Zebradish cardiac development requires a conserved secondary heart field

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SUMMARY
The secondary heart field is a conserved developmental domain in avian and mammalian embryos that contributes myocardium and smooth muscle to the definitive cardiac arterial pole. This field is part of the overall heart field and its myocardial component has been fate mapped from the epiblast to the heart in both mammals and birds. In this study we show that the population that gives rise to the arterial pole of the zebrafish can be traced from the epiblast, is a discrete part of the mesodermal heart field, and contributes myocardium after initial heart tube formation, giving rise to both smooth muscle and myocardium. We also show that Isl1, a transcription factor associated with undifferentiated cells in the secondary heart field in other species, is active in this field. Furthermore, Bmp signaling promotes myocardial differentiation from the arterial pole progenitor population, whereas inhibiting Smad1/5/8 phosphorylation leads to reduced myocardial differentiation with subsequent increased smooth muscle differentiation. Molecular pathways required for secondary heart field development are conserved in teleosts, as we demonstrate that the transcription factor Tbx1 and the Sonic hedgehog pathway are necessary for normal development of the zebrafish arterial pole.

KEY WORDS: Bmp signaling, Cardiac arterial pole, Cardiac progenitors, Hedgehog signaling, Second heart field, tbx1, Zebrafish

INTRODUCTION
In chick and mouse, cardiac progenitors are organized bilaterally in the epiblast on either side of the primitive streak (Hatada and Stern, 1994; Tam et al., 1997). Previous fate mapping in the chick and mouse epiblast has established that the cranial-to-caudal organization of cardiac progenitors before gastrulation corresponds to the cranial-to-caudal organization of the heart (Rosenquist, 1970; Yang et al., 2002). The cranially located arterial pole progenitors ingress through the primitive streak earlier than the more caudal venous pole progenitor cells (Chuai and Weijer, 2009; Cui et al., 2005). After gastrulation, these progenitors reorganize into two bilateral fields in the anterior lateral plate mesoderm (ALPM) prior to cardiogenesis, with arterial pole progenitors located medially and venous pole progenitors located laterally (Abu-Issa and Kirby, 2008; Yang et al., 2002). As cardiogenesis proceeds, these mesodermal fields fuse at the ventral midline to form a heart tube that lacks septation and chambers (Arguello et al., 1975). In chick, the cardiomyocytes in the heart tube that have undergone early differentiation typically do not proliferate while the heart tube continues to elongate (van den Berg et al., 2009). Thus, the continuous addition of cells from a progenitor population allows the developing heart to elongate and loop (Buckingham et al., 2005; de la Cruz et al., 1977).

The portion of the heart field that gives rise to the initial heart tube has been called the first heart field, whereas the second heart field is added later. In mouse, these two populations of cardiac progenitors are spatially and molecularly distinct (Kelly et al., 2001; Cai et al., 2003). Second heart field progenitors differentiate as they are added to the heart tube to give rise to the right ventricle, the outflow tract and part of the atria (Buckingham et al., 2005). The definitive arterial pole is formed from the most medial portion of the second heart field, known as the secondary heart field (Abu-Issa and Kirby, 2007; Dyer and Kirby, 2009b). In chick, this progenitor population remains in the pericardial wall caudal to the developing arterial pole until it is added to the heart tube to form the most distal outflow myocardium and the smooth muscle at the base of the great arteries (Mjaatvedt et al., 2001; Waldo et al., 2001; Waldo et al., 2005).

The organization of cardiac progenitors seen in zebrafish mirrors that observed in chick and mouse. Atrial and ventricular precursors are organized in partially overlapping zones in the lateral marginal zone (LMZ) of the blastoderm before gastrulation (Keegan et al., 2004). The atrial progenitors are located ventrally and farther from the ingressing LMZ relative to ventricular progenitors, defining the order of gastrulation. After gastrulation, the mesodermal cardiac progenitors are organized into bilateral fields with ventricular progenitors located medially relative to atrial progenitors (Schoenebeck et al., 2007).

These mapping studies in zebrafish were terminated prior to the addition of smooth muscle of the bulbous arteriosus, the most distal component of the intrapericardial arterial pole immediately cranial to the ventricular myocardium. The adult zebrafish outflow tract, with the most proximal smooth muscle surrounded by a sleeve of myocardium, is homologous to that observed in animals with a divided arterial pole (Grimes et al., 2006; Grimes et al., 2010). Zebrafish also display a low level of proliferation in early differentiating cardiomyocytes, which would necessitate the addition of cells to the heart tube after its formation (de Pater et al., 2009) (K. A. Nembrhard and M.L.K., unpublished). Although such an addition has been shown (de Pater et al., 2009), the location of arterial pole precursors has not been...
investigated. Furthermore, the incorporation of such cardiac progenitors into the smooth muscle of the bulbus arteriosus has not been explored.

