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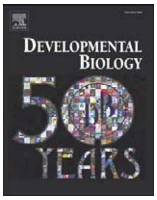

Differential requirement for BMP signaling in atrial and ventricular lineages establishes cardiac chamber proportionality

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ABSTRACT

The function of an organ relies upon the proper relative proportions of its individual operational components. For example, effective embryonic circulation requires the appropriate relative sizes of each of the distinct pumps created by the atrial and ventricular cardiac chambers. Although the differences between atrial and ventricular cardiomyocytes are well established, little is known about the mechanisms regulating production of proportional numbers of each cell type. We find that mutation of the zebrafish type I BMP receptor gene *alk8* causes reduction of atrial size without affecting the ventricle. Loss of atrial tissue is evident in the lateral mesoderm prior to heart tube formation and results from the inhibition of BMP signaling during cardiac progenitor specification stages. Comparison of the effects of decreased and increased BMP signaling further demonstrates that atrial cardiomyocyte production correlates with levels of BMP signaling while ventricular cardiomyocyte production is less susceptible to manipulation of BMP signaling. Additionally, mosaic analysis provides evidence for a cell-autonomous requirement for BMP signaling during cardiomyocyte formation and chamber fate assignment. Together, our studies uncover a new role for BMP signaling in the regulation of chamber size, supporting a model in which differential reception of cardiac inductive signals establishes chamber proportion.

Introduction

Organ function is the cumulative effect of coordinating each of an organ's individual subunits. In the vertebrate heart, the propulsion of circulation depends upon the serial contraction of each of its independent pumps, the atrial and ventricular cardiac chambers. Therefore, attainment of proper cardiac chamber proportionality is crucial for effective cardiac function.

Little is known about the mechanisms that regulate the relative sizes of the cardiac chambers. Atrial and ventricular cardiomyocytes are distinct cell types that differ both histologically and physiologically (Moorman and Christoffels, 2003). For example, in the zebrafish heart tube, chamber cardiomyocytes have distinct cell morphologies, with atrial cardiomyocytes appearing more squamous and ventricular cardiomyocytes appearing more cuboidal (Rohr et al., 2008). Additionally, zebrafish atrial and ventricular cardiomyocytes express the chamber-specific myosin heavy chains, *atrial myosin heavy chain* (*amhc*) and *ventricular myosin heavy chain* (*vmhc*) (Berdougo et al., 2003; Yelon et al., 1999). Although several transcription factors have been implicated in the regulation of chamber-specific characteristics (Bruneau, 2002), the signaling pathways that trigger initial chamber fate assignment and generate the appropriate numbers of atrial and ventricular cardiomyocytes are poorly understood.

Analysis of the mechanisms regulating chamber size is relatively convenient in the simple two-chambered zebrafish heart, in contrast to the four-chambered mammalian heart, which arises from multiple heart fields (Buckingham et al., 2005; Cai et al., 2003; Kelly et al., 2001; Meilhac et al., 2004). Fate map experiments in zebrafish reveal that cardiac progenitors are located at the lateral margin of the embryo at the onset of gastrulation (Keegan et al., 2004). Even at this early stage, chamber progenitor populations are already spatially organized. Within the lateral marginal zone of the blastula, atrial progenitors are located more ventrally, and ventricular progenitors are located more dorsally; additionally, ventricular progenitors tend to be closer to the margin than are the atrial progenitors (Keegan et al., 2004). The relative organization of chamber progenitors seems to be maintained during and after gastrulation (Keegan et al., 2004; Schoenebeck et al., 2007). Cardiac specification is thought to take place during the time window in which the migrating progenitor cells form the lateral plate mesoderm, culminating in robust expression of precardiac markers like *nkx2.5* around the 6–8 somite stage (Thomas et al., 2008). Myocardial differentiation then initiates around the 13–14 somite stage (Yelon et al., 1999), and chamber-specific gene expression is first evident at the 14 somite stage with the expression of *vmhc* (Yelon et al., 1999) and at the 19 somite stage with the expression of *amhc* (Berdougo et al., 2003).

