnic layer was devoid of expression. At HH stage 12, TWIST1 expression was present in the right but not the left sinus venosus (Figure 1B and 1F).

**Whole Mount In Situ Hybridization, Immunohistochemistry, and 3-Dimensional Reconstruction**

Whole mount in situ hybridization was carried out as previously described.14 BCIP/NBT was used as a substrate. Antisense RNA probes for chicken Wilms tumor 1 (WT1) and transcription factor 21 (TCF21) were generated from full-length cDNA clones. Histology and immunohistochemistry were carried out as previously described.13 The 3-dimensional reconstruction of the inflow tract and the PE using Amira 5.4 was performed as previously described.26 The assembly of z-stacks from the Zeiss LSM510 was done with Velocity (Perkin Elmer).

An expanded Methods section is available in the Online Data Supplement.

**Methods**

**Embryonic Manipulations**

White Leghorn eggs were incubated at 37°C until they reached the desired stage.49 The embryos were cultured using the EC culture method.29 1,1′-Dioctadecyl-3,3,3′,3′-tetrachlorofluorescein perchorlate (DiI) injections were placed into the right somatic mesoderm of Hamburger-Hamilton (HH) stage 10 embryos. To deliver shRNA constructs, 25 nL of viral particles were injected into the right coelomic cavity. Electroporation of embryos was carried out with TWIST1-pcDNA3.1 and SNAI1-pcDNA3 constructs, as previously described.30,31 For visualization of electroporated cells, either an enhanced green fluorescent protein (eGFP) vector (pCCAGGS-GFP) or a red fluorescent protein (RFP) vector (pKFPRNAIC)30 was used. Long-term fate mapping was carried out using orthotopic grafting of GFP-labeled PE in shell-less culture. Statistical analysis was performed using the 2-tailed z test or t test, and statistical significance was accepted at P<0.05.
the onset of proepicardial marker gene expression (Figure 1C and 1G). TWIST1 expression was particularly high in the right somatic mesoderm that is attached to the right inflow tract via the lateral mesocardium and which later develops into the cardinal veins (Figure 1G). At HH stage 16, when the PE has fully formed, TWIST1 expression was absent (Figure 1D and 1H).

We previously showed that SNAI1 expression is maintained in the right LPM and the right inflow tract up to HH stage 11. To test whether TWIST1 acts downstream of SNAI1, we performed gain-of-function experiments in which SNAI1 was ectopically expressed in the left LPM at HH stage 6 and resulted in an upregulation of TWIST1 in the right inflow tract (9 of 17; 53%; Online Figure I). Conversely, application of SNAI1 antisense oligonucleotides at HH stage 8 caused a loss of TWIST1 expression in the right inflow tract (12 of 19; 63%). These data suggest that TWIST1 is a downstream target of SNAI1. We performed transfection experiments in which SNAI1 was ectopically expressed in the right LPM at HH stage 6 and resulted in an upregulation of TWIST1 expression in the right inflow tract (9 of 17; 53%; Online Figure I). Conversely, application of SNAI1 antisense oligonucleotides at HH stage 8 caused a loss of TWIST1 expression in the right inflow tract (12 of 19; 63%). These data suggest that TWIST1 is a downstream target of SNAI1 in the LPM. The fact that TWIST1 expression is not maintained in the PE indicates that TWIST1 acts before the PE has fully formed.

Proepicardial Lineage Originates From the Somatic Mesoderm Layer

Although it has been proposed that PE cells are derived from the peripheral portion of the heart fields, it is worth noting that the PE is composed of different cell types (mesothelial, mesenchymal, and endothelial cells) whose developmental origin might possibly be diverse. We performed fate-mapping experiments to test the hypothesis that a subset of PE cells was actually derived from the somatic mesoderm. We first DiI-labeled somatic mesodermal cells lateral to the right sinus in cultured chick embryos at HH stage 10 and followed their fate until HH stage 16 (Online Figure II). Labeled cells were found in the pericardial fold but also on the right sinus and in the PE. The migrating cells generated a continuous track from the site of DiI injection to their different destinations. Apparently cells were migrating along the lateral mesocardium, which bridges the pleural cavity at this stage to enter the cardiac inflow tract ultimately. The DiI-labeled somatic mesoderm cells only ended up in the PE at HH stage 16 when they were located within the TWIST1 expression domain at HH stage 10 (Online Figure III). To achieve a more precise labeling, a GFP expression vector was electroporated either into the somatic mesoderm dorsal to the venous pole or into the inflow tract. When the somatic mesoderm layer was electroporated at HH stage 9 and GFP-expressing cells were followed up to HH stage 16, these cells entered the lateral mesocardium and the right sinus horn and were also found in the PE (Figure 2A–C; Online Figure II). It is worth noting that especially the outer mesothelial layer was labeled, which forms the villous protrusions of the mature PE. In contrast, electroporation of the right inflow tract at late HH stage 11 led to strong GFP expression in the right sinus and throughout the entire PE at HH stage 16, which suggests that at HH stage 11 PE progenitors are already present in the splanchnic mesoderm layer (Figure 2D–F). The combination of these 2 fate-mapping approaches using GFP and RFP expression vectors labeled 2 largely distinct cell populations (Online Figure IV). A few cells were labeled with GFP and RFP, which is likely due to the fact that some GFP-labeled somatic mesoderm cells already entered the right inflow tract at late HH stage 11 when the electroporation with the RFP expression vector was performed. To ensure that the electroporation technique used was able to label cells in a site-specific manner, somatic mesoderm was electroporated either on the left or right body side at HH stage 9 (Figure 2G–I). The embryos were harvested...
just after the onset of GFP expression. Labeled cells were only found in the somatic mesoderm layer demonstrating that tissue-specific labeling can be achieved with the electroporation technique used here.

