Tbx5 and Bmp Signaling Are Essential for Proepicardium Specification in Zebrafish

Jiandong Liu, Didier Y.R. Stainier

Rationale: The proepicardial organ (PE) contributes to the cellular diversity of the developing heart by giving rise to the epicardium as well as vascular smooth muscle cells and fibroblasts. Despite the importance of these cells in cardiac development, function and regeneration, the signals required for the specification of the PE remain largely unexplored.

Objective: We aim to identify the signaling molecules and transcription factors that regulate PE specification.

Methods and Results: Here, we present the first genetic evidence that bone morphogenetic protein (Bmp) signaling in conjunction with the T-box transcription factor Tbx5a is essential for PE specification in zebrafish. Specifically, Bmp4 from the cardiac region, but not the liver bud, acting through the type I BMP receptor Acvr1l, is required for PE specification. By overexpressing a dominant-negative form of a Bmp receptor at various embryonic stages, we determined when Bmp signaling was required for PE specification. We also found that overexpression of bmp2b right before PE specification led to the ectopic expression of PE specific markers including tbx18. Furthermore, using loss-of-function approaches, we discovered a previously unappreciated PE specification role for Tbx5a at early somite stages; this role occurs earlier than, and appears to be independent from, the requirement for Bmp signaling in this process.

Conclusion: Altogether, these data lead us to propose that Tbx5a confers anterior lateral plate mesodermal cells the competence to respond to Bmp signals and initiate PE development. (Circ Res. 2010;106:1818-1828.)

Key Words: proepicardial organ • tbx18 • tcf2l • acvr1l • tbx5 • zebrafish

An increasing body of evidence has revealed that the proepicardial organ (PE) contributes cells to the coronary vessels1–6 and thus plays a critical role in cardiac development and function. In amniotes, the PE is an extracardiac grape-like structure associated with the septum transversum and located near the venous pole of the heart.7–9 Cells of the PE migrate to the postlooped heart to form its outermost layer, the epicardium. Subsequently, a fraction of the epicardial cells give rise to the subepicardial mesenchyme through an epithelial–mesenchymal transition.1,4,10,11 These epicardial-derived mesenchymal cells invade the heart and differentiate into coronary vascular smooth muscle cells, perivascula fibroblasts and inter-myocardial fibroblasts.1–7

Recent data indicate that the epicardium plays a critical role in cardiac regeneration in zebrafish. The epicardium reactivates expression of developmental genes on partial amputation of the adult heart and participates in cardiac regeneration at least in part by apparently contributing to the new vasculature in the regenerating myocardium.12 Another study has shown that epicardial cells can be efficiently differentiated into cardiomyocytes in vitro,13 and two recent reports14,15 have claimed that the epicardium can give rise to cardiomyocytes during mouse embryonic development. Thus, the epicardium has generated substantial interest recently, especially as a potential source of new cardiomyocytes during homeostatic repair.

In addition to providing various cell types to the developing heart, the epicardium has important regulatory functions in the development of the myocardium.16–20 Targeted deletion of VCAM-1 or α4 integrin results in the absence of the epicardial layer in the mouse heart.17,18 This defect in epicardial formation in turn causes a significant reduction in the thickness of the ventricular myocardium. Migration of PE cells to form the epicardium appears to require the activity of the T-box transcription factor Tbx5.21 In chick embryos, PE cells transfected with morpholino antisense oligonucleotides for Tbx5 failed to migrate of the PE.21 Interestingly, an epicardial specific knockout of the retinoid X receptor α (RXRa) gene also leads to the thinning of the ventricular myocardial wall.22 Thus, it has been proposed that the epicardium is a source of secreted mitogenic factors that promote cardiomyocyte
proliferation. The epicardial derived signals include fibroblast growth factors, such as Fgf9, whose expression appears to be regulated by retinoic acid signaling.23

