RESEARCH ARTICLE

Myocardium and BMP signaling are required for endocardial differentiation

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ABSTRACT
Endocardial and myocardial progenitors originate in distinct regions of the anterior lateral plate mesoderm and migrate to the midline where they coalesce to form the cardiac tube. Endocardial progenitors acquire a molecular identity distinct from other vascular endothelial cells and initiate expression of specific genes such as nfatc1. Yet the molecular pathways and tissue interactions involved in establishing endocardial identity are poorly understood. The endocardium develops in tight association with cardiomyocytes. To test for a potential role of the myocardium in endocardial morphogenesis, we used two different zebrafish models deficient in cardiomyocytes: the hand2 mutant and a myocardial-specific genetic ablation method. We show that in hand2 mutants endocardial progenitors migrate to the midline but fail to assemble into a cardiac cone and do not express markers of differentiated endocardium. Endocardial differentiation defects were rescued by myocardial but not endocardial-specific expression of hand2. In metronidazole-treated myl7:nitroreductase embryos, myocardial cells were targeted for apoptosis, which resulted in the loss of endocardial nfatc1 expression. However, endocardial cells were present and retained expression of general vascular endothelial markers. We further identified bone morphogenetic protein (BMP) as a candidate myocardium-derived signal required for endocardial differentiation. Chemical and genetic inhibition of BMP signaling at the tailbud stage resulted in severe inhibition of endocardial differentiation while there was little effect on myocardial development. Heat-shock-induced bmp2b expression rescued endocardial nfatc1 expression in hand2 mutants and in myocardium-depleted embryos. Our results indicate that the myocardium is crucial for endocardial morphogenesis and differentiation, and identify BMP as a signal involved in endocardial differentiation.

KEY WORDS: Heart, Endocardium, Myocardium, Zebrafish, Nfatc1, Bone morphogenetic protein

INTRODUCTION

The endocardium forms the endothelial lining of the heart and gives rise to cardiac valves and septa (Eisenberg and Markwald, 1995). In addition to serving as a structural barrier for circulating blood cells, the endocardium has crucial roles during development that were recognized only recently. Endocardial cells are important for the migration of myocardial progenitors during cardiac cone assembly and for myocardial trabeculation (Holtzman et al., 2007; Stankunas et al., 2008). Endocardial cells have recently been shown to contribute to hematopoiesis and coronary vasculature (Nakano et al., 2013; Tian et al., 2014). Furthermore, the endocardium has multiple signaling roles during heart regeneration (Kikuchi et al., 2011; Zhao et al., 2014). Despite the crucial role for the endocardium in heart development and function, the molecular mechanisms that regulate endocardial development are still poorly understood.

Although it is difficult to study early endocardial development in mammals due to embryo inaccessibility, the zebrafish (Danio rerio) has emerged as an advantageous model system in which to study cardiovascular development (Asnani and Peterson, 2014). Endocardial cells and vascular endothelial cells of blood vessels express many of the same molecular markers, which suggests a common origin of both cell types. Indeed, recent evidence argues that endocardial cells are derived from vascular endothelial progenitors (Misfeldt et al., 2009; Milgrom-Hoffman et al., 2011). However, endocardial cells exhibit distinct migratory behavior and express specialized markers such as nfatc1 (de la Pompa et al., 1998), which shows that the endocardial and vascular endothelial cells of blood vessels are biochemically distinct. In zebrafish, endocardial precursors can be distinguished from other vascular endothelial cells as they migrate medially and posteriorly towards the midline and fuse between the 15- and 18-somite stages (Bussmann et al., 2007). Subsequently, they undergo a complex leftward movement to position the endocardial primordium on the left side of the embryo and form the lining of the primitive heart tube. This migration of endocardial progenitors is evolutionarily conserved. Bilateral fields of endocardial progenitors translocate to the midline between 7.5 and 8.5 dpc in a mouse embryo (Drake and Fleming, 2000). However, the signaling pathways that regulate specification, migration and differentiation of endocardial progenitors are poorly understood.

We have previously demonstrated that hedgehog (Hh) signaling is required for endocardial differentiation in zebrafish embryos (Wong et al., 2012). In the absence of Hh signaling, endocardial progenitors fail to migrate to the midline and do not initiate endocardial nfatc1 expression, while endothelial differentiation of blood vessels is not affected. This argues that Hh is one of the signals specifically required for endocardial differentiation. However, Hh is not sufficient to induce endocardial differentiation, suggesting that additional signals are necessary.

Endocardial and myocardial cells closely interact during development. In the absence of the endocardial progenitors in cloche mutants, myocardial cells exhibit defects in cone assembly (Holtzman et al., 2007). On the other hand, myocardium-derived
bone morphogenetic protein (BMP) signaling is crucial for epithelial-to-mesenchymal transformation (EMT) to form endocardial cushions and valves (Jiao et al., 2003; Rivera-Feliciano and Tabin, 2006; Garside et al., 2013). In addition, myocardial-derived BMP has been recently implicated in endocardial proliferation during the endocardial ballooning phase (Dietrich et al., 2014). However, myocardial requirement for the initial endocardial specification and differentiation has not been established.

