



Published in final edited form as:

*Development*. 2008 November ; 135(22): 3789–3799. doi:10.1242/dev.024083.

## Hedgehog signaling plays a cell-autonomous role in maximizing cardiac developmental potential

Natalie A. Thomas<sup>1</sup>, Marco Koudijs<sup>2,3</sup>, Fredericus J. M. van Eeden<sup>2,3</sup>, Alexandra L. Joyner<sup>1,\*</sup>, and Deborah Yelon<sup>1,†</sup>

<sup>1</sup>Developmental Genetics Program and Department of Cell Biology, Kimmel Center for Biology and Medicine, Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, NY 10016, USA <sup>2</sup>Hubrecht Institute, Developmental Biology and Stem Cell Research, Uppsalalaan 8, 3584CT, Utrecht, The Netherlands <sup>3</sup>University of Sheffield, Department of Biomedical Science, Western Bank, Sheffield S10 2TN, UK

### Abstract

Elucidation of the complete roster of signals required for myocardial specification is crucial to the future of cardiac regenerative medicine. Prior studies have implicated the Hedgehog (Hh) signaling pathway in the regulation of multiple aspects of heart development. However, our understanding of the contribution of Hh signaling to the initial specification of myocardial progenitor cells remains incomplete. Here, we show that Hh signaling promotes cardiomyocyte formation in zebrafish. Reduced Hh signaling creates a cardiomyocyte deficit, and increased Hh signaling creates a surplus. Through fate-mapping, we find that Hh signaling is required at early stages to ensure specification of the proper number of myocardial progenitors. Genetic inducible fate mapping in mouse indicates that myocardial progenitors respond directly to Hh signals, and transplantation experiments in zebrafish demonstrate that Hh signaling acts cell autonomously to promote the contribution of cells to the myocardium. Thus, Hh signaling plays an essential early role in defining the optimal number of cardiomyocytes, making it an attractive target for manipulation of multipotent progenitor cells.

### Keywords

Hedgehog; Cardiac progenitor specification; Cyclopamine; Heart development; Smoothed; Zebrafish

## INTRODUCTION

A principal goal of regenerative medicine is to convert naïve progenitor cells into specific types of differentiated cells capable of organ repair. For example, to facilitate cardiac repair,

<sup>†</sup>Author for correspondence (yelon@saturn.med.nyu.edu).

<sup>\*</sup>Present address: Developmental Biology Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA

### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/135/22/3789/DC1>

we seek efficient strategies for specifying cardiac identity and directing myocardial differentiation in uncommitted cells (Laflamme and Murry, 2005; Nerem, 2007). It is therefore desirable to elucidate the entire network of signals that govern cardiac progenitor specification in the early embryo. Which signals cooperate to establish cardiac fate, when do these signals act and where do key signaling events take place?

Previous studies have established important roles for several signaling pathways in the specification of the cardiac progenitor pool. The Fgf, Bmp and Nodal pathways play inductive roles and are thought to promote cardiac fate assignment (Brand, 2003; Solloway and Harvey, 2003; Yelon, 2001). By contrast, the retinoic acid pathway restricts the acquisition of cardiac identity (Keegan et al., 2005), and Wnt signaling promotes or represses cardiogenesis depending on the developmental stage (Klaus et al., 2007; Ueno et al., 2007). These inductive and repressive signaling pathways presumably collaborate to ensure formation of the appropriate number of cardiomyocytes. However, as the roster of relevant signals remains incomplete, it is difficult to decipher the precise recipe for creating cardiac identity.

The Hedgehog (Hh) signaling pathway regulates specification, patterning and growth of multiple embryonic organs (Ingham and McMahon, 2001), and has been implicated in cardiac development. In mouse, loss of *Shh* causes several cardiac abnormalities, including ventricular hypoplasia, septation defects and outflow tract (OFT) shortening (Chiang et al., 1996; Tsukui et al., 1999; Washington Smoak et al., 2005). Tissue-specific removal of Hh pathway components has demonstrated that Hh signaling is required within the cardiac neural crest and the second heart field (the origin of OFT myocardium) for OFT morphogenesis (Goddeeris et al., 2007; Lin et al., 2006; Washington Smoak et al., 2005), and that Hh signaling within the dorsal mesocardium is required for atrioventricular septation (Goddeeris et al., 2008).

Less is known about earlier roles that Hh may play during cardiac progenitor specification. In *Drosophila*, ectodermal Hh influences the specification and positioning of cardiac progenitor cells within the cardiogenic mesoderm (Jagla et al., 1997; Liu et al., 2006; Ponzielli et al., 2002). Mice that lack *smoothed* (*Smo*), the obligate Hh co-receptor, and mice lacking both *Shh* and Indian hedgehog (*Ihh*) exhibit more severe cardiac defects than mice lacking only *Shh* (Zhang et al., 2001). These defects include aberrant cardiac morphogenesis, reduced heart size and delayed initiation of expression of the pre-cardiac marker *Nkx2-5* (Zhang et al., 2001). Expanded *Nkx2-5* expression is observed in mice lacking the inhibitory patched 1 (*Ptch1*) receptor. Although these studies implicate Hh signaling in early steps of heart formation, it remains unclear if, when, where or how Hh signaling impacts cardiac fate assignment.