To follow the fate of PE cells of somatic mesodermal origin, we performed orthotopic grafting experiments. For this purpose, the somatic mesoderm of donor embryos at HH stage 9 were electroporated with the GFP expression vector and cultured until HH stage 17. The right sinus, including the PE, was excised and grafted orthotopically into host embryos of the same age (Figure 3A and 3B). GFP-labeled cells colonized the ventricle and participated in epicardium formation (Figure 3C and 3D). Labeled cells also invaded the ventricular wall and displayed a scattered distribution. Some GFP-positive cells were found to be localized directly adjacent to coronary vessels and expressed smooth muscle α-actin (Figure 3E and 3F). However, endothelial cells were not GFP-labeled. These data suggest that the somatic mesoderm–derived PE lineage gives rise to the epicardium, smooth muscle cells, and possibly fibroblasts.

Loss of TWIST1 in the Right Coelomic Cavity Disrupts the Formation of Proepicardial Villous Protrusions

Because progenitor cells from the right somatic mesoderm contributed to the PE and TWIST1 was expressed in this region, we investigated whether manipulation of TWIST1 expression affected PE formation. For this purpose, we generated viral Replication-Competent Avian sarcoma-leukosis virus long terminal repeat with a Splice acceptor (RCAS) constructs that express shRNAs targeted against TWIST1. With this approach, a loss of TWIST1 transcript of ≈70% was achieved (Figure 4L). To monitor the level of infection, an RCAS-GFP construct was used. The viral particles were injected into the right coelomic cavity of embryos at HH stage 8 to 10 and the embryos were cultivated up to HH stage 17 to 18. At the stage of harvest, GFP-expressing cells were found in the right coelomic cavity, the right sinus, and the PE (Online Figure V). We did not observe any discernible effect on embryonic development when the TWIST1 virus was injected after HH stage 9. However, virus injection before HH stage 9 led to a specific proepicardial phenotype. The PE of embryos in which TWIST1 was knocked down was of smaller size and malformed in comparison with the controls (39 of 65; 60%; Figure 4I–K; Online Figure V). Obviously, the formation of villous protrusions was impaired. We checked expression levels of TCF21 (also known as Epicardin, Capsulin, or Pod1), which normally is expressed throughout the PE, the inflow tract mesenchyme, and particularly in the villous protrusions (Figure 4A and 4E). In loss of TWIST1 embryos, the villous mesothelium was largely disrupted and TCF21 expression was lost on the proepicardial surface (Figure 4B and 4F) but was retained in the underlying mesenchyme. Additionally, we analyzed the expression of WT1, another established PE marker gene. The loss of TWIST1 caused alterations in the expression pattern, which resembled that of TCF21 (Figure 4C, 4D, 4G, and 4H).

To appreciate the structural changes of the proepicardial surface, whole mount specimen were scanned with the help of a confocal microscope and the z-stack was reconstructed in Velocity. In control embryos, the PE was covered with a mesothelium, which consisted of evenly distributed and densely packed epithelial cells, which formed the characteristic villous structure of the PE (Online Figure V). Loss of TWIST1, however, led to a reduction of the mesothelial layer, and epithelial cells that were left on the PE were much less dense and villous protrusion were only found in the form of irregular and isolated clusters, which resulted in an overall smaller PE. Terminal deoxynucleotidyl transferase dUTP nick-end labeling analysis revealed that these differences were not caused by enhanced apoptosis, nor was there an effect on the relative proliferation rate (Online Figure V). We used Amira 3-dimensional reconstruction to illustrate and quantify the size differences further of the PE from control and loss-of-TWIST1–treated embryos and were able to determine an ≈50% volume reduction of the PE because of the loss of extensive villous protrusions (n=3; Figure 4I–K). These experiments demonstrate that TWIST1 acts in a time window from stage HH 8 to the formation of the PE and confirm that loss of TWIST1 in the right coelomic cavity has an impact on the mesothelial portion of the PE.
We also investigated the surrounding tissues, especially the heart, for evidence of any malformations caused by the loss of TWIST1 virus, but cardiac looping and the size and shape of the heart were unaffected as was the overall growth of the embryo (Online Figure V). However, there was one additional phenotype that was detected if TWIST1 virus was injected before HH stage 9. Loss-of-TWIST1 virus-injected embryos displayed an inversion of embryonic turning (loss of TWIST1, 8 of 37, 21%; controls, 2 of 36, 5.8%; P < 0.05; Online Figure V).