In zebrafish, endocardial and myocardial fields can be separated as early as gastrulation stages (Stainier et al., 1993; Lee et al., 1994). During early somitogenesis stages, both endocardial and myocardial cells are positioned within distinct regions of the anterior lateral plate mesoderm (ALPM), with endocardial cells positioned more anteriorly than myocardial progenitors (Schoenebeck et al., 2007). Endocardial progenitors initiate migration to the midline at the 14-somite stage and coalesce into a ‘disk’, which is followed by the migration of myocardial progenitors that form a ring surrounding the disk (Stainier et al., 1993; Glickman and Yelon, 2002; Bussmann et al., 2007). Subsequently, the cardiac disk is transformed into a cardiac cone that elongates to form a tube and initiates heart beat. Intriguingly, endocardial nfatc1 expression is only initiated at 21-22 hpf, shortly after myocardial cells come into close contact with endocardial progenitors (Wong et al., 2012). Therefore, we hypothesized that the myocardium may provide a signal critical for endocardial differentiation.

In this study, we tested this hypothesis by analyzing endocardial differentiation in hand2 mutants, which are deficient in myocardial progenitors (Yelon et al., 2000). We show that hand2 function is required in myocardial progenitors for endocardial differentiation. We further used genetic ablation of myocardial progenitors to demonstrate that myocardial cells are required to maintain endocardial differentiation. And finally, we establish BMP as a candidate myocardial-derived signal required for endocardial differentiation.

RESULTS

Hand2 is required within the myocardium for endocardial differentiation

To determine the role for the myocardium in endocardial development, we analyzed endocardial differentiation in hand2 mutant embryos, which have greatly reduced numbers of myocardial progenitors (Yelon et al., 2000). Previous studies have demonstrated that endocardial precursors in hand2 mutants migrate to the midline and form the endocardial sheet similar to their wild-type siblings but exhibit defective antero-posterior spreading (Garavito-Aguilar et al., 2010). However, endocardial differentiation has not been previously analyzed in hand2 mutants.

Markers associated with endocardial differentiation, such as nfatc1, notch1b and klf2a, are expressed throughout the endocardium at 24-48 hpf stages in wild-type embryos and are subsequently enriched in the atrioventricular canal (AVC) (Westin and Lardelli, 1997; Oates et al., 2001; Walsh and Stainier, 2001; Wong et al., 2012). Endocardial expression of these markers was severely reduced or completely absent in hand2/−/− embryos at the analyzed stages of 22 hpf; 30 hpf (nfatc1) and 53 hpf (nfatc1, notch1b and klf2a) (Fig. 1A-F). Analysis of the expression of the vascular endothelial markers flt1a and kdr (Thompson et al., 1998) at 28 hpf and the endothelial reporter etv2-GFP (Proulx et al., 2010) at 22-36 hpf showed that overall vascular endothelial differentiation was not affected in hand2 mutants (Fig. 1G,H; supplementary material Fig. S1). However, the endocardial cone was absent in hand2 mutants and endocardial progenitors were present in a broad loose epithelial sheet spanning the cardiac-forming region (supplementary material Fig. S1, white dotted line). The development of the first aortic arch, which connects to the cardiac tube, did not appear to be significantly affected (Fig. 1G,H).

To understand better the nature of endocardial defects in hand2 mutants, we analyzed earlier stages of endocardial morphogenesis. The midline expression of flt1a, kdr, cdh5 and etv2-GFP in endocardial progenitors was largely unaffected in hand2 mutants at the 17-somite stage (Fig. 1I-L), in agreement with previous studies (Garavito-Aguilar et al., 2010). To visualize endocardial development during the stages of the cone formation, we analyzed endocardial and myocardial formation in double-transgenic etv2: GFP; myl7:mCherry embryos at 22-36 hpf. In hand2 mutants at 22 hpf, sparse myocardial cells were not fused at the midline but remained bilateral, while a disorganized etv2:GFP+ cell population formed an endocardial sheet that was spread throughout the medial region (supplementary material Fig. S1A,D). Between 26 and 36 hpf, hand2 mutants displayed a range of phenotypes in unfused and fused cardiomyocytes; however, the etv2-GFP cells remained spread out as a sheet throughout the cardiac region (supplementary material Fig. S1B,C,E,F). Intriguingly, an ectopic vessel was observed extending posteriorly from the endocardial sheet in hand2 mutants (supplementary material Fig. S1B,C,E,F). As described previously, cardiac cone or tube failed to assemble in hand2 mutants (Yelon et al., 2000).