The zebrafish proepicardium, like that of higher vertebrates, is a transient epithelial structure that delivers mesothelial cells to form the epicardium.24 Despite the importance of the PE in coronary and cardiac development, little is known about the molecular mechanisms that govern its initial appearance or specification. In mouse embryos, Gata4 is highly expressed in the PE and genetic ablation of Gata4 results in a complete absence of PE formation.25 PE induction also seems to involve inductive signal(s) from neighboring tissues. In the chick embryo, the PE is closely apposed to the liver bud. Quail-to-chick transplantation data indicate that the liver bud has the capacity to induce ectopic PE marker gene expression when implanted into the posterior lateral plate mesoderm.26 Nevertheless, it is unclear whether the liver primordium is necessary for PE induction. And if it is, the exact nature of the liver bud-derived signal(s) required for PE specification also remains to be identified. In this study, we provide genetic evidence for a role for myocardium (but not liver bud) derived bone morphogenetic protein (Bmp) and the T-box transcription factor Tbx5a in PE specification in zebrafish. Loss of bmp4 activity causes a significant reduction in PE marker gene expression. Likewise, PE specification is severely compromised in acvr1l (aka alk8) mutants as well as in embryos overexpressing a dominant-negative form of a Bmp receptor right before PE specification. We further show that tbx5a mutants also have severe defects in PE specification. To delineate the temporal requirement for Tbx5a in PE development, we generated a dominant negative form of Tbx5a under the heat shock promoter, and used it to reveal that Tbx5a is required at early somite stages for this process. Furthermore, our data suggest that Tbx5a functions earlier than, and independently from, the requirement for Bmp signaling in PE specification. Thus, we propose that Tbx5a activity in the anterior lateral plate mesoderm confers competence to a subset of these cells to respond to Bmp signals and initiate PE development.

Methods

Zebrafish Strains

Embryos and adult fish were raised and maintained under standard laboratory conditions.27 The mutant and transgenic lines used in this study are as follows: acvr1lmo108.28,29 bmp4st72,30 har59,31 tbx5am21,32 wnt2bbst401,33 hnf1bah2169,34 Tg(hsp70l:dntbx5a) f23 and Tg(hsp70l:dnBmpr-GFP)w30 and Tg(hsp70l:dnBmpr-GFP)y30,35 and Tg(hsp70l:dnBmpr-GFP)y30,35 and Tg(hsp70l:dnBmpr-GFP)y30,35

Generation of a Stable Dominant-Negative tbx5a Transgenic Line

The truncated version of zebrafish tbx5a was amplified with the primers AGATCTATGGCGGACAGTGAAGACACC and GCG-GCCGCTAGACAGCAGACACC and GCG-GCCGCTAGACAGCAGACACC and GCG-GCCGCTAGACAGCAGACACC. This fragment was fused in frame with a myc tagged engrailed repressor domain37 and then placed downstream of the hsp70 promoter.38

The uncut DNA was coinjected with I-sceI enzyme into one cell stage AB embryos.39 F1 founders were screened for green fluorescent protein (GFP) expression in the eye driven by the crystallin promoter in the vector, and stable transgenic lines were generated.

Non-standard Abbreviations and Acronyms

acvr1l activin A receptor, type I like
Bmp bone morphogenetic protein
dpf days postfertilization
Hand2 heart and neural crest derivatives expressed transcript 2
hpf hours postfertilization
hhex hematopoietically expressed homeobox
hnfta HNF1 homeobox Ba
Nkx2.5 NK2 transcription factor related 5
PE proepicardial organ
prox1 prospero-related homeobox gene 1
Tbx T-box
wnt2bb wingless-type MMTV integration site family, member 2Bb

Heat Shock Conditions

For the heat shock experiments, embryos obtained from outcrossing a hemizygous Tg(hsp70l:dnBmpr-GFP)24,25 fish were heat-shocked around 36 hours postfertilization (hpf) for 25 minutes at 40°C. After heat shock, the embryos were allowed to further develop in a 28°C incubator, and harvested for fixation at 57 hpf. Because GFP expression is maintained in these embryos for at least 24 hours after heat shock, hemizygous embryos expressing GFP were easily identifiable. Similarly, embryos from Tg(hsp70l:dnBmpr-GFP)w30 outcross were heat-shocked around 10 hpf or at later stages for 20 minute at 39.5°C. The embryos were allowed to further develop until harvested at 57 hpf for fixation. After in situ hybridization, the embryos were genotyped for the presence of the hsp70l:dnb5 transgene using the following primers: 5'-ATGAGCTCTCAAGATGAGCGGAG-3' and 5'-GACACCAGTGCCCTTCCAGG-3'. To hyperactivate Bmp signaling, embryos obtained from outcrossing a hemizygous Tg(hsp70l:bmp2b)29,31 were heat-shocked for 30 minutes at 37°C. After in situ hybridization, genotyping for the presence of the hsp70l:bmp2b transgene was carried out using the primers: 5'-CATGTGGACTGCTATGGTTCCATC-3' and 5'-GAGAGCGCGACCAGCGAGAG-3'.