Overexpression of TWIST1 in the Left Somatic Mesoderm Leads to Cell Invasion Into the Cardiac Inflow Tract and Induces Ectopic WT1 and TCF21

Because TWIST1 expression in the right coelomic cavity was necessary for the formation of villous proepicardial mesothelium, we wanted to corroborate further our initial hypothesis that TWIST1 is capable of mobilizing cells from the somatic mesoderm, which subsequently contribute to the PE. Therefore, we also performed gain-of-function experiments in which expression vectors encoding TWIST1 and GFP were coelectroporated into the left somatic mesoderm, which normally has a lower level of TWIST1 than the right side (Figure 1). Embryos were electroporated at HH stage 9 and incubated up to HH stage 16 and GFP expression was monitored at different stages (Figure 5A–E). Overexpression of TWIST1 in the left somatic mesoderm promoted in most embryos a strong invasion of cells into the left cardiac inflow tract and even the atrium (16 of 19; 84%), whereas in control embryos, GFP-positive cells were found in the left pericardial fold and the somatic mesoderm and scarcely in the inflow tract (14 of 14; 100%).

Rescue of TWIST1 in the Right Somatic Mesoderm Restores the Formation of Proepicardial Villous Protrusions

To validate that TWIST1 acts in the right somatic mesoderm as an important determinant for asymmetrical PE development, we performed a rescue experiment. We combined the loss-of-function approach with region-specific overexpression by electroporation. The viral TWIST1 shRNA particles were injected into the right coelomic cavity, and at the same time, the right somatic mesoderm was coelectroporated with TWIST1 and RFP constructs (Figure 6A). As previously observed, the RFP-expressing cells entered the inflow tract and were found in the PE (Figure 6B and 6C). Embryos that were treated with virus against TWIST1 but electroporated with an

Figure 4. Loss of TWIST1 has a strong impact on proepicardial development. A to D, Whole mount in situ staining of chick embryos detecting TCF21 (A and B) and WT1 (C and D) expression after infection with TWIST1 and control virus, respectively, on the right side of the embryo. E to H, Transverse sections through the inflow tract region of the embryos shown in (A–D). TWIST1 virus-infected embryos show a disrupted structure of the villous mesothelium and a loss of TCF21 and WT1 expression. I and J, 3-Dimensional reconstruction illustrates the smaller size of the proepicardium (PE; blue) in loss-of-TWIST1 embryos. K, Estimates of the PE volume using the 3-dimensional reconstruction reveal a reduction by ~50% in the loss of TWIST1 (n=3). L, Real-time PCR analysis of TWIST1 in DF1 cells, which were infected with TWIST1 or control virus demonstrating a loss of ~70% (n=5) of TWIST1 transcripts after shRNA treatment. K and L, Error bars indicate ±SD; *P<0.05. Scale bars, 150 µm.
empty expression vector (10 of 18; 55%; \( P < 0.05 \); control, \( n = 10 \)) displayed a reduction of \( TCF21 \) expression, an aberrant PE morphology, and 50% size reduction when compared with control embryos (\( n = 3 \); Figure 6D, 6E, 6G, 6H, 6J, 6K, and 6M). In addition, those disrupted PEs had difficulties to attach to the ventricle, most likely because of their smaller

![Diagram](image1)

**Figure 6. Rescue of proepicardium (PE) formation by forced expression of **\( TWIST1 \). **A**, Schematic representation of the experimental design. A \( TWIST1 \)/red fluorescent protein (RFP) plasmid was electroporated into the right somatic mesoderm layer and subsequently \( TWIST1 \)-shRNA-expressing virus particles were injected into the right coelomic cavity. **B** and **C**, The RFP signal was located close to the right sinus at Hamburger Hamilton (HH) stage 12, whereas at HH stage 17 it was localized to the PE (white arrowhead). **D** to **L**, Whole mount in situ hybridization for \( TCF21 \) in control (**D**), loss of function (**E**), and rescued embryos (**F**). **G** and **H**, Arrowheads point to the distorted PE epithelium of loss-of-function embryos and the normal-seeming epithelial surface of rescued embryos, respectively. **J** to **L**, 3-Dimensional reconstruction of the PE structure in control (**J**), loss of function (**K**), and rescued embryo (**L**). Note the similar size of the PE in control and rescued embryos. **M**, Quantification of PE volume (\( n = 3 \)). Error bars indicate \( \pm SD \); *, **\( P < 0.05 \); **n.s. Scale bars, 100 \( \mu m \).