A secondary heart field in zebrafish would require molecular regulation prior to addition to the heart tube, as is the case in mammals and birds. In the latter models, the transcription factor Isll1 has been detected in cardiac progenitors prior to differentiation (Ma et al., 2008; Yuan and Schoenwolf, 2000), whereas Bmp signaling is needed for myocardial differentiation (Prall et al., 2007; Waldo et al., 2001). Another transcription factor, TBX1, has been implicated in defects of the arterial pole in human (Chieffo et al., 1997; Merscher et al., 2001). The Sonic hedgehog (Shh) pathway is an important regulator of Tbx1 (Garg et al., 2001; Yamagishi et al., 2003), and defects in this pathway mirror those observed in Tbx1 mutants (Washington Smoak et al., 2005). Shh signaling and Tbx1 are both important for the normal proliferation of secondary heart field progenitors (Dyer and Kirby, 2009a; Kochilas et al., 2002; Xu et al., 2004).

We hypothesized that secondary heart field progenitors in zebrafish would be organized in a similar pattern to that seen in chick and mouse, and that the pathways controlling the development of these progenitors would be conserved. We show that the zebrafish has a subset of cardiac progenitors within the mesodermal heart fields that contribute to both the myocardium of the ventricle and the smooth muscle of the bulbus arteriosus. This population of cells is located in the most medial portion of the mesodermal heart fields in zebrafish and is present in the pericardial wall adjacent to the arterial pole after early heart tube formation. The molecular mechanisms involved in the formation of the zebrafish arterial pole are also broadly conserved. These conserved features of arterial pole development make zebrafish an ideal organism in which to study presegmentation development of the arterial pole.

MATERIALS AND METHODS

Embryo collection, preparation and identification

Zebrafish (Danio rerio) were maintained following published protocols (Westerfield, 1993; Nüsslein-Volhard and Dahm, 2002). Where indicated, Tübingen wild-type fish (Tu), Tg(cmlc2::GFP) (kindly provided by Deborah Yelon, UCSF), Tg(gsc::GFP) (kindly provided by Deborah Yelon), thbx1pa2955 (AB) (kindly provided by Tatjana Piotrowski, University of Utah), smd577::GFP (AB) (kindly provided by Stephen Devoto, Wesleyan University), Tg(sox10::eGFP) (kindly provided by Thomas Schilling, UC Irvine), Tg(cmlc2::GFP); thbx1pa2955 (AB) and Tg(cmlc2::GFP); smd577::GFP (AB) were used to obtain embryos. Genotyping for thbx1pa2955 (AB) histological sections was performed as described (Piotrowski et al., 2003). All embryos were anesthetized with 0.016% tricaine and fixed in 4% paraformaldehyde at 65°C for 10 minutes, then blocked in 1% BSA, 0.5% Triton X-100, 5% normal goat serum (NGS) in 1X PBS for 2 hours and then incubated with primary antibodies MF20 (Myosin, sarcomere; Developmental Studies Hybridoma Bank (DSHB), University of Iowa, IA, USA; 1:10), S46 (Slow myosin heavy chain 1, 2, 3; DSHB; 1:10) and anti-En2 (Tropoelastin 2 or Elastin b; kindly provided by Fred W. Keeley, University of Toronto; 1:1000) in blocking solution overnight at 4°C. For 39.4D5 (Isl1 homeobox; DSHB) and anti-phosphorylated (p) Smad1/5/8 (Cell Signaling) labeling, embryos were rehydrated in PBST and blocked using blocking solution containing 0.3% Triton X-100 for 2 hours and then incubated with primary antibodies MF20, CT3 (Cardiac troponin T; DSHB; 1:10), 39.4D5 (1:100) or anti-p-Smad1/5/8 (1:100) in blocking solution overnight at 4°C. Fluorescent secondary antibodies (Invitrogen; 1:200) were used for detection as appropriate.

For fluorescein labeling, fixed embryos were serially rehydrated in PBST and permeabilized as described above. Blocking was performed with 5% sheep serum and 2 mg/ml BSA in PBST. For cell tracing at later stages, Tu, Tg(cmlc2::GFP) or Tg(sox10::eGFP) embryos, with the fluorescein:rhodamine combination described above. These were kept in the dark until the 6- to 8-somite stage, and then embedded in 1% low melting point agarose (Lonza) in distilled water. Using the notochord and neural plate as references, caged dye was photoactivated as described above.