In this study, we demonstrate that Hh signaling plays an important role in driving cardiac specification in the zebrafish embryo. Using both loss-of-function and gain-of-function approaches, we demonstrate that Hh signaling promotes cardiomyocyte formation. This influence of Hh takes place during and shortly after gastrulation, when Hh signaling ensures specification of the proper number of myocardial progenitors. We use genetic inducible fate mapping in the mouse embryo to show a direct response to Hh signaling within the

myocardial progenitors. Finally, transplantation experiments in zebrafish identify a cell-autonomous role for Hh signaling in promoting the contribution of cells to the myocardial lineage. Together, these results reveal that Hh signaling has an early and direct impact on cardiac progenitors, and identify Hh as an essential component of the optimal signaling milieu for cardiac specification.

## MATERIALS AND METHODS

### Zebrafish

The following zebrafish mutations and transgenes were used: *smo*<sup>b577</sup> (Varga et al., 2001), *smo*<sup>hi1640</sup> (Chen et al., 2001), *ptch*<sup>hu1602</sup> (Koudijs et al., 2008), *ptc2/lep*<sup>tj222</sup> (Koudijs et al., 2005), *Tg(cmlc2:DsRed2-nuc)*<sup>f2</sup> (Mably et al., 2003) and *Tg(cmlc2:egfp)*<sup>wu277</sup> (Huang et al., 2003). The cardiac phenotype of embryos mutant for the null allele *smo*<sup>hi1640</sup> was found to be comparable with that of *smo*<sup>b577</sup> embryos at the 18-somite stage, at 24 hours post-fertilization (hpf) and at 48 hpf. All zebrafish work followed protocols approved by the NYU School of Medicine IACUC.

### Generation of maternal-zygotic *smo* embryos

Germline replacement chimeras were generated as previously described (Ciruna et al., 2002). Donor embryos were generated from an intercross of fish heterozygous for *smo*<sup>b577</sup>, and wild-type embryos were used as hosts. At 24 hpf, host embryos were screened for the presence of donor-derived *Tg(nanos:gfp)*-expressing cells, and the identity of the corresponding donor embryo for each host was determined phenotypically at this stage. Three fertile female chimeras were successfully raised out of 67 *Tg(nanos:gfp)*-positive hosts. *MZsmo* embryos presented the same characteristic morphology seen in zygotic *smo*, including U-shaped somites, mild cyclopia and ventral tail curvature.

### Cyclopamine treatment

Embryos in their chorions were treated with 75  $\mu$ M CyA (Toronto Research Chemicals) in egg water, diluted from a 10 mM stock in ethanol. Unless stated otherwise, CyA was added at 40-50% epiboly. Wash-out experiments were conducted by replacing the embryo media three times with fresh egg water, which has been shown to restore Hh signaling after CyA exposure (Chen et al., 2001). For fate-mapping experiments, dechorionated embryos were treated with 50  $\mu$ M CyA. Control embryos for the CyA treatments were exposed to 0.0075% ethanol in egg water.

### RNA injections

Embryos were injected with 100 pg of zebrafish *shha* mRNA (Ekker et al., 1995) at the one-cell stage.

### Immunofluorescence and cardiomyocyte counting

MF20 and S46 whole-mount immunofluorescence of embryos was conducted as previously described (Alexander et al., 1998; Yelon et al., 1999). Cardiomyocyte counting using the transgene *Tg(cmlc2:DsRed2-nuc)* was conducted as previously described (Schoenebeck et

al., 2007). To generate embryos for counting, *Tg(cmlc2:DsRed2-nuc)<sup>+/-</sup>; smo<sup>b577/+</sup>* fish were intercrossed to generate zygotic *smo* mutant embryos and were crossed to *MZsmo* germ line chimeras to generate *MZsmo* embryos.

### In situ hybridization

In situ hybridization was conducted as previously described (Berdougo et al., 2003; Concordet et al., 1996; Thompson et al., 1998; Yelon et al., 1999). Mutant embryos were identified after imaging via PCR genotyping; protocols are available upon request. To count cells at 18-somite or 22-somite stages, we scored cells positive for the NBT/BCIP precipitate in each heart field (see Fig. S1 in the supplementary material). Individual cells are easily identified as the precipitate is excluded from the nucleus and the cells are arranged in epithelial sheets, typically one cell thick (Trinh and Stainier, 2004).

### Fate mapping with caged fluorescein

Fate-mapping experiments in tier 1 of the 40% epiboly embryo were conducted using previously described protocols (Keegan et al., 2004). In each experiment, we labeled neighboring blastomeres along the embryo margin. After recording the positions of labeled blastomeres, individual embryos were placed in half-dram glass vials with egg water. For CyA-treated embryos, 50  $\mu$ M cyclopamine was added at this stage. To enhance identification of labeled cardiomyocytes, we performed in situ hybridization for *cmlc2* prior to detection of the fluorescein lineage tracer (Keegan et al., 2004).

### Genetic inducible fate mapping

Fate mapping in mouse embryos was conducted as described previously (Ahn and Joyner, 2004). *Gli1CreER<sup>T2/+</sup>; R26R/R26R* males were mated with Swiss Webster females to generate *Gli1CreER<sup>T2/+</sup>; R26R/+* embryos. Noon on the day of a plug was designated embryonic day (E) 0.5. To initiate labeling at ~E7.0, TM was administered by gavage at 5 pm on E6.5. For fate mapping of Gli1-derived cells between ~E8.0 and E9.0, TM was given at 5 pm on E7.5. Hearts (E18.5) or upper bodies (E15.5) were dissected in ice-cold PBS, fixed in 4% paraformaldehyde in PBS for 20 minutes, cryoprotected and embedded in OCT (Blaess et al., 2006). Sections (12  $\mu$ m) of the dissected tissues were cut on a cryostat, and  $\beta$ -galactosidase staining and nuclear fast red counterstaining were performed (Ahn and Joyner, 2004). All mouse work followed protocols approved by the NYU School of Medicine IACUC.