![Diagram](image2)

**Figure 5. Overexpression of **\( TWIST1 \) **leads to cell invasion of the cardiac inflow tract and ectopic marker gene expression.** **A**, **B**, and **D**, Forced expression of \( TWIST1 \)/green fluorescent protein (GFP) in the left somatic mesoderm leads to an enhanced invasion of GFP-positive cells into the left cardiac inflow tract (white arrowhead) in comparison with control embryos (**C**) in which GFP-positive cells are only found in the pericardial fold (P). **E**, Immunohistochemistry detection of VMHC demonstrates that the GFP-positive cells that invaded the heart are not cardiac myocytes. A indicates atrium; and V, ventricle. **F** to **Q**, Forced expression of \( TWIST1 \) induces ectopic expression of \( TCF21 \) and \( WT1 \) in the left sinus horn in comparison with controls. **G** and **M**, GFP-positive cells in the left sinus horn (white arrows). **J**, **K**, **O**, and **Q**, Blue arrows indicate ectopic \( TCF21 \) and \( WT1 \) expression domains in the left sinus horn. Scale bars, 150 \( \mu m \).
size (Figure 6E, 6H, and 6K). However, when embryos were treated with virus against TWIST1 and at the same time subjected to forced expression of TWIST1 in the right somatic mesoderm, the proepicardial surface was significantly less disrupted (12 of 21, 57%; P<0.05; Figure 6F, 6I, 6L, and 6M). In such embryos, the proepicardial expression of TCF21 was not diminished and the villous outgrowth was largely restored to the point that they were able to attach to the ventricular surface. The rescued PEs displayed an overall larger volume in comparison with that of loss-of-function embryos and reached almost the size of controls (Figure 6J–M). These experiments demonstrate that expression of TWIST1 in the right somatic mesoderm is crucial for the invasion of mesothelial progenitor cells into the inflow tract and for the proper formation of proepicardial villi.

**Somatic Mesodermal Cells Require TWIST1 to Participate in PE Formation**

To corroborate further that TWIST1 is essential for the migration of somatic mesodermal progenitor cells to the sinus horn and their participation in PE formation, embryos injected with control GFP-expressing virus were electroporated with an RFP control construct. Under these conditions, the PE was found to harbor cells that coexpressed GFP and RFP (n=12; Figure 7A–C; Online Figures VI and VII). In TWIST1 loss-of-function embryos that were electroporated with an RFP/control vector, no labeled cells were found in the PE but were readily detected in the somatic mesoderm (n=15; Figure 7D–F; Online Figures VI and VII). In rescue embryos, TWIST1 shRNA/GFP virus was injected, but in this case combined with forced expression of TWIST1 in the somatic mesoderm using an RFP/TWIST1 expression vector. Under these conditions, cells were found in the PE that were co-labeled with GFP and RFP (n=18; Figure 7G–I; Online Figures VI and VII).

**Discussion**

Formation of the epicardium and the coronary vasculature is preceded by the development of the PE, an accumulation of progenitor cells at the venous pole of the heart. In this study, we provide evidence for a novel origin of a subpopulation of proepicardial cells in the chick embryo. We show that there is a contribution of somatic mesodermal cells to the mesothelial portion of the PE that forms the typical villous protrusions. Currently, it is thought that the PE originates from the periphery of the heartforming fields in the LPM and is part of an early cardiac progenitor lineage. This was concluded from fate-mapping experiments and Cre-recombinase studies in which Isl1- and Nkx2.5-Cre labeled cells were found in the PE. The PE is composed of an outer layer of mesothelial cells and an inner core of mesenchymal cells, which are most likely derived from the cardiogenic mesoderm of the inflow tract. Our experiments indicate that there is a common origin of the superficial proepicardial mesothelium and pericardial cells, which are derived from the somatic mesoderm. In a previous study, we already proposed a close relation of both tissues because they form a continuous layer inside the pleural cavity and coexpress many genes like T-box transcription factor 18 and WT1.

In the chick, we found progenitor cells that reside in the somatic mesoderm to enter the cardiac inflow tract via the right lateral mesocardium and participate in PE formation. Because the PE forms on the right side in several vertebrate species, asymmetrical cell invasion might be conserved. We identified the transcription factor TWIST1 to be responsible for this right-sided invasion of the inflow tract. The knockdown of TWIST1 in the right somatic mesoderm disrupted the formation of proepicardial villi, whereas forced expression of TWIST1 in the left somatic mesoderm promoted cell invasion into the left inflow tract and ectopic expression of TCF21 and WT1. Therefore, we conclude that TWIST1 is necessary and sufficient for PE induction. It must be noted, however, that forced expression did not lead to the formation of a mature PE displaying villous outgrowth on the left body side. This might be because of the fact...
that in our experiment the somatic mesoderm cells maintained TWIST1 expression, whereas normally TWIST1 is downregulated before PE formation commences. Inhibition of bone morphogenetic protein (BMP) causes a massive reduction of proepicardial marker gene expression, and it was proposed that BMP is an essential inducing signal. On the basis of our new data, the function of BMP could also be interpreted in a different way. Possibly the symmetrical BMP2 expression domains in both sinus horns is involved in recruiting sufficient numbers of somatic mesoderm progenitor cells by chemotraction. In support of this assumption, it has recently been shown that PE cells are attracted to the heart by a BMP gradient.