GFP signal amplification

For 14-somite stage Tg(cmlc2::GFP) embryos, photoactivation of cardiac progenitors was conducted as described above for the 7-somite stage. Embryos were dehydrated in methanol after fixation and kept at ~20°C overnight; then serially rehydrated in PBST. Embryos were permeabilized as described above, and then blocked in 10% NGS and 2% BSA in PBST, followed by incubation in rabbit anti-GFP (Invitrogen; 1:1000) overnight at 4°C. After washing in PBST, embryos were incubated in anti-rabbit IgG HRP (Invitrogen; 1:1000) for 1.5 hours at room temperature. After further washing with PBST, the TSA-Plus Tetramethylrhodamine System (Perkin Elmer) was applied according to the manufacturer’s instructions with the fluorescein-conjugated tyramide at 1:1000 in the reaction buffer.

Histology

Labeled embryos were dehydrated in methanol, transferred into Histoclear (National Diagnostics) for 20 minutes and embedded in paraffin at 56°C. The cooled blocks were then sectioned as described (Grimes et al., 2006).

In situ analysis

After rehydration into PBST, embryos were permeabilized as described above, then hybridized with a digoxigenin-conjugated riboprobe for hand2 at 67°C overnight. The hand2 construct was kindly provided by Deborah Yelon (UCSF) (Schoenebeck et al., 2007). Following hybridization, embryos were rinsed and transferred into PBST, then incubated in anti-
digoxigenin antibody (Roche; 1:5000) in 2% sheep serum and 2 mg/ml BSA overnight at 4°C. The \( \text{hand2} \) expression domain was visualized using NBT/BCIP staining.

RESULTS

Zebrafish arterial pole precursors are clustered dorsally among ventricular progenitors prior to gastrulation

To establish the location of arterial pole progenitors in the zebrafish blastoderm we took advantage of the Tg(\( \text{gsc}::\text{GFP} \)) line, which expresses GFP in the Goosecoid homeobox transcription factor domain, allowing clear identification of the dorsal axis prior to gastrulation (Doitsidou et al., 2002). Adopting similar fate-mapping strategies to those of Keegan et al. (Keegan et al., 2004), caged fluorophores were used as lineage tracers to identify cells of the blastula that contribute to the most cranial myocardium of the ventricle and the smooth muscle of the bulbus arteriosus. By activating caged fluorophores with laser photolysis in no more than one or two neighboring blastomeres and recording their position within the blastula, the contribution of these labeled cells to the arterial pole was assessed at 72 hpf. As previously published (Keegan et al., 2004), atrial and ventricular progenitors were found in distinct populations of cells in the first four tiers of cells in the LMZ at 40% epiboly. Ventricular myocardial progenitors were found to reside between 55° and 140° from dorsal in tiers one through three, with atrial precursors between 75° and 160° from dorsal in tiers two through four (Fig. 1). In addition to the identification of atrial and ventricular progenitors, we identified cells that populated the myocardium at the distal outflow tract of the ventricle, restricted to the first and second tiers between 55° and 105° from dorsal (Fig. 1). No labeled cells were detected in the smooth muscle of the bulbus arteriosus at 72 hpf. As excessive proliferation might lead to dilution of the fluorescein in photolabeled cells, we looked for smooth muscle progenitors at a later stage in development.

Arterial pole precursors are located mediocranially in the zebrafish heart fields

As we were unable to identify smooth muscle progenitors in the zebrafish heart fields we took advantage of the Tg(\( \text{gsc}::\text{GFP} \)) line, which expresses GFP in the Goosecoid homeobox transcription factor domain, allowing clear identification of the dorsal axis prior to gastrulation (Doitsidou et al., 2002). Adopting similar fate-mapping strategies to those of Keegan et al. (Keegan et al., 2004), caged fluorophores were used as lineage tracers to identify cells of the blastula that contribute to the most cranial myocardium of the ventricle and the smooth muscle of the bulbus arteriosus. By activating caged fluorophores with laser photolysis in no more than one or two neighboring blastomeres and recording their position within the blastula, the contribution of these labeled cells to the arterial pole was assessed at 72 hpf. As previously published (Keegan et al., 2004), atrial and ventricular progenitors were found in distinct populations of cells in the first four tiers of cells in the LMZ at 40% epiboly. Ventricular myocardial progenitors were found to reside between 55° and 140° from dorsal in tiers one through three, with atrial precursors between 75° and 160° from dorsal in tiers two through four (Fig. 1). In addition to the identification of atrial and ventricular progenitors, we identified cells that populated the myocardium at the distal outflow tract of the ventricle, restricted to the first and second tiers between 55° and 105° from dorsal (Fig. 1). No labeled cells were detected in the smooth muscle of the bulbus arteriosus at 72 hpf. As excessive proliferation might lead to dilution of the fluorescein in photolabeled cells, we looked for smooth muscle progenitors at a later stage in development.