### Transplantation

Blastula stage transplantations were conducted as previously described (Ho and Kane, 1990; Parker and Stainier, 1999). Between 3 and 4 hpf, 10-20 donor cells were transplanted from the margin of a donor embryo and placed into the margins of two wild-type host embryos. Donor embryos carried the *Tg(cmlc2:egfp)* transgene to allow evaluation of cardiac contribution at 2 days post-fertilization. To ensure that both donor populations (wild-type and *MZsmo*) were exposed to the same experimental conditions, including developmental stage at time of transplant, we generated clutches that were made up of 50% wild-type (*smo<sup>+/-</sup>*) and 50% *MZsmo* donor embryos by crossing a germ line chimera *MZsmo* female

to a *smo*<sup>+/-</sup> male carrying two copies of *Tg(cmlc2:egfp)*. The genotype of each donor embryo was determined post-transplant at 24 hpf. For experiments with a lineage tracer, 1 nl of fluorescein-dextran (MW 10,000, Invitrogen), from a 5 mg/ml stock in Phenol Red with 0.2 M KCl, was injected into donor embryos.

## Imaging

Images were captured with Zeiss Axiocam digital cameras on Zeiss Axioplan and M2Bio microscopes and were processed with AxioVision (v4.6.3 and v3.0.6), Adobe Photoshop 7 and Adobe CS3 software, except for live fate mapping images, which were captured with MetaMorph Imaging software (v6.1r3, Universal Imaging).

## Statistical analyses

Statistical tests were run with GraphPad Prism v.4, Microsoft Excel, and as by Zar (Zar, 1999). To compare the means of cell counting data sets, we used two-tailed, unpaired *t*-tests. To compare transplant contribution frequencies, we used the normal approximation of the  $\chi^2$  test to evaluate the difference between the two proportions. Because of the smaller number of experiments conducted, we used Fisher's exact test to compare the frequencies of labeling myocardial progenitors in our fate-mapping studies.

## RESULTS

### Hedgehog signaling promotes cardiomyocyte formation

The zebrafish genome appears to contain a single *smoothened* homolog, which is maternally provided, ubiquitously expressed during gastrulation stages and widely expressed during early somitogenesis (Chen et al., 2001; Varga et al., 2001). To investigate the involvement of Hh signaling in cardiac development, we inhibited Smoothened function, both genetically and pharmacologically, and analyzed the resulting cardiac phenotypes.

First, we examined the phenotypes caused by two different *smoothened* mutant alleles, *smo*<sup>b577</sup> and *smo*<sup>hi1640</sup> (Chen et al., 2001; Varga et al., 2001): in both cases, mutant embryos exhibit small, dysmorphic, improperly looped hearts at 48 hpf (Fig. 1A,B; N.A.T. and D.Y., unpublished). The mutant ventricle appears compact and tubular, and the mutant atrium is misshapen. However, these phenotypes may not reflect all functions of Hh during heart formation, owing to residual signaling activity provided by maternally supplied *smoothened* (Chen et al., 2001; Varga et al., 2001). To deplete Hh signaling activity in the early embryo, we generated maternal-zygotic *smo*<sup>b577</sup> mutants (*MZsmo*) via germline replacement (Ciruna et al., 2002). As a complementary approach, we treated embryos with cyclopamine (CyA), a pharmacological inhibitor of Smoothened (Chen et al., 2002), from the 30% epiboly stage, prior to the initiation of gastrulation. Both approaches consistently yield cardiac chambers that are reduced in size and misshapen. The hearts of *MZsmo* mutant embryos resemble slightly smaller versions of zygotic *smo* mutant hearts (Fig. 1B,C), whereas CyA treatment causes a greater and more variable disruption of cardiac morphology and size (Fig. 1D). The exacerbation of the cardiac phenotype in CyA-treated embryos might be attributable to as yet uncharacterized off-target effects of CyA, as the *smo*<sup>b577</sup> allele is thought to be a

functional null that disrupts protein folding and localization (Barresi et al., 2000; Eberhart et al., 2006; Feng et al., 2006; Varga et al., 2001).

Circulation in embryos deficient in Hh signaling is disrupted due to defects in dorsal aorta formation (Chen et al., 1996; Lawson et al., 2002), and alterations in circulation can impact cardiac chamber morphogenesis (Auman et al., 2007). Thus, to determine whether the cardiac chamber defects simply reflected defects in morphogenesis, we quantified the number of cardiomyocytes at 52 hpf. Examining all three loss-of-function scenarios, we found significant reductions in the number of cardiac cells (Fig. 1E; see Table S1 in the supplementary material). In all cases, the number of ventricular cardiomyocytes is more affected than the number of atrial cardiomyocytes; for example, CyA-treated embryos display 65% fewer ventricular cardiomyocytes and 56% fewer atrial cardiomyocytes than are found in wild-type embryos. Altogether, the consistent trend of reduced cardiac cell number in embryos with disrupted Hh signaling demonstrates a role for Hh in promoting cardiomyocyte formation.