In this study, we demonstrate that TWIST1 is a downstream target of SNAI1, which we previously implicated in the induction of a right-sided PE. In this earlier study, we have shown that the NODAL–PITX2 pathway does not interfere with asymmetrical proepicardial marker gene expression. This now becomes more plausible because PITX2 is not expressed in the somatic mesoderm layer; therefore, overexpression of PITX2 on the right side could not interfere with the right-sided bias in the somatic mesoderm that is generated by TWIST1. Others have shown that loss of SNAI1 is causing the inversion of embryonic turning in mice. Interestingly, the knockdown of TWIST1 also causes aberrant embryonic turning, suggesting a conserved pathway that regulates embryonic turning in amniotes. Clockwise axial rotation in chick and mouse embryos has been associated with differential proliferation; however, asymmetrical cell migration and cell shape changes may also be involved. Significantly, embryonic turning like asymmetrical PE development is not affected by the loss of PITX2. TWIST1 has been repeatedly reported to induce epithelial–mesenchymal transition in cooperation with SNAI1 and to promote cell invasion during cardiac regeneration, valve formation, and the migration of cardiac neural crest cells. Particularly relevant in this context is the fact that TWIST1 seems to be crucial for mobilizing extracardiac cell populations like cardiac neural crest cells and epicardium-derived cells because our own observations suggest a similar role for TWIST1 in early PE development. Our data from the chick might also be of significance for other vertebrates because TWIST1 is expressed in the murine pericardium and somatic mesoderm.

The idea that the PE consists of multiple progenitor cells with diverse origins is corroborated by various studies. Distinct populations of endothelial cells within the PE have been observed that share marker gene expression with endothelial cells from the liver or the inflow tract. Expression of Scleraxis and Semaphorin 3D defines proepicardial subcompartments, which contribute to the coronary vascular endothelium and are distinct from the T-box transcription factor 18/WT1 expressing cell population, which gives rise to fibroblast and smooth muscle cells. TCF21-expressing cells seem to be fate-restricted to the cardiac fibroblast population. Therefore, the idea of distinct cellular compartments with differing molecular profiles in the PE is particularly interesting because we observed a loss of WT1- and TCF21-expressing villous mesothelium after knockdown of TWIST1. In addition, our long-term fate-mapping revealed a contribution of GFP-labeled somatic mesodermal cells to the epicardium, smooth muscle cells and possibly fibroblasts. However, endothelial cells were not labeled in this fate-mapping approach, suggesting that endothelial cells are not derived from the somatic mesoderm but most likely have different origins as discussed above. Our concept that a subpopulation of the mesothelial portion of the PE is derived from the somatic mesoderm underlines the fact that the PE represents a focal point for different extracardiac progenitor cells that accumulate at the venous pole. The mesothelium is hereby of special importance because it represents a highly invasive and multipotent extracardiac cell type, which forms the epicardium and thereby drives the maturation of the embryonic heart.

The conclusion that TWIST1 is cell-autonomously required for somatic mesoderm migration is inferred from experiments involving knockdown of TWIST1 with a virus applied to the coelomic cavity and thus may also have had an impact on splanchnic mesoderm or the PE. We consider this interpretation, however, to be unlikely given that TWIST1 is undetectable in these tissues by in situ hybridization. In most cases, TWIST1 is involved in the control of epithelial–mesenchymal transition and thereby promoting cell migration. However, it is also possible that TWIST1 induces expression of signaling molecules that promote cell invasion noncell-autonomously. There is ample evidence for a role of different signaling molecules in PE formation. To distinguish between a cell-autonomous and a nonautonomous role of TWIST1, somatic mesoderm–specific gene ablation will ultimately be required.

We have provided ample evidence for the existence of a cell population in the somatic mesoderm that participates in PE development. We now propose a novel model for the formation of the proepicardial mesothelium in which TWIST1 acts downstream of the right-sided FGFR/SNAI1 pathway and is involved in cell mobilization of pericardial progenitor cells that contribute to asymmetrical PE formation in the chick (Figure 8). The translation of left-right signaling into biased cellular mobility, which ultimately impacts on asymmetrical organogenesis, is a mechanism that might also be observed in other vertebrates.
Acknowledgments
We thank Angela Torlopp for carefully reading the manuscript and Ursula Herbst-Brand for technical assistance.

Sources of Funding
This work was supported by Imperial College London and the Magdi Yacoub Foundation.

Disclosures
None.

References
What Is Known?