In Situ Hybridization and RNA Injections

Single or double whole-mount in situ hybridizations were performed as previously described,40 using the following probes: tbr1,41 tcf21,42 emlc2,42 tbs532,43,44 bmp4,45,46 and acvr1l29 tcf21 mRNA (20 pg) was injected into one-cell stage wild-type or acvr1l mutant embryos. The acvr1l mutant embryos were easily identifiable by the lack of pectoral fin buds.

Results

Development of the Proepicardium in Zebrafish

It is well documented that the PE in avian and mouse embryos becomes morphologically distinguishable as a cluster of cells located in close proximity to the sinus venosus of the looping-stage heart.7–9 To examine the progression of PE development in zebrafish embryos, we focused on the heart region and found that an extracardiac cluster of spherical cells which bears morphological resemblance to the PE was first detectable by light microscopy around 48 hpf. This cluster appears at the pericardial surface of the yolk and is located at the level of the atrioventricular junction (Figure 1A).24 By 72 hpf, this cluster has grown in size and has come in contact with the myocardium to initiate the formation of the epicardium (Figure 1B).
To determine the identity of this extracardiac cell population, we examined the expression of the common PE marker genes *tbx18* and *tcf21* relative to that of the myocardial marker *cmlc2* using double in situ hybridization. At 48 hpf, *tbx18* and *tcf21* expression is detected in the region between the developing atrium and ventricle (Figure 1C and 1D). Lateral views at the same stage show that the *tbx18* positive cells appear close to the atrioventricular junction and adjacent to the pericardial cavity (Figure 1E). No expression of *tbx18* or *tcf21* is observed in the heart region at 36 hpf (data not shown). By 4 days postfertilization (dpf), the *tbx18* and *tcf21* positive cells are no longer organized in a cluster, but rather spread over the heart to cover the myocardium (Figure 1F through 1I). Therefore, the expression pattern of the PE marker genes *tbx18* and *tcf21* corresponds precisely to the physical appearance of the extracardiac cell population, indicating their proepicardial identity.

**Bmp Signaling Is Essential for PE Specification**

In vitro culture studies have suggested that Bmp signaling regulates the differentiation of the proepicardial cells. In chick embryos, reducing Bmp signaling levels by implantation of Noggin soaked beads into the sinus venosus region appears to reduce PE marker gene expression. However, the Bmp ligand and receptor potentially implicated in PE formation have not been identified. To determine whether Bmp signaling is indeed required for PE development, we first examined PE formation in *acvr1l* (activin A receptor, type I-like) zebrafish mutants. *acvr1l* encodes a type I Bmp receptor and it is expressed both maternally and zygotically. Zygotic *acvr1l* mutants display a weak dorsalized phenotype as shown by the absence of ventral tail fin formation. We found that in *acvr1l* mutants the expression of *tbx18* and *tcf21* in the heart region was completely absent, whereas *tbx18* expression in the pectoral fin and *tcf21* expression in the pharyngeal arch appeared unaffected (Figure 2B and 2D).

To define the temporal requirement for Bmp signaling in the specification of the PE and circumvent the function of Bmp signaling in dorsoventral patterning, we heat-shocked hemizygous Tg(hsp70l:dnBmpr-GFP) embryos and their wild-type siblings at 36 hpf, a stage before the formation of the PE and the onset of PE marker gene expression. We found that when examined at 57 hpf the expression of *tbx18* and *tcf21* was significantly reduced in the heart region of the zebrafish.
heat-shocked transgenic embryos (Figure 2F, 2G, 2H, 2J, 2K, and 2L, arrows; severe reduction: 17/23 for \textit{tbx18}, 19/27 for \textit{tcf21}; almost absent 6/23 for \textit{tbx18}, 8/27 for \textit{tcf21}) compared to their heat-shocked wild-type siblings. These data indicate that Bmp signaling occurring after 36 hpf is essential for PE specification.