Fibronectin (fn1) expression has been suggested as one of the earliest markers for endocardial progenitors, observed as early as the 10-somite stage in the anterior lateral plate mesoderm (ALPM) (Trinh and Stainier, 2004; Palencia-Desai et al., 2011; Wong et al., 2012). However, the identity of all fn1-expressing cells in the ALPM region has not been clear. Interestingly, increased fn1 expression has been previously reported in hand2 mutants (Garavito-Aguilar et al., 2010). To analyze further the effect of the hand2 mutation on endocardial morphogenesis, we performed in situ hybridization and fn1:mCherry-NTR reporter analysis (Wang et al., 2013). fn1 expression was not significantly affected in hand2 mutants at the 12-somite stage, prior to the migration of endocardial progenitors to the midline (Fig. 1M). However, at the 17-somite and 20-somite stages, the midline population of fn1-expressing cells appeared reduced, while strong bilateral fn1 expression was observed in hand2 mutants (Fig. 1N,O). As previously demonstrated, the midline population of fn1-expressing cells was absent in cloche mutants (Trinh and Stainier, 2004) (Fig. 1P), suggesting that this population of fn1+ cells corresponds to the endocardial progenitors. However, cloche mutants retained bilaterally located fn1+ cells in the ALPM region (Fig. 1P). Analysis of fixed fn1:mCherry-NTR; myl7:GFP and fn1:mCherry-NTR; kdr:GFP double-transgenic embryos showed that at the 17- to 22-somite stages, most endocardial and some myocardial progenitors were positive for fn1:mCherry-NTR expression, while at 42 hpf fn1:mCherry-NTR expression was observed in both endocardium and myocardium (supplementary material Fig. S2). As observed by confocal microscopy, hand2 mutants at the 22- to 24-somite stages exhibited a reduced population of fn1:mCherry-NTR cells at the midline and showed intense bilateral fn1:mCherry-NTR expression that largely overlapped with the endothelial etv2:GFP expression but not with the myocardial myl7:GFP expression (supplementary material Fig. S3). These results argue that fn1 expression in the ALPM region at these stages is largely restricted to the endocardial progenitors. In hand2 mutants, these progenitors do not coalesce into the endocardial tube at the midline but instead remain as a sheet that encompasses lateral and medial domains of the cardiac-forming region.
It has been shown that hand2-expressing cells in the ALPM region correspond to the myocardial progenitors (Schoenebeck et al., 2007). However, it is possible that hand2 may also be expressed in the endocardial progenitors at low levels that are difficult to detect by in situ hybridization. Indeed, the hand2:GFP transgenic reporter line (Kikuchi et al., 2011) displays GFP expression in both myocardial and endocardial layers (supplementary material Fig. S4A,B). To determine which cardiac tissue requires hand2 function during endocardial differentiation, we tested whether hand2 myocardial or endocardial expression was sufficient to restore nfatc1 expression in hand2 mutants. Transient hand2 expression under myocardial myl7 promoter (supplementary material Fig. S4C) resulted in a partial rescue of nfatc1 expression in hand2 mutant embryos. Forty-seven percent of myl7:hand2-mCherry DNA-injected hand2−/+ embryos displayed moderate levels of nfatc1 expression and 11% displayed robust nfatc1 expression (n=73), whereas 100% of non-injected hand2−/+ embryos showed weak or no nfatc1 expression (n=21) (Fig. 2A-E,K). Because transient endocardial/endocardial specific hand2 expression using fli1a:hand2-mCherry construct did not show significant rescue of nfatc1 expression (data not shown), a stable line was established using To2 transgenesis (supplementary material Fig. S4D). Eighty-eight percent of fli1a:hand2-mCherry; hand2−/+ embryos had none or weak nfatc1 expression and 12% displayed moderate (still greatly reduced) nfatc1 expression level (Fig. 2F-J,L). Continuous hand2 expression under the control of the fli1a promoter resulted in embryonic lethality during later stages, most likely due to hand2 overexpression in the vasculature, where it is not normally expressed (data not shown). Overall, these results argue that hand2 function is required in the myocardium for endocardial differentiation.

**Genetic ablation of the myocardium results in the loss of endocardial differentiation**

To further test the role for the myocardium in endocardial morphogenesis, we used a genetic cell ablation model in which cardiomyocytes specifically express nitroreductase (NTR) (Curado et al., 2007). Upon treating embryos with the prodrug metronidazole (MTZ), cardiomyocytes develop cytotoxic products that directly kill only the NTR-expressing cells (Curado et al., 2007; Pisharath and Parsons, 2009). To perform cardiomyocyte ablation, a new Tg(myl7: mCherry-NTR)993 line was established that expresses mCherry-NTR fusion protein under the myocardial myl7 promoter (Huang et al., 2003). To confirm induction of apoptosis in the myocardial cell layer, immunohistochemistry against caspase 3 was performed in myl7:
mCherry-NTR; kdrl:GFP transgenic embryos treated with MTZ. Although the treatment was started at the tailbud stage, no significant defects were observed at 24 hpf or earlier stages (data not shown). At 36 hpf, intense caspase 3 expression was present in the myocardial cell layer (supplementary material Fig. S5). No changes in the myocardial mCherry or endocardial GFP expression were apparent in MTZ-treated embryos at 30 and 40 hpf stages (Fig. 3A,B). However, pericardial edema was apparent in MTZ-treated embryos as early as 30 hpf, indicating a decreased cardiac function (supplementary material Fig. S6). At 50 hpf there were clearly fewer mCherry-expressing cardiomyocytes, and the cardiac tube appeared partially collapsed and dysmorphic (Fig. 3C). By 72 hpf, very few mCherry-positive cardiomyocytes remained in MTZ-treated embryos (Fig. 3D). Interestingly, in the absence of the myocardium, the endocardial