- Proepicardium formation in many vertebrates is asymmetrical and under the control of a right-sided signaling pathway.
- A currently popular model proposes that the proepicardium and the venous pole myocardium have a common embryonic origin. Specific combinations of signaling factors allocate cells to either lineage.
- The proepicardium hosts progenitor cell populations that form the epicardium, the subepicardial mesenchyme, and epicardium-derived cells, which develop into the coronary vasculature, cardiac interstitial, and perivascular fibroblast.

What New Information Does This Article Contribute?

- The transcription factor TWIST1 is transiently expressed in a progenitor population in the somatic layer of the lateral plate mesoderm that contributes cells to the proepicardium.
- Loss- and gain-of-function experiments show that TWIST1 is required for normal proepicardium formation and mobilization of the cells to the developing heart.
- This TWIST1-positive cell population gives rise to the epicardium, smooth muscle, and fibroblast lineages, but not to endothelial cells.

Novelty and Significance

The proepicardium is considered a multipotential cell population, which can give rise to many different cell populations. It is now firmly established that the proepicardium is made up of several cell populations, which differ in their developmental origin and function. The findings might have considerable implications for understanding cardiac development, regeneration, and fibrosis in the diseased heart. This work also establishes that 2 pathways exist that control left-right asymmetrical development of the heart. One pathway, which acts on the left side of the embryo in the splanchnic mesoderm layer, involves NODAL and PITX2 genes, which control several processes in morphogenesis, including the asymmetrical positioning of the large arteries or the sinoatrial node. On the right side of the embryo, a different pathway involving FGF8, SNAI1, and TWIST1 genes is active in the somatic mesoderm layer. We demonstrate that this right-sided pathway determines asymmetrical proepicardium formation by mobilizing TWIST1-positive cells, which migrate into the venous pole myocardium and participate in proepicardium formation. This right-sided pathway also seems to control embryonic turning. We speculate that the right-sided pathway controls additional aspects of asymmetrical morphogenesis of the heart.
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_Circ Res._ 2013;113:1128-1137; originally published online September 9, 2013; doi: 10.1161/CIRCRESAHA.113.301347

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Expanded Methods Section

Generation of retroviral constructs
The shRNA oligos directed against chicken TWIST1 were designed using the design tool from Invitrogen (http://rnadesigner.invitrogen.com/rnaiexpress/). Three shRNA oligonucleotides and three scrambled control oligonucleotides were designed\(^1\). Twist1 a: GATCCA CATCGA CTTCCT CTACCA GGTCTT TTCAAGA GAAAG ACCTGG TAGAGGA.

Twist1 b: GATCC GTCCA AGATGG CAAGCT GCAGCT ATGTCA AGAGAC ATAGC TGCAGC TTGCCA TCTTGG ATTTT TGC.

Twist1 c: GATCC GTCGAC TTCCTC TACCAG GTCTT ACATTC AAGAGATGTAAG ACCTGG TAGAGGAAGT CGATT TTTGC, control 1: GATCCG CCTCGT TATCATC ACTCCGT TACATTC AAGAGAT GTAACGG AGTGATG ATAACGA GGCAAA AAGC, control 2: GATCCG GCCAGC GAACGT TTTACG CTACAGA GGTGTTT CAAGAGA ACACCT CTGTGAC GTAAGG TGCTCC TGAATC GC.

The oligonucleotides were cloned via BamH1 and Not1 into the pENTR3c-H1 vector (Sheri Holmen, Van Andel Institute, Grand Rapids). A Gateway cloning step (Gateway Vector Conversion Reagent System, Invitrogen) was used to generate an RCAS construct, which harbours the shRNA expressing sequence and the H1 promoter. RCAS viruses were propagated in DF1-cells and virus titre was assessed using a serial dilution\(^2,3\). Real Time PCR analysis was performed using a TaqMan probe against chicken TWIST1 (Applied Biosystems)\(^4\).