To determine whether Bmp signaling is sufficient to induce PE marker gene expression, we overexpressed \textit{bmp2b} by heat shocking hemizygous Tg(hsp70: \textit{bmp2b})\textit{fr13} embryos at 36 hpf to hyperactivate Bmp signaling. In embryos with ubiquitous \textit{bmp2b} expression, we observed ectopic \textit{tbx18} expression (19/25) around the heart region, whereas \textit{tcf21} expression appeared unaffected (Figure 3B and 3C and data not shown).

The weak PE-inducing activity of Bmp2b signaling suggests that it might cooperate with other signaling pathways to promote PE formation. This observation is consistent with a prior study showing that the liver bud, but not the lung bud, has the capacity to induce ectopic PE marker gene expression although both primordia express high levels of Bmp family member genes. The lack of response of \textit{tcf21} to \textit{bmp2b} overexpression prompted us to examine whether \textit{tcf21} overexpression could induce \textit{tbx18} expression in the heart region. To this end, we injected 20 pg of \textit{tcf21} mRNA into one-cell stage wild-type embryos, and analyzed them at 57 hpf. Interestingly, overexpression of \textit{tcf21} expanded \textit{tbx18} expression in the heart region. In \textasciitilde 60% (16/25) of the \textit{tcf21} overexpressing embryos, two clusters of \textit{tbx18} positive cells were observed, each of which was about the same size as the cluster seen in wild-type embryos. Because both Bmp signaling and \textit{tcf21} can induce ectopic \textit{tbx18} expression, we wanted to investigate the relationship between Bmp signaling and \textit{tcf21} in regulating \textit{tbx18} expression.

We injected \textit{tcf21} mRNA into embryos from \textit{acvr1l} heterozygote in crosses and examined \textit{tbx18} expression at 57 hpf. We found that overexpression of \textit{tcf21} in embryos that lacked pectoral fin formation, a phenotype observed in \textit{acvr1l} mutants, did not rescue the loss of \textit{tbx18} expression in the heart region, suggesting that the \textit{tbx18} inductive properties of Tcf21 rely on Bmp signaling.

**The Liver Is Not Required for PE Induction in Zebrafish**

A prior study has shown that the liver bud can induce ectopic PE marker gene expression in chick embryos; however, it remains unclear whether the hepatic tissue is essential for PE induction. Attempts to address this issue genetically have been hampered by the fact that in addition to a complete absence of liver formation, loss of \textit{Foxa1; Foxa2} function in mouse embryos also causes a significant developmental delay of many internal organs at a stage when the PE starts to
form. In zebrafish, \textit{wnt2bb} has been recently identified as a positive regulator of hepatoblast specification. In \textit{wnt2bb} mutants, development of the liver is significantly delayed. To explore a potential inductive role of the liver primordium on PE formation, we crossed a \textit{wnt2bb}^{-/-} female with a \textit{wnt2bb}^{-/-} male and analyzed PE marker gene expression at 57 hpf. Interestingly, \textit{tbx18} and \textit{tcf21} expression in the heart region appear unaffected (Figure 4A and 4B). Because some hepatoblasts are present in \textit{wnt2bb} mutants at 57 hpf, we examined \textit{hnf1b}a (previously known as \textit{tcf2}, \textit{hnf1b}, \textit{vhnf1}) mutants to further test the requirement of the liver bud in PE formation. The expression of the earliest hepatic marker \textit{acvr1l} in wild-type embryos of 48 hpf and mutant embryos, we examined the expression pattern of acvr1l in wild-type embryos (Figure 5D and 5F) (19/21 for \textit{tbx18} and 16/17 for \textit{tcf21}), whereas their expression in the neighboring tissues did not appear to be affected (data not shown). These data indicate that \textit{bmp4} regulates \textit{tbx18} and \textit{tcf21} expression in the heart region. To determine whether the effect of \textit{Bmp4} could be mediated through the Bmp receptor \textit{Acvr1l}, because PE marker gene expression in the cardiac region is completely absent in \textit{acvr1l} mutant embryos, we examined the expression pattern of \textit{acvr1l} in wild-type embryos of 48 hpf and found that \textit{acvr1l} appears to be ubiquitously expressed in the cardiac region, as well as neighboring tissues, although its expression level seems to be relatively low in the cardiac region (data not shown).