Contribution to the arterial pole is delayed in early heart tube formation

To assess the timing of secondary heart field contribution to the zebrafish heart, we examined the location of the arterial pole progenitor population during the early differentiation of cardiac cells at the 14-somite stage in Tg(\( \text{cmlc2}::\text{GFP} \)) fish (\( \text{cmlc2} \) is also known as \( \text{myl7} \) – Zebrafish Information Network), which express GFP in the myocardium (Huang et al., 2003). GFP becomes detectable in differentiating cardiac cells at the 14-somite stage through immunohistochemical amplification of the signal. Cells photoactivated in grid zone F at the 7-somite stage, which contributed exclusively to the arterial pole and the ventricle, were not GFP positive, suggesting that they remained undifferentiated. These cells were dorsal to the population of GFP-positive cardiac cells in Tg(\( \text{cmlc2}::\text{GFP} \)) zebrafish at the 14-somite stage (Fig. 3A-C), as the GFP-positive cardiomyocytes moved towards the midline to form the cardiac cone. Cells labeled in grid zones B and H, which contributed to both the atrium and the ventricle, could be found both within and outside the GFP-positive domain at the 14-somite stage (Fig. 3D,E).

Given that \( \text{Isl1} \) has been observed in differentiated cardiac tissue in chick (Yuan and Schoenwolf, 2000), we examined \( \text{Isl1} \) expression in the cells that form the heart in zebrafish. As the cardiogenic fields approached the dorsal midline at the 14-somite stage, we observed \( \text{Isl1} \) in an area ventral and lateral to the trigeminal placode as is the case in the cardiac tissue in the GFP-positive domain in Tg(\( \text{cmlc2}::\text{GFP} \)) fish (Fig. 3F-H). \( \text{Isl1} \) is robustly expressed in the trigeminal placode also, making it a reliable landmark at the 14-somite stage (Thisse et al., 2004). In transverse sections, we observed \( \text{Isl1} \) in the layer of differentiated cardiomyocytes and in the mesodermal cells dorsal to them (Fig. 3J), where labeled cells that contribute to the arterial pole were visible at this stage (Fig. 3A-E), with no observable coelom in between. At 30 hpf, cells from grid zone F were within the branchial region, typically in the pericardial wall, but had not been incorporated into the heart tube (Fig. 3K).

Fig. 1. Arterial pole progenitors at 40% epiboly. Fate map of arterial pole progenitors in the zebrafish embryo at 40% epiboly. Shown are the first four tiers of the lateral marginal zone (LMZ) for the left and right sides of the embryo, dorsal to the right (0°). The equatorial circumference of the embryo has been divided into 3° segments, each segment corresponding approximately to a single cell diameter. The fate map is presented with the embryo flattened for simplicity. Dots represent experiments in which caged fluorophores were activated in single cells and colors indicate the position of their progeny at 72 hpf.
Progenitors progressively move into the arterial pole after heart tube formation

Cells that contribute to the arterial pole did not differentiate during initial heart tube formation and remained in the branchial region during looping, which is reminiscent of secondary heart field progenitors in chick embryos (Waldo et al., 2005). We therefore predicted that we could label cells in the branchial region and observe them in the myocardium from 48 hpf, when myocardial addition nears completion. We labeled cells in the dorsal pericardial wall adjacent to the arterial pole with the lipophilic dye DiI at 24, 30 and 36 hpf and observed incorporation into the ventricle at both 48 and 72 hpf. In these experiments, we observed cells in the myocardium in 82% (19/23) of embryos injected with DiI at 24 hpf, 62% (18/29) of embryos injected at 30 hpf, and 55% (11/20) of embryos injected at 36 hpf. Cells labeled at 30 and 36 hpf were generally restricted to the most cranial portion of the ventricle at the developing outflow tract, whereas cells labeled earlier migrated further into the ventricle (Fig. 4; data not shown). The presence of these DiI-labeled cells in the myocardium was confirmed by confocal imaging of the hearts (Fig. 4C,F).