### Hedgehog signaling is required for the initial establishment of cardiomyocytes

The observed reduction in cardiomyocyte number could be the result of an early shortage of myocardial progenitors, inefficiency of myocardial differentiation and/or later defects in the proliferation or survival of differentiated cardiomyocytes. To determine when the cardiac deficit originates, we evaluated the expression of *nkx2.5* in myocardial progenitors (Schoenebeck et al., 2007). Compared with wild-type embryos, *MZsmo* mutants and CyA-treated embryos have smaller fields of *nkx2.5*-expressing cells in the anterior lateral plate mesoderm (ALPM), as well as decreased intensity of *nkx2.5* expression (Fig. 2A-C), suggesting an early defect in myocardial progenitor specification. Evidence of a myocardial deficiency persists after terminal differentiation is under way. At the 18-somite stage, the number of *cmlc2*-expressing differentiated cardiomyocytes and the intensity of *cmlc2* expression are decreased in *MZsmo* mutants and CyA-treated embryos (Fig. 2D-F). Similar defects were apparent when examining chamber-specific gene expression (Fig. 2G-L).

For a more quantitative understanding of the extent of the early cardiomyocyte deficit, we counted the number of cells present prior to heart tube assembly. In these studies, we used CyA treatment to generate large numbers of embryos deficient in Hh signaling. Quantification of the reduction in *cmlc2*, *vmhc* or *amhc* demonstrated similar effects on both ventricular and atrial lineages. CyA-treated embryos displayed ~50% of the number of *cmlc2*-expressing cells, 50% of the number of *vmhc*-expressing cells and slightly more than half the number of *amhc*-expressing cells seen in wild-type embryos (see Table S2 in the supplementary material). Thus, reduction of Hh signaling causes a significant early reduction in the number of ventricular and atrial cardiomyocytes, comparable in scale with the deficits observed in the cardiac chambers at 52 hpf.

### Ectopic and amplified activation of Hedgehog signaling increases cardiomyocyte numbers

Having shown that a loss of Hh signaling reduces cardiomyocyte numbers, we then asked whether increased Hh signaling is sufficient to generate extra cardiomyocytes. We carried out gain-of-function experiments in which we induced ectopic Hh signaling, through



exogenous expression of *shh*, or increased endogenous Hh signaling levels, through mutation of *patched1* (*ptc1*) and *patched2* (*ptc2*). Injection of zebrafish *shh* (hereafter referred to as *shh*) mRNA at the one-cell stage induces ectopic expression of Hh target genes (Barth and Wilson, 1995; Concordet et al., 1996; Ekker et al., 1995; Krauss et al., 1993; Lewis et al., 1999). In *ptc1;ptc2* double mutants, the loss of both Ptc gene products results in overactive Hh signaling throughout the embryo, including in the ALPM, where *ptc2* is normally expressed at low levels (Koudijs et al., 2008). In both gain-of-function scenarios, we found a subtle expansion of *nkx2.5* expression (Fig. 3A-C) and a more evident expansion of *cmlc2* expression (Fig. 3D-F). Examination of *vmhc* and *amhc* expression revealed similar expansions of both genes, indicating that both chamber lineages are sensitive to increased Hh signaling (Fig. 4A-D).

The early expansion of the cardiomyocyte population in embryos with increased Hh signaling could lead to an increase in the number of cells in the cardiac chambers; alternatively, the cardiomyocyte surplus might not be maintained. Quantification of cells in the cardiac chambers of *shh*-injected embryos revealed an increased number of atrial cardiomyocytes (Fig. 4G; see Table S1 in the supplementary material). However, although the ventricles of *shh*-injected embryos often appeared enlarged, the increase in ventricular cell number was not statistically significant (Fig. 4E-G). As *shh*-injected embryos display an excess of *vmhc*-expressing cells at earlier stages (Fig. 4A,B), there may be additional consequences of ectopic Hh signaling that affect ventricular cardiomyocyte maintenance. Combining these results with our loss-of-function data, we conclude that the level of Hh signaling activity is an important determinant of the number of cardiomyocytes that arise from the heart fields.

### Hedgehog signaling is required prior to myocardial differentiation

To determine when Hh signaling is required to promote cardiomyocyte formation, we administered CyA during discrete periods of development. First, we added CyA at different stages and allowed embryos to develop until the 18-somite stage, when we evaluated the number of *vmhc*-expressing ventricular cardiomyocytes. We analyzed *vmhc* instead of *cmlc2* in order to simplify quantification, as our data indicated that atrial and ventricular populations respond similarly to reduced Hh signaling (Figs 1, 2). Addition of CyA at the dome or germ ring stage caused a reduction of more than half of the number of ventricular cardiomyocytes at the 18-somite stage (Fig. 5A,B; see Table S3 in the supplementary material). Treatment with CyA at successively later stages significantly reduced the number of ventricular cardiomyocytes; however, the extent of cardiomyocyte loss decreased with later application of CyA (Fig. 5A,B; see Table S3 in the supplementary material). Finally, adding CyA at the 10-somite stage did not change the number of ventricular cardiomyocytes (Fig. 5A,B; see Table S3 in the supplementary material). These data suggest that the requirement for Hh signaling during cardiomyocyte formation begins during gastrulation and ends by the 10-somite stage.