Embryonic manipulations
White Leghorn eggs were incubated at 37°C in a moist atmosphere until they reached the desired stage\(^5\). The embryos were cultivated using the EC culture method\(^6\) in which a filter paper aperture is placed on the embryo to maintain the tension of the vitelline membrane. The embryos were cut out around the filter paper and inversely placed on an 35mm dish filled with agar/albumin. Dil was prepared as a 5mM stock solution in ethanol and used as a 5µM solution in PBS. Dil injections of 5nl were placed into the right somatic mesoderm of HH stage 10 embryos, incubated for 2 min and then excess Dil was removed by pipetting PBS on the embryo and draining it afterwards.\(^7\) The delivery of shRNA expressing constructs was carried out by injecting 25 nl of viral particles into the right coelomic cavity. The embryos were subsequently incubated overnight. Electroporation was carried out by injecting 2µg/µl DNA (Twist1-pcDNA3.1, Tom Jessel, Columbia University, New York and Snai1-pcDNA3, Angela Nieto, Instituto de Neurociencias, Alicante), which was subsequently transfected by 4 pulses of 9V with a duration of 250ms. The visualization of transfected cells was achieved by using either GFP (pCAGGS)\(^8\) or RFP (pRFP-RNAIC, Ark Genomics) expressing vectors. The loss of SNAI1 was achieved using antisense oligonucleotides, which were applied in a concentration of 2mM in 20% pluronic gel.\(^9\) The long-term fate mapping of somatic mesodermal cells was achieved by transplantation of the GFP-labelled PE cluster into a host embryo. The host embryos were cultivated up to HH stage 17 in shell-less culture by transferring the entire egg content into a plastic weighing boat (diameter 10cm) and sealing the top carefully with plastic foil. The labelled PE cluster was excised and transferred to the host using a scaffold made of a small piece of folded aluminium foil. This scaffold was also used to stabilize the transplant in close proximity to the host heart. The embryos were then incubated up to an additional four days. BrdU labelling was achieved by using 1mM BrdU in PBS. Embryos were incubated for 3 h, then fixed and processed for paraffin histology and immunohistochemistry. Paraffin sections were cooked in 10mM sodium-citrate including 0.05% Tween (pH 6) followed by 15 min of denaturation in 2N HCl. BrdU antibody (Roche) was used at 1:100 and incubated overnight at 4°C. The smooth muscle actin antibody (Abcam) was used at 1:400, the anti GFP and anti RFP antibodies (Abcam) were
used at 1:200, all of them were incubated overnight at 4°C. Statistical analysis was performed using the two-tailed z-test or t-test and statistical significance was accepted at P<0.05.

Whole mount in situ hybridization, Immunohistochemistry and 3D reconstruction

Whole mount in situ hybridization was initiated by rehydrating the embryos from 100% methanol in which they were stored at -20°C. The embryos were subjected to hydrogen peroxide bleaching followed by a short proteinase K treatment and refixation with PFA/glutaraldehyde before being prehybridized for 2 hrs. The hybridization with digoxigenin-labelled probes detecting TCF21, WT1 or TWIST1 mRNA was performed overnight at 70°C. After extensive washing, the embryos were blocked in 10% sheep serum and subsequently incubated with an anti-digoxigenin alkaline phosphatase-conjugated antibody (1:4000). After extensive washing to remove residual antibody, the embryos were finally stained with BCIP/NBT as substrate to visualize the mRNA as blue precipitation. The embryos were subjected to paraffin histology and 10 µm sections of the desired regions were generated. Whole mount immunohistochemistry was performed similar to the in situ hybridization protocol but the initial RNA hybridization was replaced by an overnight incubation with the primary antibody and the proteinase K and the refixation steps were omitted. A GFP-antibody from Abcam (ab290) was used in a dilution of 1:400 to visualize GFP-expressing cells. Electroporated embryos were counterstained using a 1:1000 dilution of WGA-Alexa 555, mounted on chambered slides in Mowiol and imaged with a Zeiss confocal LSM510 microscope. The reconstruction of the PE cell cluster with AMIRA was done by using a stack of paraffin sections of the inflow tract at the desired stage. The illustration with Volocity was done by importing and assembling a z-stack of images obtained from the confocal microscope Zeiss LSM510.