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Fig. 2. Hand2 function is required within the myocardium for endocardial nfatc1 expression. (A-E) Transient overexpression of a hand2-2A-mCherry construct under the myocardial-specific myl7 promoter results in a partial rescue of nfatc1 expression in hand2 mutants. In situ hybridization analysis of nfatc1 expression at 40 hpf. In uninjected wild-type embryos, nfatc1 is expressed throughout the endocardium with a higher concentration at the AV boundary (A, arrowhead). Endocardial nfatc1 expression is absent in uninjected hand2 mutants (B, arrowhead). (C-E) Representative embryos within different categories of rescued endocardial nfatc1 expression (arrowheads). nfatc1 expression is also observed in pharyngeal arches. (F-J) Endothelial/endocardial-specific overexpression of hand2-2A-mCherry under the fli1a promoter fails to rescue endocardial nfatc1 expression in hand2 mutants, as analyzed by in situ hybridization at 26 hpf. (F,G) Non-transgenic (fli1a:hand2-mcherry−) embryos in wild-type (F) and hand2−/− (G) background. (H-J) fli1a:hand2-mCherry-positive embryos in wild-type (J) and hand2−/− (H,I) background. Eighty-nine percent of fli1a:hand2-mCherry+; hand2−/− embryos had little or no nfatc1 expression (H) and 11% had moderate nfatc1 expression, not significantly different from non-transgenic (fli1a:hand2-mCherry−) embryos (see L). Arrowheads indicate endocardial nfatc1 expression. (K,L) Percentages of myl7:hand2-mCherry and fli1a:hand2-mCherry embryos and their siblings in wild-type and hand2−/− background that showed different levels of nfatc1 expression.
endocardial differentiation in vitro, and in endocardial proliferation during the endocardial ballooning in zebrafish embryos (Mifeldt et al., 2009; Dietrich et al., 2014). However, a BMP requirement for early endocardial differentiation in vivo has not been established.

To test the role for BMP signaling during endocardial development, embryos were treated with the highly specific BMP inhibitor LDN193189 (Cuny et al., 2008; Cannon et al., 2010), and subsequently analyzed for endocardial nfatc1 and myocardial myl7 expression. Treatments that started prior to the tailbud stage (10 hpf) resulted in strong reduction of both endocardial and myocardial marker expression (data not shown), consistent with the previously established role of BMP in inducing cardiac mesoderm (Zhang and Bradley, 1996; Marques and Yelon, 2009; de Pater et al., 2012). However, treatment starting at the tailbud stage resulted in only a minor reduction of myocardial myl7 expression, as well as the defects in the elongation and jogging of the cardiac tube, consistent with the known requirement of BMP in heart tube rotation and differentiation of late forming cardiomyocytes (Smith et al., 2008; de Pater et al., 2012), whereas nfatc1 expression was greatly reduced or nearly completely absent (Fig. 5E-H,R). Overall vascular patterning was also normal in LDN193189-treated embryos at 24 hpf (supplementary material Fig. S7). Furthermore, the endocardial tube was collapsed but often was easily identifiable in BMP-inhibited myl7:GFP; kdr:mCherry transgenic embryos (Fig. 5I-L). LDN193189 treatment starting at later stages of 12 and 17 somites (15 hpf and 17.5 hpf) resulted in significant, albeit progressively smaller, inhibition of nfatc1 expression (Fig. 5M-P). Later LDN193189 treatment starting at 24 hpf failed to significantly inhibit endocardial nfatc1 expression at 44-48 hpf (data not shown). Although these results may suggest that BMP signaling is required only for the initiation of endocardial nfatc1 expression and not its maintenance, we cannot exclude the possibility that LDN193189 is only partially effective in inhibiting BMP signaling at later stages, when BMP expression levels may be higher. To confirm the results obtained using chemical BMP inhibitor, we performed inhibition of BMP signaling using heat-shock inducible expression of the dominant-negative BMP receptor (DNBMPR)-GFP fusion protein (Pyati et al., 2005). When DN-BMPR-positive embryos were heat-shocked at the tailbud stage, 37% of them showed greatly reduced or absent expression of nfatc1 when compared with the normal nfatc1 expression pattern in 94% of their heat-shocked siblings that were negative for the DNBMPR transgene (Fig. 5S,T). These results argue that BMP signaling is specifically required to initiate endocardial differentiation.

To test whether induction of BMP signaling was sufficient to initiate endocardial differentiation in myocardial-deficient embryos, we used the heat-shock inducible hsp70:bmp2b line (Chocron et al., 2007), crossed into the hand2 mutant background. Embryos were heat-shocked at the 16-somite stage and then analyzed at 24 hpf for nfatc1 expression. Significant induction of nfatc1 expression was observed in hsp70:bmp2b; hand2−/− embryos (Fig. 6A-C). Ubiquitous bmp2b induction did not rescue cardiac cone formation, however; instead, nfatc1 expression was observed in scattered cells within the cardiac-forming region (Fig. 6C). Intriguingly, the sheet of undifferentiated endocardial progenitors was observed in the same area in hand2−/− embryos (supplementary material Fig. S1). Fluorescent in situ hybridization/immunohistochemistry analysis for nfatc1 and Cdh5/VE-cadherin expression confirmed the endocardial identity of nfatc1-positive cells in hsp70:bmp2b; hand2−/− embryos (Fig. 6D-I). A partial rescue of nfatc1 expression was also observed in myocardium-depleted MTZ treated myl7:mCherry-NTR; hsp70: BMP2b embryos when subjected to heat-shock for BMP2b
induction (Fig. 6J-L). These results argue that BMP induction is sufficient to initiate endocardial differentiation in hand2−/− and myocardial ablated myl7:mCherry-NTR embryos.