To confirm the importance of this time interval, we took advantage of the fact that CyA inhibition of Hh signaling is reversible (Chen et al., 2001). To inhibit and then restore signaling, we exposed embryos to CyA for defined time intervals; subsequent media

changes allowed significant restoration of Hh signaling within 2 hours, as assessed by recovery of *ptc1* expression (see Fig. S2 in the supplementary material). Consistent with our prior results (Fig. 5A,B), inhibition of Hh signaling from the dome stage until the six-somite stage has the same effect as continuous inhibition from the dome stage until the 18-somite stage (Fig. 5C,D; see Table S4 in the supplementary material). Together, our data suggest that formation of a normal number of cardiomyocytes requires continual Hh signaling during gastrulation and early somitogenesis stages. Interestingly, this time period leads up to the initiation of robust expression of *nkx2.5* around the six-somite stage (Schoenebeck et al., 2007) and far precedes the onset of myocardial differentiation (Yelon et al., 1999). Thus, this temporal requirement suggests that Hh signaling may influence progenitor specification.

### Hedgehog signaling regulates the specification of myocardial progenitors

Although our timing experiments suggested that Hh signaling might impact the specification of myocardial progenitors, it was also possible that the cardiomyocyte reduction in embryos with depleted Hh signaling represented a deficit in the proliferation of myocardial progenitors and their progeny, as Hh promotes cell proliferation in many developmental contexts (Ingham, 1995; Roy and Ingham, 2002). In order to distinguish between these possibilities, we monitored myocardial progenitors in CyA-treated embryos by labeling cells in the lateral marginal zone (LMZ) at 40% epiboly. In prior fate-mapping studies, we have shown that the LMZ contains a mix of multipotent mesendodermal progenitor cells, including myocardial progenitors (Keegan et al., 2004). Examining the fates of cells from the LMZ allows the determination of myocardial progenitor distribution and productivity (Keegan et al., 2004). By comparing experiments in wild-type and CyA-treated embryos, we sought to determine if Hh signaling influences myocardial progenitors via an effect on progenitor specification and/or proliferation.

In each experiment, we labeled neighboring blastomeres in tier 1 (the first row of cells above the blastoderm margin at 40% epiboly) via photoactivation of caged fluorescein-dextran (Fig. 6A). After recording the location of the labeled cells, we raised embryos until 48 hpf. CyA was administered to individual embryos subsequent to blastomere labeling, allowing us to monitor any potential alterations to progenitor distribution or proliferation in response to a loss of Hh signaling. We scored whether labeled cells contributed to the myocardium and counted the number of labeled cardiomyocytes (Fig. 6B,C). As we sampled blastomeres in tier 1, we found ventricular myocardial progenitors but not atrial myocardial progenitors (Fig. 6D,E; Tables S5-S7 in the supplementary material), consistent with our prior demonstration of separate spatial origins for ventricular and atrial lineages (Keegan et al., 2004). We compared the frequency of finding ventricular progenitors in wild-type and CyA-treated embryos, focusing on experiments labeling cells between 50°-125° from dorsal, where ventricular progenitors are known to reside (Keegan et al., 2004). We found wild-type myocardial progenitors at a frequency of 48%: out of 33 labeling experiments in wild-type embryos, we labeled a myocardial progenitor on 16 occasions (Fig. 6D,E). By comparison, we labeled a myocardial progenitor in eight out of 35 such experiments in CyA-treated embryos, a frequency of 23% (Fig. 6D,E). Thus, CyA treatment significantly reduced the frequency of labeling a ventricular progenitor to half that of wild type, indicating that inhibition of Hh signaling results in a reduced density of myocardial progenitors.



We next tested whether reducing Hh signaling also affects the production of cardiomyocytes by myocardial progenitors by comparing the number of labeled cardiomyocytes found in our experiments in wild-type and CyA-treated embryos. Our previous fate-mapping studies suggested that an individual myocardial progenitor at 40% epiboly typically produces four or five cardiomyocytes by 48 hpf (Keegan et al., 2004). We found no significant difference in progenitor productivity following CyA treatment: we detected an average of 4.6 cardiomyocytes per wild-type progenitor and an average of 4.5 cardiomyocytes per CyA-treated progenitor (Fig. 6E; see Table S5 in the supplementary material). Thus, extrapolating from our tier 1 fate map data, we conclude that reduced Hh signaling causes a reduction in myocardial progenitor specification, but not in progenitor proliferation.

### **Complementary changes in endothelial patterning in response to altered Hedgehog signaling**

Given the reduction in myocardial progenitors observed in CyA-treated embryos, we next asked whether this loss correlates with an expansion of another embryonic lineage. We focused on lineages that originate near the myocardial progenitors (Keegan et al., 2004), including the endocardial, anterior endothelial, myeloid, pectoral fin mesenchyme and pharyngeal mesendodermal lineages. No obvious gains were evident from gross morphological assessment or from our fate-mapping experiments. However, careful examination of molecular markers revealed slight alterations in endothelial patterning. Specifically, we noticed a broadened common cardinal vein (CCV) in *smo* mutants and CyA-treated embryos (Fig. 7A,B; N.A.T. and D.Y., unpublished). Conversely, *shh*-injected embryos seem to lack the CCV (Fig. 7C). Prior to vessel formation, subtle changes in endothelial progenitor marker expression reveal similar phenotypes (see Fig. S3 in the supplementary material).