**Hand2 and Tbx5a Are Essential for PE Formation**

In chick and mouse embryos, the PE is closely apposed to the liver primordium and the sinoatrial myocardium; thus, both tissues have been postulated to play a role in PE induction. In zebrafish embryos, although the PE does not appear to be in close proximity to the liver when it arises, it does not rule out the possibility that the PE progenitors are in contact with the hepatoblasts at an earlier developmental time. Because our genetic data indicate that signal(s) from the liver bud are not essential for PE specification, we focused on the heart region to identify the Bmp ligand(s) involved in this process. We examined the expression pattern of several Bmp family members at the time of PE formation and earlier. \textit{bmp2a} and \textit{bmp2b} appear not to be expressed in the heart at 36 hpf and 48 hpf (Figure 5A and 5B). In contrast, we found that \textit{bmp4} was expressed in the heart, mainly in the outflow tract, atrioventricular junction, and sinoatrial myocardium by 48 hpf. The expression of \textit{bmp4} in the cardiac region (Figure 5A, arrow and arrowheads) is consistent with a potential role in PE specification. To determine whether \textit{bmp4} is involved in PE formation, we examined PE marker gene expression in \textit{bmp4} mutants and found a severe reduction of \textit{tbx18} and \textit{tcf21} expression in the heart region at 57 hpf (Figure 5D and 5F). In \textit{tbx18} mutants, it is possible that the PE progenitors are in contact with the liver bud. However, \textit{tbx18} and \textit{tcf21} mutants to further test the requirement of the liver bud in PE formation, as assessed by \textit{acvr1l} mRNA expression in the cardiac region is completely absent in \textit{acvr1l} mutant embryos, we examined the expression pattern of \textit{acvr1l} in wild-type embryos of 48 hpf and found that \textit{acvr1l} appears to be ubiquitously expressed in the cardiac region, as well as neighboring tissues, although its expression level seems to be relatively low in the cardiac region (data not shown).
possible that the observed absence of PE formation is secondary to the myocardial defects.