Table 1. Summary of mapped cells in the mesodermal heart fields

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In total, 111 cells were mapped.
A/V, atrioventricular canal; OFT, outflow tract.
* Bulbus arteriosus (BA) and myocardium (M) at the arterial pole.
† Only the myocardium of the arterial pole.
* Labeling of smooth muscle only was never observed.
Fig. 3. Cells that contribute to the arterial pole remain outside the early heart tube. (A) Diagram of a transverse section of a 14-somite embryo, with dorsal to the top, showing the neural tube (NT, light blue) with differentiating myocardium (red) in the ALPM overlying the yolk (Y, yellow). (B) Whole-mount 14-somite embryo in caudal view, dorsal to top. Labeled cells from grid zone F (black) were observed in the splanchnic mesoderm of Tg(cmlc2::GFP) zebrafish that expresses GFP in differentiated cardiomyocytes (orange). (C) Transverse section oriented as in A showing labeled cells (black) relative to differentiating myocardium (red). (D,E) Cells from grid zone B (black) can be seen in the differentiated myocardium (red) at 14 somites in whole mount (D) and in section (E). (F) Diagram of a 14-somite embryo from the left side, dorsal to top, showing the position of the cardiac progenitors (red), the eye (E, pink), the trigeminal placode (Tg, light blue) and the yolk (yellow). (G,H) A 14-somite embryo oriented as in F, showing Isl1 (H, green) and cardiomyocytes (G, orange). The trigeminal placode and the non-differentiating portion of the heart field are Isl1 positive. Arrows indicate the anterior and posterior extent of cmic2::GFP or Isl1 signal in G and H, respectively. (I,J) Transverse sections of embryos at the 14-somite stage at the level of the trigeminal placode. Isl1 (green) can be barely detected in the nuclei (blue) of differentiating myocardium (arrowhead) but more brightly in the nuclei of cells in the adjacent splanchnic mesoderm (arrow). (K) Dorsal view of a 30 hpf zebrafish. Cells uncaged from grid zone F at the 7-somite stage (black, arrows) can be seen at some distance from the heart tube (orange). A, atrium; V, ventricle. Scale bars: 10 μm.

**Progenitors adjacent to the arterial pole express second heart field markers**

In mouse, the transcription factor Isl1 is expressed in the mesoderm of the pericardial wall after differentiation of the first heart field has begun, in a population that continues to proliferate and remains contiguous with the heart tube (Cai et al., 2003). Isl1 was detected in the zebrafish branchial region at 24, 36 and 48 hpf, in cells adjacent to the arterial pole (Fig. 5A-C’). These cells extended from the pericardial wall into the developing ventricle. Isl1 was largely absent at the arterial pole by 72 hpf (Fig. 5D,D’).

Bmp signaling in the secondary heart field has been linked to the differentiation of cardiac progenitors that are being added to the arterial pole in mammals and birds (Prall et al., 2007; Dyer et al., 2010). We observed Bmp signaling activity at the arterial pole of zebrafish embryos using an antibody for phosphorylated (p) Smad1/5/8, which are intracellular effectors of the Bmp pathway that are phosphorylated when Bmp signaling is active (Faure et al., 2000). pSmad1/5/8 staining was strong in cells adjacent to the arterial pole between 24 and 48 hpf (Fig. 6A-C’), but appeared to be restricted to a subset of Isl1-expressing cells closest to the arterial pole myocardium up until 48 hpf (Fig. 6D). Bmp signaling was also evident in the most proximal cells of the bulbus arteriosus at 72 hpf (Fig. 6E,E’).

**Bmp promotes myocardial differentiation from arterial pole progenitors**

To determine the role of Bmp signaling we knocked down Bmp-driven phosphorylation by treating embryos with dorsomorphin, which specifically blocks Bmp-mediated phosphorylation of Smad1/5/8 in zebrafish (Yu et al., 2007). Embryos were treated with 40 or 20 μM dorsomorphin from 24 to 72 hpf, or with DMSO as a control. Exposure of the embryos to 40 μM dorsomorphin resulted in a dramatic truncation of the ventricle, with subsequent expansion of the smooth muscle of the bulbus arteriosus (Fig. 7A-C). Development of the head muscles was also significantly delayed after dorsomorphin treatment and appeared comparable to the head musculature normally observed at 52 hpf (Fig. 7C,D). As smooth muscle appears at 52 hpf, we also treated embryos with dorsomorphin from 24 hpf to 48 hpf to observe any effects on the ventricle exclusively. The ventricle appeared to be smaller and looping was perturbed in the absence of Bmp signaling (Fig. 7E,F), but the effect was milder than that previously observed at 72 hpf. We verified the dorsomorphin-induced inhibition of Bmp signaling by treating embryos with 40 μM dorsomorphin or DMSO from 24 to 30 hpf and subsequently probing with anti-pSmad1/5/8. As expected, Smad1/5/8 phosphorylation at the arterial pole was significantly reduced at 30 hpf (Fig. 7G,H).