These data suggest the possibility that Hh signaling influences the choices made by multipotent cardiovascular progenitor cells that are able to become either myocardium or particular subsets of endothelium (Kattman et al., 2007; Martin-Puig et al., 2008; Wu et al., 2008). Alternatively, these endothelial patterning defects could reflect aberrant morphogenesis of the anterior vessels, as defects in trunk vessel morphogenesis are seen in *shha* mutants (Brown et al., 2000; Gering and Patient, 2005). In favor of the former hypothesis, we note that addition of CyA from dome stage until the 6-somite stage is sufficient to broaden the CCV (N.A.T. and D.Y., unpublished). Even so, it remains possible that the complementary changes in myocardial and endothelial lineages in response to altered Hh signaling reflect independent functions of Hh, rather than transformations between myocardial and endothelial fates. Precise identification of the fates exchanged when Hh signaling is altered awaits a more detailed understanding of the lineage relationships, gene expression patterns and morphogenetic behaviors of specific subsets of anterior endothelial cells.

### **Myocardial progenitors in the mouse embryo directly respond to Hedgehog signaling**

Hh signaling might take place directly within myocardial progenitors; alternatively, Hh signaling might be received elsewhere and influence myocardial progenitors indirectly. During the interval when Hh signaling is required for cardiac specification, myocardial

progenitors are not immediately adjacent to sources of Hh ligands in the zebrafish embryo. During gastrulation stages, myocardial progenitors are found in lateral mesendodermal territories (Keegan et al., 2004; Stainier et al., 1993), whereas *shha* and *shhb* are expressed in dorsal mesoderm (Ekker et al., 1995; Krauss et al., 1993). During early somitogenesis stages, cardiac progenitors are located in bilateral fields of the ALPM (Fishman and Chien, 1997; Schoenebeck et al., 2007), and Hh ligands are found in midline structures (Concordet et al., 1996; Currie and Ingham, 1996; Krauss et al., 1993; Lewis et al., 1999). Although it is known that Hh can act as a long-range signal in some contexts (Goetz et al., 2002), we have been unable to detect *ptc1* or *gli1* expression in the regions of the zebrafish embryo that contain myocardial progenitors (N.A.T. and D.Y., unpublished), although *ptc2* expression has been reported at low levels in the ALPM (Koudijs et al., 2008). Difficulties detecting *ptc1* or *gli1* may be due to limitations in detecting low levels of gene expression via in situ hybridization.

As another approach to examine whether myocardial progenitor cells receive Hh signals, we pursued genetic inducible fate mapping (GIFM) in the mouse embryo (Joyner and Zervas, 2006). GIFM enables labeling of cells directly responding to Hh signaling during discrete periods of development owing to an inducible Cre recombinase expressed from the *Gli1* locus (*Gli1-CreER<sup>T2</sup>*) (Ahn and Joyner, 2004). As *Gli1* is a direct transcriptional target of Hh signaling and is not expressed in the absence of Hh signaling (Bai et al., 2002; Dai et al., 1999; Lee et al., 1997; Marigo et al., 1996), *Gli1* expression indicates a positive response to Hh signaling. Upon tamoxifen (TM) administration, Cre recombination activity is induced within 8-12 hours in *Gli1*-expressing cells, and remains active for ~24 hours (Hayashi and McMahon, 2002; Zervas et al., 2004). Recombination of the *R26R* reporter transgene permanently marks these *Gli1*-derived cells and their progeny with *lacZ*. However, owing to mosaic expression of Cre, particularly at the low doses of TM used, the GIFM technique does not mark all of the Hh-responding cells in a given tissue (Ahn and Joyner, 2004; Joyner and Zervas, 2006; Zervas et al., 2004).

Use of a *Ptch1<sup>LacZ</sup>* allele in mouse has demonstrated *Ptch1* expression in the OFT at E9.5 and in the dorsal mesenchymal protrusion within the atria at E10.5 (Goddeeris et al., 2008; Washington Smoak et al., 2005). However, it is unknown whether mouse myocardial progenitors directly respond to Hh signaling prior to myocardial differentiation (Zhang et al., 2001). We therefore labeled Hh-responding cells from ~E7.0-E8.0 by administering TM at 5 pm on E6.5; this period encompasses the formation of the cardiac crescent and the transition to a linear heart tube (Fig. 8A) and precedes by one full day the reported *Ptch1<sup>lacZ</sup>* expression in the lengthening OFT (Washington Smoak et al., 2005). When labeled cells were analyzed at E18.5, we found *Gli1*-derived cardiomyocytes in all embryos examined (Fig. 8B-D). Furthermore, we observed *Gli1*-derived cardiomyocytes in all four chambers (see Table S8 in the supplementary material). These GIFM experiments indicate that myocardial progenitors directly receive and respond to Hh signaling during early stages of heart formation in the mouse embryo, potentially beginning as early as E7.0.