The T-box transcription factor Tbx5a has also been reported to play a critical role in cardiac and pectoral fin development in zebrafish. Similar to hand2, tbx5a is expressed in the lateral plate mesoderm, but its expression is restricted to a narrower domain extending from the precardiac mesoderm to the pectoral fin bud. The major cardiac morphogenetic defect of tbx5a mutants at the stage when the PE starts to form in wild-type is a failure to complete cardiac looping (Figure 6G and 6H), but otherwise the mutant heart appears morphologically intact before it eventually deteriorates to exhibit a string-like morphology. These milder cardiac phenotypes observed in zebrafish tbx5a mutants are likely attributable to functional redundancy between tbx5a and its newly identified paralog tbx5b. To determine whether tbx5a function is required for PE formation, we examined the expression of PE marker genes at 52 hpf and found that the expression of tbx18 and tcf21 in the heart region was barely detectable in tbx5a mutant embryos (Figure 6C and 6F). Altogether, these data suggest that tbx5a might have a direct function for PE specification, rather than affecting PE formation indirectly through its role in myocardial development. To further explore tbx5a function in PE formation, we generated a putative dominant negative form of Tbx5a by fusing the Drosophila Engrailed repressor domain to a truncated Tbx5a containing its N terminus and T-box domains. We heat-shocked hemizygous Tg(hsp70l:dntbx5a)f23 embryos at various developmental stages and found that overexpression of the dominant negative Tbx5a around 10 hpf resulted in mild cardiac edema with relatively normal heart morphogenesis and reduction in the size of the pectoral fin at 52 hpf (Figure 6I, compare the right 2 embryos with their wild-type sibling on the left; and Figure 6P through 6S). These data are consistent with a role for tbx5a in cardiac and pectoral fin development. Importantly, these embryos also showed a
Figure 6. Hand2 and Tbx5a are required for the induction of proepicardial marker gene expression. A through F, Wild-type, hand2, or tbx5a mutant embryos were analyzed for tbx18 (A through C) or tcf21 (D through F) expression at 52 hpf. tbx18 (B and C) and tcf21 (E and F) expression (arrows) is completely absent in hand2 (B and E) and tbx5a (C and F) mutants. G and H, cmic2 expression in 52 hpf tbx5a mutant embryos and their wild-type siblings. Cardiac components including the ventricle, atrium, and inflow tract are formed properly although cardiac looping is delayed. H through O, Tg(hsp70l:dntbx5a)f32 hemizygous embryos heat-shocked at 10 hpf showed mild cardiac edema (H, arrows), with relatively normal cardiac morphogenesis (P and Q) and reduction of the pectoral fins (R and S) by 52 hpf. These embryos also exhibited a significant reduction of PE marker gene expression (18/22 for tbx18 and 21/25 for tcf21) (K and N). By contrast, Tg(hsp70l:dntbx5a)f32 hemizygous embryos exhibited wild-type levels of tbx18 and tcf21 expression when heat-shocked at 24 hpf (L and O).
significant reduction in the PE marker gene expression (Figure 6K and 6N; 18/22 for tbx18 and 21/25 for tcf21). By contrast, Tg(hsp70I:dnTbx5a) embryos exhibited wild-type levels of tbx18 and tcf21 expression when heat-shocked after 24 hpf (Figure 6L and 6O). This requirement for Tbx5a at early somite stages in PE specification is in sharp contrast to its role in PE cells migration toward the myocardium. Thus, we have revealed a previously unappreciated role for Tbx5a during PE formation and propose that tbx5a functions at early somite stages to provide competence to a subset of lateral plate mesodermal cells to differentiate into the PE lineage.

The above data show that manipulating Bmp signaling and Tbx5a activity both affect PE marker gene expression. These observations raise the possibility that Bmp signaling and Tbx5a might function in the same pathway to regulate PE specification. To test this possibility, we monitored the expression of bmp4 in the cardiac region including the atrioventricular and sinoatrial myocardium of tbx5a mutants. However, we did not find any significant changes in the level of bmp4 expression in the mutants (Figure 7A and 7B). Conversely, we examined tbx5a expression in acvr1l mutants at early somite stages because tbx5 is required at these stages for PE specification. The expression of tbx5a in the lateral plate mesoderm in acvr1l mutants at the 10-somite stage appears unaffected compared to wild-type (Figure 7C and 7D). These data suggest that Bmp signaling and Tbx5a function independently and further corroborate the idea that they are required at different times to regulate PE formation.

Discussion
The PE is known to be the primary extracardiac source for several cardiac cell types, including cardiac fibroblasts and coronary smooth muscle cells. In addition, the PE derived epicardium secretes mitogenic factors that promote ventricular cardiomyocyte proliferation during development. In this study, we focused on the earliest step of PE development and presented genetic evidence that Bmp signaling is essential for PE specification in zebrafish. We also revealed a critical role for Tbx5 in PE specification. Loss of Tbx5 function leads to the absence of PE formation. Based on its expression pattern and the heat shock experiments that blocked Tbx5 function at various stages, we propose that Tbx5 is required at early somite stages to provide competence to a subset of tbx5 expressing mesodermal cells to become PE cells at a later developmental stage.

Evolutionary Conservation and Differences During PE Development
PE formation has been investigated in mouse and chick embryos, but there are only limited studies on PE development in other model systems. Our data suggest that in zebrafish embryos, the appearance of the PE coincides with the transition of the heart from a primitive linear heart tube to a postlooped two-chambered pumping organ. The PE cells are clustered into a cauliflower like structure which is positioned close to the sinus venosus and at the level of the atrioventricular junction. These observations suggest that the PE in zebrafish arises in a similar fashion to that in mouse and chick. Strikingly, PE development is also conserved at a molecular level. The PE marker genes tbx18, tcf21 and wt1 identified in mouse and chick also appear to be expressed in the zebrafish PE. However, differences in PE formation between zebrafish, mouse and chick do exist. In zebrafish, mouse and chick embryos, the PE is located adjacent to the developing liver bud. The close apposition of the liver to the PE suggests an inductive role for the liver primordium in PE formation. In fact, Ishii et al. showed that liver bud implantation could induce ectopic PE marker gene expression in chick embryos. In zebrafish, however, the liver primordium seems to be dispensable for PE develop-
Bmp Signaling Is Required for PE Specification