**Arterial pole progenitors in zebrafish are distinct from the neural crest population**

Cardiac neural crest contributes to the septation of the arterial pole in animals with a divided systemic and pulmonary circulation (Kirby et al., 1983; Kirby et al., 1985), as well as to the innervation of the heart (Kirby and Stewart, 1983), but not the myocardium, during embryonic development. In zebrafish, two studies have found a significant contribution of neural crest to the myocardium (Li et al., 2003; Sato and Yost, 2003). To determine whether our tracing studies had inadvertently labeled migrating neural crest cells, we utilized Tg(sox10::eGFP) fish (Carney et al., 2006). Sox10 is active in all neural crest cells during the early stages of migration from the neural tube in zebrafish (Antonellis et al., 2008). In Tg(sox10::eGFP) fish, GFP expression was restricted to
the neural tube at the 7-somite stage (see Fig. S1A-C in the supplementary material). Cells in the cranial neural crest dispersed out towards the ALPM in three migrating streams by the 14-somite stage (see Fig. S1D-F in the supplementary material). Arterial pole progenitors in grid zone F (Fig. 2B) were photoactivated at the 7-somite stage in the Tg(sox10::eGFP) zebrafish and, in five embryos analyzed, the labeled arterial pole progenitors were distinct from the GFP-positive cells that expressed sox10 (see Fig. S1C,F in the supplementary material).

The addition of myocardial cells from the branchial region to the heart tube is reduced in mutants with arterial pole defects

Both the transcription factor Tbx1 and the Shh signaling pathway have been established as important components of normal arterial pole development in animals with systemic and pulmonary circulations (Dyer and Kirby, 2009a; Kochilas et al., 2002; Xu et al., 2004; Washington Smoak et al., 2005). To determine whether Shh signaling and Tbx1 play similar roles in zebrafish, we studied arterial pole development in strains with a hypomorphic mutation in smo (Varga et al., 2001), an obligatory member of the Hedgehog signaling pathway, and a nonsense mutation in tbx1 (van gogh) (Piotrowski et al., 2003).

All the components of the arterial pole are visible and well defined by 72 hpf (Fig. 8A-C). Anti-Eln2 effectively labels the bulbus arteriosus during embryonic development in zebrafish (Miao et al., 2007) and provides a strong signal at this time. In smo mutants, looping did not occur and the ventricle was significantly reduced in size at 36 and 48 hpf. The smooth muscle of the bulbus arteriosus did not typically appear in these mutants by 72 hpf (Fig. 8D-F). At 96 hpf, smooth muscle was visible in only five of 16 smo mutants in section, with only two possessing a bulbus arteriosus-like structure (data not shown). In tbx1 mutants, the ventricle appeared smaller at 36 hpf and was significantly smaller than in wild-type siblings at 48 hpf. Even though the ventricle was reduced, the heart appeared to loop partially. At 72 hpf, the ventricle remained small and the bulbus arteriosus was reduced in size and dysmorphic (Fig. 8G-I). To assess any changes in the differentiation of arterial pole progenitors in the mutant embryos, we looked at Bmp signaling as a gauge of the number of differentiating cells at the arterial pole. We counted the cells that were positive for pSmad1/5/8 at the arterial pole in serial sections.
DISCUSSION

In the epiblast of chick and mouse, the craniocaudal location of cardiac progenitors is collinear with their organization in the heart tube (Hatada and Stern, 1994; Tam et al., 1997). Because gastrulation occurs craniocaudally, the most cranial epiblast cells, which represent the arterial pole progenitors, are the first to gastrulate. Although a dorsal midline primitive streak does not exist in zebrafish, the location of cardiac progenitors in the blastoderm is comparable because the first tier of cells gastrulate first with the subsequent tiers following (Warga and Kimmel, 1990). Thus, our data support the work of Keegan et al. (Keegan et al., 2004) and show that the location of myocardial progenitors within the blastoderm and their order of gastrulation are consistent among all vertebrate models studied. Our data show that arterial pole progenitors represent a subset of the ventricular myocardial progenitors in the blastoderm, which would be among the first cardiac progenitor cells to undergo gastrulation. Although we did not observe arterial pole smooth muscle progenitors in the blastula, none of our tracing experiments found labeled smooth muscle cells in the absence of labeled myocardium and this supports the idea that some smooth muscle precursors are at least located in the vicinity of myocardial precursors.