**DISCUSSION**

In this study, we demonstrate the signaling requirement of myocardial cells during endocardial differentiation. hand2 mutant embryos, which are deficient in myocardial cells, lack the markers of differentiated endocardial cells but retain general vascular endothelial marker expression. Genetic ablation of myocardial cells upon nitroreductase/MTZ treatment results in the loss of endocardial nfatc1 expression. We further establish BMP as a candidate myocardium-derived signal, required for endocardial differentiation.

Prior to the 14-somite stage, endocardial cells are present bilaterally in the ALPM and express the same markers as other vascular endothelial cells of blood vessels. At this stage, there are no known markers that can be used to differentiate endocardial cells versus other vascular endothelial cells. fibronectin (fn1) expression is observed in endocardial progenitors but not in other vascular endothelial progenitors, prior to their migration towards the midline. But fn1 expression is not restricted to endocardial progenitors and is observed also in other cell types such as myocardial progenitors, as we demonstrate in this study. Therefore, it is reasonable to consider that endocardial progenitors have not yet initiated their differentiation prior to their migration towards the midline and are similar to other vascular endothelial cells.

After endocardial progenitors have arrived at the midline, myocardial progenitors initiate their migration. After the endocardial progenitors are surrounded by the myocardial cells, they initiate nfatc1 expression starting at about 22 hpf (Wong et al., 2012). In addition to nfatc1, other markers such as klf2a and notch1b are also specifically expressed in the endocardial cells and not expressed in the majority of vascular endothelial cells (Vermot et al., 2009). Because of this unique marker profile, endocardial cells can be considered as a distinct lineage separate from other vascular endothelial cells, and nfatc1 expression can be considered as one of the earliest markers of endocardial differentiation. Although our results argue that endocardial cells include a subtype of vascular endothelial cells, both cell types can be converted into each other. Thus, BMP signaling is required to establish endocardial identity within a subset of vascular endothelial cells. Furthermore, BMP expression is sufficient to initiate nfatc1 expression in a subset of vascular endothelial cells in hand2 mutants. These results support a model where endocardial cells are derived from vascular endothelial lineage, in agreement with other recent studies (Bussmann et al., 2007; Schoenebeck et al., 2007; Milgrom-Hoffman et al., 2011).

Our previous work has implicated Hh signaling in endocardial morphogenesis. Upon inhibition of Hh signaling, fn1 expression in
endocardial progenitors, their migration to the midline and differentiation was also severely inhibited yet overall vascular endothelial differentiation was not affected (Wong et al., 2012). Although Hh signaling has also been implicated in myocardial differentiation (Thomas et al., 2008), our previous work argued that Hh has distinct roles in endocardial and myocardial morphogenesis (Wong et al., 2012). However, overexpression of Hh homologs, while perturbing endocardial morphogenesis, was not sufficient to induce ectopic endocardial differentiation, which suggested that additional signals were necessary. Here, our results suggest that myocardial-derived BMP provides another crucial signal necessary for endocardial differentiation (Fig. 7).

As we demonstrate in this study, hand2 mutant embryos display a nearly complete loss of expression of nfatc1 and other specialized endocardial markers. Expression of general vascular endothelial markers such as kdr1 and cdh5 is retained in the endocardial progenitors, which argues that endocardial differentiation is specifically affected in hand2 mutants. Because the few remaining myocardial progenitors fail to extend into the cardiac cone, endocardial morphogenesis is also defective in hand2 mutants. One could argue that endocardial differentiation fails as an indirect consequence of defective morphogenesis. However, our previous experiments demonstrated that midline convergence and cardiac tube formation is not necessary for endocardial differentiation. In sox32 knockdown embryos, both endocardial and myocardial precursors remain positioned bilaterally and fail to form the heart tube, yet endocardial progenitors still initiate nfatc1 expression (Wong et al., 2012). These results also support our hypothesis that the myocardium provides a signal involved in endocardial differentiation. In sox32 morphants, endocardial progenitors are positioned bilaterally, adjacent to the myocardial cells; therefore, they are expected to receive the endocardial-inducing signal.

In the experiments that involve genetic ablation of myocardial cells, nfatc1 expression was initiated normally but subsequently lost from MTZ-treated embryos. Because myl7:NTR expression is weak prior to 24 hpf, and it is expected that additional time is needed for NTR and the toxic compounds to accumulate, apoptosis is not observed until 36 hpf. This is in line with the previous study, in which

**Fig. 5. BMP signaling is required for endocardial differentiation.** (A-D) As analyzed by in situ hybridization, myocardial bmp4 expression is greatly reduced in hand2−/− embryos (B,D) when compared with their siblings at 22 hpf (A,B) and 30 hpf (C,D). (E-H) Endocardial nfatc1 is greatly downregulated in BMP inhibitor LDN193189-treated embryos at 22 hpf (E,F) and 26 hpf (G,H) when compared with 0.1% DMSO-treated controls. (I-L) mCherry and GFP fluorescence are observed in the endocardium and myocardium, respectively, in 24 hpf fixed myl7:GFPP; kdr1:mCherry embryos that were treated with 10 μM LDN193189 from the the tailbud stage onwards (I,K, merged channels; J,L, mCherry channel only). Arrowheads indicate endocardial tube. (M-P) Treatment of embryos with BMP inhibitor LDN193189 starting at the 12- and 17-somite stages results in significant inhibition of nfatc1 expression at 26-28 hpf, as analyzed by in situ hybridization. Treatment starting at the 12-somite stage resulted in more significant downregulation of nfatc1 expression than the treatment starting at the 17-somite stage. (Q,R) Myocardial myl7 expression at 30 hpf is dysmorphic but does not show significant downregulation in the embryos treated with LDN193189 starting at the tailbud stage. (S,T) Heat-shock-inducible expression of DnBMPR-GFP results in significant downregulation of nfatc1 expression. hsp70:dnBMPR-positive embryos and their siblings were heat-shocked at the tailbud stage for 60 min and analyzed at 27 hpf.
heart damage was observed at 48 hpf, 24 h after MTZ treatment was initiated (Curado et al., 2007). As we show here, there is a high correlation between the loss of nfact1 expression and the loss of myocardial progenitors. This suggests that continuous signaling from the myocardium is required for the maintenance of endocardial differentiation. This suggests that endocardial identity is not a stable state and is reversible. In the absence of surrounding myocardium, endocardial progenitors revert to vascular endothelial cells.