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Recent work in zebrafish supports an emerging idea regarding the acquisition of chamber identity: the signals responsible for cardiac induction may also be responsible for the subdivision of the heart field into atrial and ventricular territories. These studies demonstrated that mild inhibition of cardiac inductive signals can lead to differential reduction of atrial and ventricular chambers. For example, although complete inhibition of Nodal signaling eliminates all cardiac mesoderm (Gritsman et al., 1999), modest loss of Nodal signaling results in reduced heart size with a particularly striking reduction of the ventricle (Reiter et al., 2001). Fate mapping experiments revealed that Nodal promotes ventricular fate assignment in blastomeres nearest to the margin (Keegan et al., 2004), consistent with a vegetal–animal gradient of Nodal signaling that positions the highest levels of Nodal signaling closest to the margin (Chen and Schier, 2002). A similar trend is observed in experiments inhibiting the FGF signaling pathway, which has also been implicated in promoting cardiac induction (Dunwoodie, 2007). Zebrafish *fgf8* mutants form small hearts with disproportionately small ventricles (Reifers et al., 2000), and temporal inhibition of FGF signaling has demonstrated its particular importance for ventricular cardiomyocyte formation during gastrulation stages (Marques et al., 2008), consistent with a dorsal–ventral gradient of FGF signaling that correlates with the more dorsal location of ventricular progenitors (Fürthauer et al., 1997; Keegan et al., 2004). Together, these studies suggest that differential reception of cardiac inductive signals within a uniform pool of cardiac progenitors could dictate chamber identity assignment.

BMP signaling has a conserved role in cardiac induction (Zaffran and Frasch, 2002). In *Drosophila*, *dpp* mutant embryos lack expression of *tinman*, an essential cardiac transcription factor gene, and do not form a dorsal vessel (Frasch, 1995). Conversely, ectopic Dpp can induce formation of ectopic *tinman*-expressing cells (Yin and Frasch, 1998). In chick, exogenous application of BMP also induces expression of the *tinman* homolog *NKX2-5* (Nakajima et al., 2002; Schlange et al., 2000; Schultheiss et al., 1997). Additionally, excess BMP signaling in mouse *Nkx2-5* mutants is thought to be responsible for the over-specification of cardiac progenitors (Prall et al., 2007). As in *Drosophila*, severe loss of cardiac tissue is also observed when BMP signaling is inhibited in vertebrates. In frog, inhibition of BMP signaling results in the absence of a morphologically recognizable heart (Shi et al., 2000). In mouse *Bmp2* mutants, expression of *Nkx2-5* is reduced or absent (Zhang and Bradley, 1996), and conditional removal of *Bmpr1* from precardiac mesoderm with a *Mesp-1 Cre* driver dramatically reduces the heart field (Klaus et al., 2007). Similarly, zebrafish *swirl* (*bmp2b*) mutants lack expression of *nkx2.5* (Kishimoto et al., 1997; Nguyen et al., 1998; Reiter et al., 2001).

While strong reduction of BMP signaling severely compromises heart development, mild reduction results in formation of a dysmorphic heart (Chocron et al., 2007). Zebrafish *lost-a-fin* (*laf*) mutants exhibit a weak dorsalized phenotype characterized by loss of the ventral tail fin, due to disruption of the type I BMP receptor gene *alk8* (Bauer et al., 2001; Mintzer et al., 2001). Maternal–zygotic *laf* mutants are morphologically similar to *swirl* (Mintzer et al., 2001). In contrast to the dramatic loss of cardiac tissue observed in *swirl* mutants, zygotic *laf* mutants form hearts with abnormal atrial morphology (Chocron et al., 2007). Because of the correlation between the relatively ventral location of atrial progenitors and the ventral–dorsal gradient of BMP signaling during gastrulation stages (Tucker et al., 2008), we hypothesized that the abnormal atrial morphology in *laf* mutants could reflect a role for BMP in atrial cardiomyocyte generation.