After gastrulation, we show that a common progenitor pool within the mesodermal cardiogenic fields gives rise to the smooth muscle and myocardial cells that comprise the definitive arterial pole of the zebrafish, as has been shown in chick (Hutson et al., 2010). In fate-mapping studies, the secondary heart field has been localized to the mediocaudal edge of the cardiogenic field in chick (Abu-Issa and Kirby, 2008). By contrast, our data show a mediocranial location of the secondary heart field progenitors in the cardiogenic field in zebrafish. This difference reflects the manner in which the heart tube is formed in these species. In chick, heart fields fold 120°–130° along with the coelom during migration towards the midline, bending ventrally to reorient towards the midline (Abu-Issa and Kirby, 2008). As the zebrafish is an anamniote, folding of the coelom and the heart fields does not occur. Instead, the heart fields merge to form a cardiac cone that becomes a tube by a process of asymmetric involution of the right side of the cone (Rohr et al., 2008; Stainier et al., 1993).

For the heart to continue to elongate and loop after early heart tube formation, second heart field progenitors must remain undifferentiated until the appropriate time for addition to the myocardium of the heart tube. The arterial pole progenitors that we have identified differentiate after heart tube formation and can be observed in the pericardial wall at 30 hpf, similar to what is observed in chick and mouse (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). Previously, de Pater et al. demonstrated...
that cells are added to the poles of the heart tube between 24 and 48 hpf in zebrafish (de Pater et al., 2009). We have observed cells labeled at the pericardial wall being incorporated into the arterial pole in the same time frame. This is consistent with the idea of a true secondary heart field in zebrafish. Additionally, after acceptance of this article for publication, Lazic and Scott (Lazic and Scott, 2011) showed that cells adjacent to and within the developing cardiac arterial pole express mef2c. In the absence of this zebrafish homolog of Mef2c, ventricular cell number is reduced significantly at 48 hpf.

The arterial pole of the zebrafish includes the most cranial myocardium of the ventricle and the large smooth muscle bulbus arteriosus that connects to the ventral aorta (Grimes and Kirby, 2009). The bulbus arteriosus is thought to protect the gills from high blood pressure arising at the ventricle and to prevent backflow (Albrecht et al., 2003; Santer, 1985). Although the function of this vessel differs from that of the base of the great arteries of mammalian and avian hearts, its developmental origin seems to be at least partially derived from the heart fields. The interface of the zebrafish bulbus arteriosus with the most cranial myocardium at the outflow is strikingly similar to that in other vertebrates (Grimes et al., 2006). The homology of the secondary heart field and the resulting arterial pole structure suggests that the molecular mechanisms involved are conserved.

To explore these mechanisms, we first looked at Isl1 as an early marker of the second heart field and showed that it is expressed in zebrafish in the mesenchyme adjacent to the arterial pole. In mice, Isl1 is restricted to the second heart field after the first heart field begins to differentiate, and Isl1 expression ceases as cells are added to the growing heart tube (Cai et al., 2003; Ma et al., 2008). In the zebrafish, Isl1 is expressed by differentiating myocardial cells that are in the process of forming the cardiac cone, as well as in mesoderm where undifferentiated cardiac cells are located at the 14-somite stage. This is similar to the enduring expression of Isl1 observed in the fusing heart tissue of the chick early heart tube (Yuan and Schoenwolf, 2000). Whereas knockdown of isl1 produces no phenotype at the arterial pole in zebrafish (de Pater et al., 2009), the isl1-null mouse has a dramatic arterial and venous pole phenotype (Cai et al., 2003). This suggests non-conservation of Isl1 function in zebrafish heart development or, alternatively, that another gene fulfills the same function but is yet to be recognized as a result of the ancient polyploidy event that took place in teleosts (Semon and Wolfe, 2007; Van de Peer et al., 2003).

We have also shown that Bmp signaling is highly active in arterial pole progenitors and in the myocardium, particularly in a subset of Isl1-positive progenitors, and also in smooth muscle closest to the ventricle at 72 hpf. Exogenous Noggin, an inhibitor of Bmp signaling, has been shown to delay differentiation in cultured secondary heart field progenitors (Waldo et al., 2001).