## Receipt of Hedgehog signaling is required cell autonomously for effective contribution to myocardium

Although we found that mouse myocardial progenitors receive Hh signals, GIFM alone could not address whether Hh signaling is required within these cells. We conducted transplantation experiments in zebrafish to test the cell autonomy of the requirement for Hh signaling during cardiomyocyte formation. Blastomeres were transplanted at the sphere stage, using donor embryos carrying the transgene *Tg(cmlc2:egfp)* (Huang et al., 2003) to facilitate assessment of myocardial contribution. To determine whether wild-type cells and *MZsmo* cells have the same ability to become cardiomyocytes, we transplanted either wild-type or *MZsmo* donor cells into wild-type hosts (Fig. 9A-E). Our data indicated that *MZsmo* donor cells contributed to host myocardium less frequently than did the wild-type donor cells (Fig. 9A). In wild-type-to-wild-type transplants, donor-derived cells contributed to the myocardium 17% of the time (29 out of 173 transplants). By contrast, *MZsmo* donor cells transplanted into wild-type hosts contributed to the myocardium only 9% of the time (13 out of 148 transplants), a significant difference from the effectiveness of wild-type donor cells. In six independent transplantation sessions, we found considerable variability in the exact frequency of myocardial contribution (see Table S9 in the supplementary material). However, wild-type donor cells consistently had a higher frequency of myocardial contribution than did *MZsmo* donor cells (see Table S9 in the supplementary material).

One explanation for the inefficient myocardial contribution of *MZsmo* cells might be a general problem with transplanted cell survival. However, in additional experiments, we labeled donor cells with a fluorescein-dextran lineage tracer and found that *MZsmo* cells contribute robustly to many lineages in wild-type hosts. For example, we found that wild-type and *MZsmo* donor cells were equally capable of fast muscle contribution (Fig. 9F-H). This result is consistent with previously reported normal contribution to wild-type host fast muscle by zygotic *smo* donor cells (Barresi et al., 2000). Thus, we conclude that the survival of *MZsmo* donor-derived cells is not generally compromised.

We next sought to determine whether there were any differences in how *MZsmo* and wild-type donor cells contribute to the myocardial lineage, apart from their differences in contribution frequency. We found that both wild-type and *MZsmo* donor cells were capable of contributing to either cardiac chamber (see Fig. S4 in the supplementary material). In host embryos with donor-derived ventricular cardiomyocytes, the average number of wild-type donor-derived cardiomyocytes ( $3.6 \pm 0.6$ ) was comparable with the average number of *MZsmo* donor-derived cardiomyocytes ( $2.8 \pm 0.4$ ) (see Fig. S4 in the supplementary material). Similarly, in the atrium, the numbers of wild-type and *MZsmo* donor-derived cardiomyocytes were nearly identical (wild-type,  $2.6 \pm 0.6$ ; *MZsmo*,  $2.8 \pm 0.6$ ; Fig. S4). Thus, consistent with our zebrafish fate-mapping results (Fig. 6E), it appears that wild-type and *MZsmo* cells differ in their ability to become myocardial progenitor cells, but, once committed to the lineage, they are equally effective in producing cardiomyocytes. Together, our results indicate that receipt of Hh signaling is required cell autonomously for full competence to contribute to myocardium.

## DISCUSSION

In this work, we demonstrate that embryonic levels of Hh signaling are crucial to establishing a heart of the appropriate scale. We propose a model in which Hh signaling acts early on naïve cells possessing cardiac developmental potential. Successful receipt of signaling over a sufficient time interval maximizes the likelihood that such a cell becomes a myocardial progenitor, and the number of myocardial progenitors is among the key determinants of the number of cardiomyocytes in the heart. Thus, Hh signaling scales heart size by influencing cardiac specification rather than by acting as a mitogen.

Our data provide a new level of resolution of the role of Hh signaling during heart development. Prior studies characterizing the cardiac phenotypes of *Smo* and *Ptch1* knockout mice indicated a relationship between Hh signaling and heart size, but the nature of this connection – when and where this signaling occurred and the cellular basis for the observed cardiac phenotypes – remained unclear (Chiang et al., 1996; Goodrich et al., 1997; Zhang et al., 2001). In the zebrafish embryo, we demonstrate that Hh signaling acts during early stages to directly promote myocardial progenitor specification. The direct requirement for Hh signaling, rather than involvement of a relay, is interesting, given the apparent distance between the myocardial progenitors and the known sources of *shha* and *shhb* at the embryonic midline. This may be an example of long-range Hh signaling, akin to that observed in *Drosophila* imaginal discs, vertebrate neural tubes, vertebrate limb buds or zebrafish endoderm, where Hh ligands are known to act at significant distances from their origins (Briscoe et al., 2001; Chung and Stainier, 2008; Gallet et al., 2006; Lewis et al., 2001; Zeng et al., 2001). In mouse, it seems likely that *Ihh* could be relevant to the specification of myocardial progenitors, as it is expressed in the anterior endoderm adjacent to the cardiac crescent (Zhang et al., 2001); additionally, the cardiac phenotype of *Shh*<sup>-/-</sup> *Ihh*<sup>-/-</sup> mice resembles the *Smo*<sup>-/-</sup> phenotype more closely than the *Shh*<sup>-/-</sup> phenotype (Chiang et al., 1996; Zhang et al., 2001).