It has been well established that BMP function in the myocardium is required for the epithelial-mesenchymal transformation (EMT) within the endocardium, leading to the formation of endocardial cushions (EC) and valves. Myocardial-specific BMP2, BMP4 and endothelial-specific knockout of Alk2 and Alk3 all result in EMT or EC morphogenesis defects (Jiao et al., 2003; Wang et al., 2005; Park et al., 2006; Rivera-Feliciano and Tabin, 2006). Our results implicate BMP signaling in an earlier stage of the endocardial differentiation.
morphogenesis, when endothelial progenitors acquire their identity and initiate expression of specialized markers such as nfact1. It is not entirely clear whether the role of BMP during EMT is separate from an earlier BMP role in endocardial differentiation. It is possible that EMT defects previously observed in BMP inhibited embryos were caused as a secondary consequence because the overall endocardial identity had been perturbed. In mouse Alk3 knockout embryos, no EC cushion morphogenesis was observed, but nfact1 expression in the heart was not affected (Park et al., 2006), suggesting that BMP roles are separate during endocardial differentiation and EMT. Similarly, Alk2 endothelial-specific mouse knockout embryos have been shown to retain nfact1 expression (Wang et al., 2005). It is likely that there is a functional redundancy between different BMP receptors during endocardial differentiation; while LDN193189 has likely that there is a functional redundancy between different BMP balancing phase (Dietrich et al., 2014). Blood flow was shown to been implicated in endocardial proliferation during the cardiac further dissect the role of BMP during endocardial morphogenesis.

specific BMP receptors involved in endocardial differentiation and expression is indirect. Future studies will be needed to identify the specific BMP receptors involved in endocardial differentiation and further dissect the role of BMP during endocardial morphogenesis.

Recently, blood flow and myocardial-derived BMP signaling have been implicated in endocardial proliferation during the cardiac ballooning phase (Dietrich et al., 2014). Blood flow was shown to affect endocardial cell area size and morphology. It has been previously shown that blood flow-sensitive kif2a expression can regulate expression of genes such as notch1b in the atrio-ventricular canal, as well as myocardial bmp4 expression (Vermot et al., 2009). Our previous work has also demonstrated that myocardial bmp4 and endocardial nfact1 and notch1b expression is misregulated during the later phase of cardiac ballooning in actc1as young mutants that have abnormal blood flow (Glenn et al., 2012). Therefore, it is possible that blood flow and mechanical forces may contribute to the maintenance of endocardial differentiation and nfact1 expression during later stages. However, nfact1 expression is initiated prior to blood circulation, therefore the initiation of nfact1 expression would not be affected by blood flow.

Multiple zebrafish BMP homologs, including BMP2b, BMP3, BMP4 and BMP10, are known to have expression in the myocardium (Chin et al., 1997; Wang et al., 2007; Schoenebeck et al., 2012; Laux et al., 2013). It is likely that two or more homologs may function redundantly during endocardial differentiation. Although the myocardium is a likely source of BMP, we cannot also exclude other tissues that may secrete BMP during endocardial differentiation.

In summary, we have demonstrated the requirement for myocardium and BMP signaling during endocardial differentiation. These results will be important for our understanding of molecular mechanisms that govern distinct lineage formation during heart development and may promote further development of therapeutic approaches aimed at regenerating endocardial-derived tissues such as valves and septa.

MATERIALS AND METHODS

Zebrafish lines

The following zebrafish lines were used for experiments: Tg(kdrl:EGFP)545 (Jin et al., 2005), Tg(kaa1a:EGFP)14 (Lawson and Weinstein, 2002), Tg(my17:EGFP)304 (Huang et al., 2003), Tg(my17:memCherry)222 (Palencia-Desai et al., 2011), Tg(hand2:EGFP)162 (Kikuchi et al., 2011), Tg(fn1:mCherry-NTR)111 (Wang et al., 2013), Tg(hsp70:bmp2b)45f (Chocron et al., 2007), Tg(hsp70:dnBMPR-GFP) (Prati et al., 2005) and Tg(eve2:EGFP)10 (Proulx et al., 2010). The myl7 promoter was previously reported as cmlc2. Mutant lines used were hand2o (Yelon et al., 2000) and cloche (Stainier et al., 1995). The Tg(fli1a:hand2-2A-mCherry)10 founder was used in all experiments involving this line.