**Fig. 8. Zebrafish tbx1 and smo mutants display arterial pole defects.**

(A-C) Ventral view of whole-mount wild-type zebrafish, with cranial to the top. The heart is looped by 36 hpf and the bulbus arteriosus (En2) is formed by 72 hpf. Ventricle (Mf20, red); atrium (S46, yellow). (D-I) In smo (D-F) and tbx1 (G-I) mutants, cardiac development is impaired. (J) Sagittal sections from the left show pSmad1/5/8 (green and arrows) near the arterial pole of the wild-type heart (CT3) at 30 hpf. (K,L) In the smo (K) and tbx1 (L) mutants, pSmad1/5/8 is significantly reduced. (M) Quantification of pSmad1/5/8-positive cells in wild-type versus smo and tbx1 mutants. Numbers in bars indicate the number of fish sampled. *, P=1.23×10^{-3}. Error bars indicate s.d. Scale bars: 5 μm.

**Fig. 9. Labeled cells in the pericardial wall are not incorporated into the developing heart in smo and tbx1 mutants.**

(A-C,G-I) Cells labeled with Dil (pseudocolored green) in the branchial region are incorporated into the myocardium (pseudocolored red) in wild-type zebrafish hearts after labeling at 24 hpf (A-C) and 30 hpf (G-I). Dil colocalized with the myocardium in wild-type fish at 48 and 72 hpf (arrows). (D-F,J-L) This incorporation of cells does not occur in most smo (D-F) or tbx1 (J-L) mutants. The green cells that appear to overlap with red cardiac cells (K) do not overlap with the myocardium 24 hours later (L). All embryos expressed cmlc2-GFP. Scale bar: 25 μm.
data are also consistent with the observation of restricted Bmp2/4 expression in the mesoderm adjacent to the arterial pole after myocardial addition has ceased in chick (Somi et al., 2004). Previous work has shown that Bmp signaling drives myocardial differentiation in vivo in chick (Tirosh-Finkel et al., 2010). Bmp signaling also induces myocardial differentiation in chick secondary heart field explants, which can separately be induced to differentiate as smooth muscle with exogenous FgF8 (Hutson et al., 2010). We confirm that Bmp signaling drives myocardial differentiation in zebrafish and further show that loss of Bmp signaling increases the size of the smooth muscle bulbus arteriosus, suggesting that the progenitor pool is temporarily restricted in that a reduced contribution to the arterial pole myocardium results in an enhanced contribution to the bulbus smooth muscle.

In addition to these markers, we have also explored the roles of Hedgehog signaling and Tbx1 transcripational regulation. Tbx1 is expressed in the mesoderm and endoderm of the pharyngeal region and is known to be necessary for myocardial and endocardial addition to the arterial pole (Chapman et al., 1996; Huyhn et al., 2007). The Shh pathway is also known to regulate Tbx1 activity in the pharyngeal region through Forkhead transcription factors (Yamagishi et al., 2003). Smoothened is a transmembrane protein that is activated in the presence of Hedgehog ligands and is required for all Hedgehog signaling. We used the smo mutant, in which all Hedgehog signaling is eliminated (Varga et al., 2001).

Recent work has shown that the Shh pathway promotes proliferation in the chick secondary heart field progenitors in vivo (Dyer and Kirby, 2009a). Additionally, the loss of Hedgehog signaling has been shown to reduce the size of the heart fields in the zebrafish blastula (Thomas et al., 2008). Given the lack of secondary heart field structures in the zebrafish mutants that we examined, our data support the preliminary investigations of Grimes and Kirby (Grimes and Kirby, 2009) and indicate that the role of these genes in arterial pole development is conserved in teleosts.

In conclusion, our data demonstrate conservation of all the steps in the development of arterial pole progenitors prior to septation. Abnormal development of secondary heart field progenitors has been shown to underlie many conotruncal defects that are seen in neonates (Hutson et al., 2006; Ward et al., 2005). The conservation of the pathways needed for arterial pole development in an organism that lacks outflow septation establishes the importance of these pathways in tissue patterning and strongly suggests that the process of elongation of the arterial pole preceded, and plays a key role in, the process of outflow septation in evolutionary history.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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References


Table 2. Summary of Di-labeled cells at the pericardial wall that moved into the ventricle

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Labeled at:</th>
<th>24 hpf</th>
<th>30 hpf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>82%</td>
<td>62%</td>
<td></td>
</tr>
<tr>
<td>smo</td>
<td>17%</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>tbx1</td>
<td>–</td>
<td>14%</td>
<td></td>
</tr>
</tbody>
</table>

The percentage of wild type, smo and tbx1 mutants that incorporated any Di-labeled cells into the myocardium following Di labeling at 24 or 30 hpf.