Here, we analyze the effects of BMP signaling on cardiac chamber size in the zebrafish embryo. We find that *laf* mutants exhibit a significant reduction in the number of atrial cardiomyocytes. Atrial tissue reduction occurs before the heart tube forms and results from inhibition of BMP signaling during cardiac progenitor specification stages. Conversely, high levels of BMP signaling result in a preferential increase of atrial size. Through mosaic analysis, we show that BMP signaling is cell autonomously required for cardiomyocyte formation and chamber identity acquisition. Together, our data reveal a crucial role for BMP signaling in regulating cardiac chamber proportion through differential effects on atrial and ventricular progenitor populations.

Materials and methods

Zebrafish

We employed the following zebrafish mutations and transgenes: *lost-a-fin*^{sk42} (*laf*/*alk8*), *Tg(cmlc2:DsRed2-nuc)*^{f2} (Mably et al., 2003), *Tg(cmlc2:egfp)*^{twu277}(Huang et al., 2003) and *Tg(hsp701:dnBmpr-GFP)*^{w30} (Pyati et al., 2005). The *laf*^{sk42} mutation is a recessive lethal allele that was identified in a screen for ethylnitrosourea-induced mutations that affect cardiac chamber morphology (Auman et al., 2007). *laf*^{sk42} fails to complement *laf*^{tm110b}, and the phenotypes of *laf*^{sk42} and *laf*^{tm110b} mutants appear indistinguishable (Bauer et al., 2001; Chocron et al., 2007; Mintzer et al., 2001; Mullins et al., 1996). All zebrafish work followed protocols approved by the NYU School of Medicine IACUC.

Immunofluorescence and cell counting

We counted cardiomyocytes in embryos carrying the transgene *Tg(cmlc2:DsRed2-nuc)*, using immunofluorescence to detect DsRed in cardiomyocyte nuclei and to detect atrial myosin heavy chain (Amhc) in atrial cells, as described previously (Marques et al., 2008). Student's *t*-test (homocedastic, two-tail distribution) was used to analyze the differences between the means of cell number data sets.

In situ hybridization

Anti-sense probes for *amhc*, *vmhc, nkx2.5, hand2*, and *gata5* were used as previously described for in situ hybridization (Berdougo et al., 2003; Chen and Fishman, 1996; Reiter et al., 2001; Yelon et al., 1999, 2000).

Dorsomorphin treatments

A 10 mM stock of dorsomorphin (Calbiochem, #171261) in DMSO was diluted to a working concentration of 5 μM in E3 medium (Nusslein-Volhard and Dahm, 2002), a concentration previously described to cause dorsalization (Yu et al., 2008). Up to 10 embryos were treated in a final volume of 1 mL in a glass vial. Vials were kept on a nutator in the dark at 28.5 °C. Control embryos were treated with a corresponding dilution of DMSO.

Heat shock conditions

Embryos from outcrosses of fish heterozygous for *Tg(hsp701:dnBmpr-GFP)* were exposed to heat shock at desired stages through placement in 40 mL of embryo medium in a Petri dish on top of a covered heat block for 1 h at 37 °C. Following heat shock, transgenic embryos were identified by *gfp* expression and body morphology. Transgenic embryos heat shocked during gastrulation exhibit a previously described mild dorsalization, while embryos heat shocked at the 8-somite stage are grossly comparable to wild-type (Pyati et al., 2005, 2006).

RNA injections

alk8CA and *alk8(K232R)* mRNAs were in vitro transcribed from previously described plasmids using the SP6 mMessage mMachine System (Ambion) (Bauer et al., 2001). Embryos were injected at the 1-cell stage with 15 pg of *alk8CA* mRNA or 20 pg of *alk8(K232R)* mRNA. Injection of either mRNA causes a range of phenotypes;