References


Online Figure I. *TWIST1* is regulated by *SNAI1* in the lateral plate mesoderm. *SNAI1* and GFP expression constructs were co-electroporated into the left somatic mesoderm. (A,B) Detection of GFP in the left lateral plate mesoderm in (A) control and (B) *TWIST1*-expressing embryos. (C,D) Subsequently, these embryos were subjected to whole mount in situ hybridization to detect *TWIST1* in (C) control and (D) *SNAI1*-expressing embryos. Forced expression of *SNAI1* induces ectopic expression of *TWIST1* on the left side of the embryo (blue arrowheads). (E,F) Treatment with antisense morpholinos directed against *SNAI1* on the right side leads to the loss of the *TWIST1* expression domain (red arrow) in the right inflow tract region.
Online Figure II. Fate mapping of proepicardial progenitor cells in the somatic mesoderm. 
(A) DiI labelling of somatic mesodermal cells lateral to the right inflow tract at HH stage 10. 
(B) At HH stage 16 DiI-positive cells are present in the pericardial fold (P), the right sinus, and the PE. An asterisk marks the application point of the DiI label, white arrows indicate direction of migration. 
(C, D) A GFP-expressing vector (pCAGGS) was electroporated at HH stage 9 into the right somatic mesoderm and at HH stage 16 GFP-positive cells are found in the right lateral mesocardium (LM) and the right pericardial fold (P) but also in the right sinus and the PE. In both panels an assembly of confocal z-stacks are shown. For visualization purpose, the embryos were counterstained with WGA-Alexa 555. Scale bars in (C, D) represent 100µm. White arrows indicate direction of migration, A: atrium.
Online Figure III. Fate mapping of PE progenitor cells in relation to the *TWIST1* expression domain in the somatic mesoderm. (A-D) Dil labelling of somatic mesoderm along the mediolateral axis at a distance of 340 µm to 620 µm relative to the embryonic midline of HH stage 10 embryos. (E-G) At HH stage 16, labelled cells can be found in the PE when the Dil label was placed within a region that is located in a distance between 340-540 µm from the embryonic midline. (H) Cells that were labelled at a distance of 620 µm relative to the midline do not contribute to the PE. (I) Summary of the positions of the 4 labelled regions ranging from 340 to 620 µm at HH stage 10. (J) Section of a HH stage 10 embryo that was subjected to *TWIST1* in situ hybridization. The asterisk marks the notochord at the embryonic midline. The *TWIST1* expression domain in the somatic layer spans from about 320 to 600 µm distance relative to the embryonic midline (blue bars on the scale bar). Red lines demarcate the positions of the Dil labelling in the embryos depicted in (A-D). Dil labelling of somatic mesoderm from 340 to 540µm lies within the *TWIST1* expression domain.
Online Figure IV. Double electroporation of right somatic mesoderm and inflow tract. (A) The somatic mesoderm was electroporated at HH stage 9 with a GFP expression vector and at HH stage 11+ the inflow tract was electroporated with an RFP expressing construct. (B) At HH stage 16 GFP- and RFP-labelled cells can be found in the PE. (C) The confocal stack shows that the GFP- and RFP-positive cell populations in the PE are largely non-overlapping except for few cells that express both GFP and RFP (white arrows). (D) 3D-reconstruction confirms the labelling of two mainly distinct cell populations in the PE. A few GFP/RFP labelled cells (white arrows) indicate that cells that were GFP-labelled in the somatic mesoderm also received the RFP construct after migrating into the inflow tract. Scale bar in C and D represent 50µm.
Online Figure V. Loss of *TWIST1* leads to malformation of the proepicardial surface and inverted embryonic turning. (A,B) The infection of the right coelomic cavity with *TWIST1*-shRNA and GFP-expressing viral particles leads to smaller and morphologically distorted PEs (arrowheads). GFP expression can be found in the right coelom, the right inflow tract and the PE. (C,D) Assembly of confocal z-stacks of whole mount specimen stained with DAPI and WGA-Alexa 555 reveals a disrupted PE surface with smaller villous protrusions and reduced cell density after loss of *TWIST1*. View shows the right sinus (RS) and atrium (A). The PE is highlighted in blue. (E,F) Loss of *Twist1* on the right side has no effect on cardiac looping. (G,H,I) Tunel staining reveals no increase in apoptosis in the PE after loss-of-*TWIST1*. *p>0.5*, scale bars: 50µm. (J-L) BrDU incorporation revealed no significant relative decrease of proliferation in loss-of-*TWIST1* embryos. *p>0.6*, scale bars: 20µm. (M) The loss of *Twist1* (left embryo) has no effect on embryo size and overall development in comparison to control treatment (right embryo). (N-P) Loss of *Twist1* on the right side leads to a significant increase in embryos displaying inverted embryonic turning (21% (n=8/37) in comparison to 5.8% (2/36) in control embryos. * p<0.05).
Online Figure VI. Co-localization of virus-injected GFP-expressing and electroporated RFP-expressing cells. (A,B) In control embryos, the electroporation of somatic mesodermal cells at HH stage 9 leads to the presence of RFP-positive cells in the PE, which also display GFP-labelling (arrowheads). (C,D) In loss-of TWIST1 embryos, the somatic mesodermal cells that have been electroporated with RFP/control vector are not present in the PE (arrowhead) and are only found in the somatic mesoderm. (D) The GFP-positive cells in the somatic mesoderm in which TWIST1 has been knocked down display co-labelling with RFP, which suggests that RFP-positive cells remain in the somatic mesoderm due to the loss-of TWIST1. (E,F) In TWIST1-rescued embryos, RFP-labelled cells, which are also GFP-positive are found in the PE. Scale bars: (A,C,E) 100µm, (B,F) 20µm, (D) 50µm.
Online Figure VII. Schematic summary of the fate mapping approach under loss-of-function and rescue-of-function conditions. The right coelomic cavity has been subjected to GFP/virus injection and electroporation with an RFP vector at HH stage 9. Control embryos were treated with GFP-expressing control virus and electroporated with RFP/control vector. This leads to the presence of RFP-positive cells in the PE. Loss-of-function embryos were treated with TWIST1 shRNA/GFP expressing virus and electroporated with RFP/control vector. After this treatment no cells are found in the PE but the cells remain in the somatic mesoderm. The rescue embryos were infected with the same TWIST1 shRNA/GFP-expressing virus but in this case electroporated with an RFP/TWIST1 expressing vector, which appears to be sufficient to remobilize somatic mesodermal cells resulting in a contribution of those cells to the PE. (LM: lateral mesocardium, PE: proepicardium, RS: right sinus, SOM: somatic mesoderm).