The myl7:mCherry-NTR construct was generated by cloning a 900-bp fragment of the myl7 promoter (Huang et al., 2003) upstream of a promoterless mCherry-Nitroreductase fusion construct. Linearized DNA (200 pg) was injected into one-cell-stage embryos and individual transgenic carrier adults were selected by screening for fluorescent progeny. Five Tg(myl7:mCherry-NTR) founders were recovered with nearly identical expression patterns and levels. Tg(myl7:mCherry-NTR)195 exhibited the strongest expression and thus was employed for these studies.

Embryos were incubated at 28.5°C for analysis at 24 hpf and later stages, and at 23.5°C for analysis during somitogenesis stages. Embryos were staged as described previously (Kimmel et al., 1995). Embryos were treated with 1-phenyl-2-thiourea (PTU) to inhibit pigment formation for stages 24 hpf and beyond. Alternatively, 30-hpf embryos were left to develop normal pigment, then fixed in BT-Fix (Westerfield, 2007) overnight at 4°C and bleached with a 2% KOH/6% H2O2 solution for 3-5 min, followed by a PBST rinse, a 10 min post-fix in BT-Fix and dehydration in an ethanol gradient to 100% ethanol prior to in situ hybridization. At least two independent replicates were performed for all experiments shown.

Design of fli1a:hand2-2A-mCherry and myl7:hand2-2A-mCherry constructs

fli1a:hand2-2A-mCherry and myl7:hand2-2A-mCherry constructs were engineered using standard Gateway cloning protocols (Invitrogen) by combining either the 478 p5E-filEp (Addgene Plasmid #31160) (Villefranc et al., 2007) or a p5E-myl7 5′ entry clone (a gift from J. Waxman, Cincinnati Children’s Hospital Medical Center, OH, USA) with the hand2 middle and 2A-mCherry 3′ entry clones. The hand2 middle entry clone was created by PCR amplification of the ORF of the hand2 gene with attB1 and attB2 sites attached to the primers and eliminating the native stop codon. The 3′ entry clone was Addgene Plasmid #26031, 543 p3 E 2A-mCherry-Pa and the Destination vector was #394 pDestTol2pA2 (Villefranc et al., 2007). Each of the final constructs (25 pg-50 pg) was co-injected with 25 pg of tol2 RNA into Tg(my17:GFP;hand2o) and heterozygous genotype of the hand2 mutation, and then genotyped for expected Gateway construct integration. Embryos from a single Tg(fli1a:hand2-2A-

mCherry)10 founder were used in all experiments involving this line.

Genotyping of zebrafish mutants

hand2 mutants were identified between 24 and 30 hpf by downregulation of myl7-GFP expression and observed cardiac biffida (Yelon et al., 2000; Huang et al., 2003). cloche mutants were identified by downregulation of the posterior domain of gata1-GFP expression (Stauini et al., 1995; Long et al., 1997; Sumanas et al., 2005). For hand2 mutants younger than 24 hpf, post in situ hybridization genotyping was performed as previously described (Yelon et al., 2000). Tissue pieces from single embryos were digested overnight at 55°C in 20 µl of the following digestion buffer: NEB Standard PCR buffer 50 µl of 10×, 1.5 µl of 100% Tween-20, 1.5 µl of 100% NP-40, 10 µl of 10 mg/ml proteinase K and 437 µl of nuclease-free water. The next morning, digestion was followed by inactivation of Proteinase K; 94°C for 10 min, then 4°C for 2 min. PCR was performed using four primers as previously described (Yelon et al., 2000).

In situ hybridization

In situ hybridization was performed as previously described (Jowett, 1999) or in an Invantis Bioanalytical Instruments BioLane HT1-16V automated in situ hybridization machine. nfact1, fn1 (Wong et al., 2012), fli1/kdrl (Thompson et al., 1998), edh5 (cadherin 5) (Larson et al., 2004), hand2 (Yelon et al., 2000), cmlc2/myl7 (Yelon et al., 1999), notch1b (Westin and Ladelli, 1997), kif2a (Oates et al., 2001) and fli1 (Thompson et al., 1998) DIG-labeled probes were synthesized as described.

BMP inhibitor chemical treatment

LDN195389 (Stemgent) was stored at −20°C as a 10 mM stock in DMSO. Stock or pure DMSO was diluted 1000 fold in 1× Danieau’s buffer (Barbas et al., 2007). Embryos were treated with LDN or vehicle control in the dark on a rocking platform at 28°C during the first 24 hpf.
Embryos were manually dechorionated or chorions were ripped open during treatment to allow better drug penetration.

MTZ genetic ablation
Embryos were collected and dechorionated by pronase between the tailbud and one-somite stage. Five embryos were directly transferred, in the dark, to each well in a 24-well tissue culture plate, containing 2 ml of treatment solution: 0.1% DMSO/1X PTU/E3 medium (Cold Spring Harbor Protocols, recipe for E3 medium for zebrafish embryos; doi:10.1101/pdb.rc666449) or with or without 10 mM monodiazolone (Sigma M3761). Plates were covered with foil and incubated at 28.8°C for 24-72 h.