In this study, we have revealed an essential role for Bmp signaling in PE specification. A complete loss or significant reduction of PE marker gene expression was observed in acvr1l mutants as well as in embryos in which Bmp signaling was blocked around 36 hpf. The reduction of PE marker gene expression by blocking Bmp signaling at 36 hpf in zebrafish is somewhat reminiscent of the loss of PE marker gene expression observed after implanting Noggin beads into the sinus venosus region of HH stage 11 chick embryos, suggesting that the role of Bmp signaling in PE specification, or maintenance of PE cell identity, is conserved. In chick embryos, bmp2, which is expressed in the sinoatrial myocardium, and bmp4, which is expressed in the PE cells, are likely to be the source of Bmp signaling, although specific knockdown studies need to be carried out to test this hypothesis.48

In zebrafish, we found that bmp4 is expressed in the atrioventricular junction and sinoatrial myocardium, and that loss of Bmp4 activity results in a reduction PE marker gene expression, indicating that bmp4 in zebrafish might be the functional ortholog of bmp2 or bmp4 in chick during PE specification.

A recent study by van Wijk et al59 suggests that in chick, the role of Bmp signaling in PE specification may be more complicated. This study shows that proepicardial cells and inflow myocardial cells originate from a common progenitor pool. The segregation of these progenitors into the proepicardium or inflow myocardium lineages appears to involve an opposing effect of BMP and FGF signaling; Bmp signaling promotes inflow myocardium formation, whereas FGF signaling favors PE formation. This reported difference in the role of BMP signaling in PE specification may in part reflect the multiple roles it plays during PE development, as is observed during pancreas development.60

In summary, we provided the first genetic evidence that Bmp4 and its receptor Acvr1l are essential for PE formation in zebrafish. In this study, we also showed that Tbx5a is required at early somite stages for PE specification. Therefore, we propose that Tbx5a is necessary to confer competence to anterior lateral plate mesodermal cells, thereby allowing some of them to respond to Bmp signals to initiate PE specification. Given that the reactivation of the adult epicardium is critical for cardiac regeneration in zebrafish,12 our study may provide a new paradigm to stimulate the epicardium to respond to cardiac injury for functional repair.

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Disclosures

None.

References

Proepicardial Organ Specification in Zebrafish


Novelty and Significance

What IsKnown?

● The proepicardium (PE)/epicardium provides precursor cells that differentiate into coronary vascular smooth muscle cells, fibroblasts, and, possibly, endothelial cells and cardiomyocytes during cardiac development.
● The PE-derived epicardium is a source of secreted factors that stimulate cardiomyocyte proliferation.
● The PE/epicardium-derived cells appear to play an important role during cardiac tissue homeostasis and regeneration.

What New Information Does This Article Contribute?

● Our study shows that Bmp4, which is expressed in the developing heart, functions through the type I Bmp receptor Acvr1l to regulate PE development.
● Our findings reveal a previously unappreciated role for Tbx5 at early somite stages for PE specification.

Despite the importance of PE in cardiac development, function, and regeneration, the signaling pathways that regulate the development of PE remain unidentified. In this study, we focused on the earliest step of PE development and our work represents the first in vivo identification of signaling molecules and transcription factor that regulate PE formation. We identified myocardial Bmp4 as a positive regulator for PE specification. In addition, we found that Tbx5 plays a critical role in PE specification. Based on the expression pattern of these factors and the heat shock experiments that blocked the activity of Tbx5 or Bmp signaling at various stages, we propose that Tbx5 is necessary to confer competence to anterior lateral plate mesodermal cells, thereby allowing some of them to respond to Bmp signals to initiate PE specification. Given that the reactivation of the adult epicardium is critical for cardiac regeneration in zebrafish, our study provides a new paradigm for stimulating the epicardium to respond to cardiac injury for functional repair.
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