Apoptosis and immunofluorescence analysis
For apoptosis analysis, embryos were fixed for 3 h at room temperature in 1% PFA, washed, blocked in SBS (saponin blocking solution: 0.2% saponin (Sigma, S4521)/2 mg/ml BSA/10% lamb serum/1× PBS) and stained with the following antibody cocktails: (1) 1:400 rabbit anti-human cleaved caspase 3 (BD Pharm, 559565); (2) 1:1200 anti-rabbit Alexa-647 (Invitrogen, A21245) plus 1:40 mouse anti-chicken myosin sarcomere IgG2b bioreactor supernatant (MF-20 DSHB); and (3) 1:2000 goat anti-rabbit:Alexa-594 (Invitrogen, A21244) plus 1:400 rabbit anti-GFP Alexa-488 (Invitrogen, A21311). Antibodies were incubated for 4 h at room temperature or overnight at 4°C in SBS. All washes were carried out for 30 min total in either PBST (PBS, phosphate-buffered saline+0.2% Tween 20) or in 0.2% saponin/PBS. Fluorescent transgenic embryos were prepared for imaging by a light fixation in BT-Fix for 1 h at room temperature or alternatively with 1% formaldehyde/PBS solution for 4 h at 4°C.

Fluorescent in situ hybridization and Immunohistochemistry
Hsp70:Bmp2b; hand2−/− embryos and their siblings were heat-shocked at the 16-somite stage as described below and fixed in 2% paraformaldehyde/PBS solution overnight. Ethanol dehydration and standard chromogenic in situ hybridization for nfatc1 was performed as described above using NBT/BCIP as a color substrate. The yolk was manually removed with surgical forceps, followed by wash in PBST, a brief fix with 2% PFA and another wash in PBST (PBS+0.2% Tween-20). Embryos were permeabilized with 0.5% TritonX-100/PBST for 30 min at room temperature and blocked for 2 h with 0.1% TritonX-100/10% lamb serum/1% BSA/PBST. Embryos were incubated in anti-VE-cadherin (1:200) (generously provided by Markus Affolter, Biozentrum, University of Basel, Switzerland) overnight at 4°C and washed at room temperature for a total of 45 min in TNT solution (0.1 M Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween-20) followed by 4 h in PBST. Samples were then incubated in (1:200) goat anti-rabbit:Alexa-594 (Invitrogen, A11012) overnight at 4°C, washed four times in PBST, dehydrated in a stepwise gradient to 100% methanol and stored at 4°C overnight. Before imaging, the embryos were rinsed in PBST and flat-mounted in Vector Shield medium. Images were captured using Nikon confocal A1Rsi inverted microscope (40× objective) at the CCHMC Confocal Imaging Core facility. Confocal imaging of NBT/BCIP substrate fluorescence was performed as previously described (Schumacher et al., 2014).

Image capture and processing
Ventral flat mounting of in situ hybridization stained or fixed transgenic embryos was done by manually deyolking in PBST with fine forceps followed by fine brushing of yolk granules in 30% glycerol with an eyelash brush. Lightly fixed embryos were monitored under fluorescent light to ensure no damage to cardiac tissue occurred during deyolking. Embryos were positioned in 30% glycerol or in 3% methylcellulose under a bridged coverslip. In some cases, the hatching gland was dissected for a better view of the heart. Images were captured with an Axioimager Z1 (Zeiss) compound microscope with Axioacam color camera or monochrome cameras (Zeiss). Images in different focal planes were combined using the Extended Focus module within Axiovision software (Zeiss). Image levels were adjusted using Adobe Photoshop CS5 to increase the contrast. For confocal imaging, live embryos were embedded in low-melting-point agarose and imaged using Nikon confocal A1Rsi inverted microscope (10× and 20× objectives) at the CCHMC Confocal Imaging Core facility.

Heat-shock experiments
To heat-shock Tg(hsp70l:dnBmpr-GFP) and control embryos, two embryos were placed into each 200 µl PCR tube with ~100 µl of fish water. Embryos were kept at 28°C, subjected to heat-shock at the 16-somite stage for 30 min at 37°C and cultured at 28°C until 30 hpf.

For Tg(hsp70l:dnBmpr-GFP) heat-shock experiments were obtained by outcrossing heterozygous Tg(hsp70l:dnBmpr-GFP) fish (Pyati et al., 2005) to wild-type fish to keep transgene copy number consistent. Embryos were heat-shocked at the tailbud stage for 60 min at 37°C using a PCR machine. Transgene-positive embryos were identified at 24 hpf by the loss of ventral tail fin phenotype that has been previously described (Pyati et al., 2005). Transgene-negative embryos served as controls.

To analyze endocardial differentiation in Tg(myl7:mCherry-NTR); Tg(hsp70l:dnBmpr2b) embryos, embryos from a myl7:mCherry-NTR+/−; hsp70l:dnBmpr2b−/− cross were subject to MTZ treatment as described above. Embryos were subjected to three consecutive 30-min heat shocks at 37°C at 6-h intervals starting at the 18-somite stage and subsequently fixed for in situ hybridization analysis at 44 hpf.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
S.P.-D. performed the experiments, analyzed data and edited the manuscript; M.S.R., J.A.S., Q.V.T., A.L.K., M.P.C. and K.B. performed experiments and analyzed data; S.S. designed the study, performed experiments, analyzed data and wrote the manuscript; N.C.G. and J.W. provided critical reagents; and K.D.P. and D.Y.R.S. provided critical reagents and gave feedback on the manuscript.

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Supplementary material
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