Hh signaling is not absolutely essential for cardiomyocyte formation; normal cardiomyocytes develop, albeit in reduced numbers, when Hh signaling is depleted. It seems unlikely that the role of Hh signaling is to prevent cardiac apoptosis, as caspase inhibitors fail to rescue the *smo* mutant cardiac phenotype (Z. Garavito-Aguilar and D.Y., unpublished). The alternative fate of the lost cardiac progenitors in the absence of Hh signaling is not yet clear, although the observed responsiveness of the CCV to Hh signaling suggests one intriguing avenue for future investigation. Overall, we were struck by our observation that loss of Hh function consistently produces quantitative phenotypes that represent a 50% reduction from wild type. In *MZsmo* and CyA-treated embryos, we found ~50% of the normal number of cardiac cells at 2 days post-fertilization and a 50% reduction in cells expressing *cmlc2*, *vmhc* or *amhc* at earlier stages (see Tables S1 and S2 in the supplementary material). We encountered ventricular progenitors half as frequently in our CyA-treated fate-mapping experiments (Fig. 6E) and found *MZsmo*-derived cardiomyocytes at half the wild-type frequency in transplantation experiments (Fig. 9A). One possibility is that loss of a specific subpopulation of cardiomyocytes accounts for the missing 50%. However, we have not encountered evidence in support of that idea, as we see that both atrial and ventricular cardiomyocytes are significantly affected. Instead, the partial reduction

of cardiac cell number presumably reflects the mechanism by which Hh signaling promotes specification.

There are multiple ways in which Hh signaling could directly impact the competence of potential myocardial progenitors. One possibility is that Hh signaling could influence the movement of myocardial progenitors, such that its absence would prevent some cells from receiving the signals necessary for the establishment of cardiac fate. The cardiac defects in zebrafish embryos with altered *Agtr11b*/Apelin signaling have been attributed to a similar mechanism (Scott et al., 2007; Zeng et al., 2007). Hh signaling has been shown to influence cell migration and motility in several developmental contexts, including axon guidance, and germ cell, endothelial cell and cardiac neural crest cell migration (Charron and Tessier-Lavigne, 2005; Deshpande et al., 2001; Goddeeris et al., 2007; Hochman et al., 2006; Washington Smoak et al., 2005). However, loss of Hh signaling has not been associated with an effect on general gastrulation movements in zebrafish (Solnica-Krezel et al., 1996; van Eeden et al., 1996), and we have not observed any obvious ALPM migration defects in *MZsmo* or *CyA*-treated embryos (N.A.T. and D.Y., unpublished).

We favor an alternate model in which Hh signaling works in combination with other signaling pathways that confer cardiac identity, potentially including the Fgf, BMP and Nodal pathways. One mechanism by which Hh signaling could affect the degree of competence imparted to a naïve cell would be to act in a serial fashion with other inductive signals. For example, Hh signaling could impart enhanced ability to respond to any of the complement of specification signals that combine to confer cardiac fate. This mechanism is reminiscent of the situation during chondrogenesis, in which Hh signaling is required to alter the competence of somitic mesoderm to differentiate in response to BMP signaling (Murtaugh et al., 1999; Zeng et al., 2002). Alternatively, instead of acting serially, Hh signaling may act in parallel with other required signals to promote cardiac fate assignment through regulation of shared target genes. The parallel pathways could then partially compensate for a loss of Hh signaling. To determine the precise nature of the relationship between Hh signaling and the competence to form myocardium, it will be necessary to identify the targets downstream of Hh/Gli signaling that impact cardiac specification.

Intriguingly, our gain-of-function studies are consistent with a combinatorial signaling model. We observed an increase in cardiomyocyte number in embryos with increased Hh signaling, although only within the ALPM and not at separate, ectopic sites. These data suggest that the myocardial developmental potential within the heart field is labile in response to Hh signaling levels, but that Hh signaling alone is not sufficient to induce cardiac fate assignment. Additionally, gain-of-function studies in P19 cells have shown that overexpression of *Shh* or *Gli2* can induce cardiomyocyte formation (Gianakopoulos and Skerjanc, 2005). However, the induction of a cardiac program required cell aggregation, suggesting that Hh signaling might cooperate with another signal induced only after aggregation in order to trigger cardiomyocyte formation. In future work, it will be interesting to define whether a comparable signal functions in the embryo.

Based on the combination of our studies in zebrafish and mouse, it is likely that Hh signaling plays a conserved, cell-autonomous role in promoting myocardial progenitor

specification. Signaling pathways that are required cell autonomously for cardiac specification have great potential for use in cell-based therapies for cardiac repair, as they will provide the ability to optimize the production of cardiomyocytes from multipotent cells in vitro (Chen et al., 2007), a mode of producing cardiomyocytes that remains relatively inefficient (Laflamme and Murry, 2005). Our finding of a cell-autonomous role for Hh signaling suggests that manipulation of this pathway could be used to more effectively tilt the balance towards cardiac fate assignment in undifferentiated cells. It would be particularly interesting to determine whether Hh signaling can influence the developmental potential of recently identified populations of mammalian multipotent cardiovascular progenitors (Kattman et al., 2007; Kattman et al., 2006; Martin-Puig et al., 2008; Moretti et al., 2006). In the future, a fuller understanding of how Hh signaling interfaces with additional contributing signals will facilitate our ability to realize the goals of regenerative medicine.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We thank J. Schoenebeck, A. Schier and members of the Yelon laboratory for discussions and support; C. Levine, S. Blaess and S. Ahn for assistance with GIFM techniques and reagents; P. Huang for the *smo* genotyping protocol; and S. Tay and B. Conrad for advice on statistical analyses. This work was supported by the March of Dimes (D.Y.), by NIH R01 HL069594 (D.Y.) and by NIH R01 HD35768 (A.L.J.). NAT received support from the NYU Graduate Training Program in Developmental Genetics (NIH T32 HD007520).

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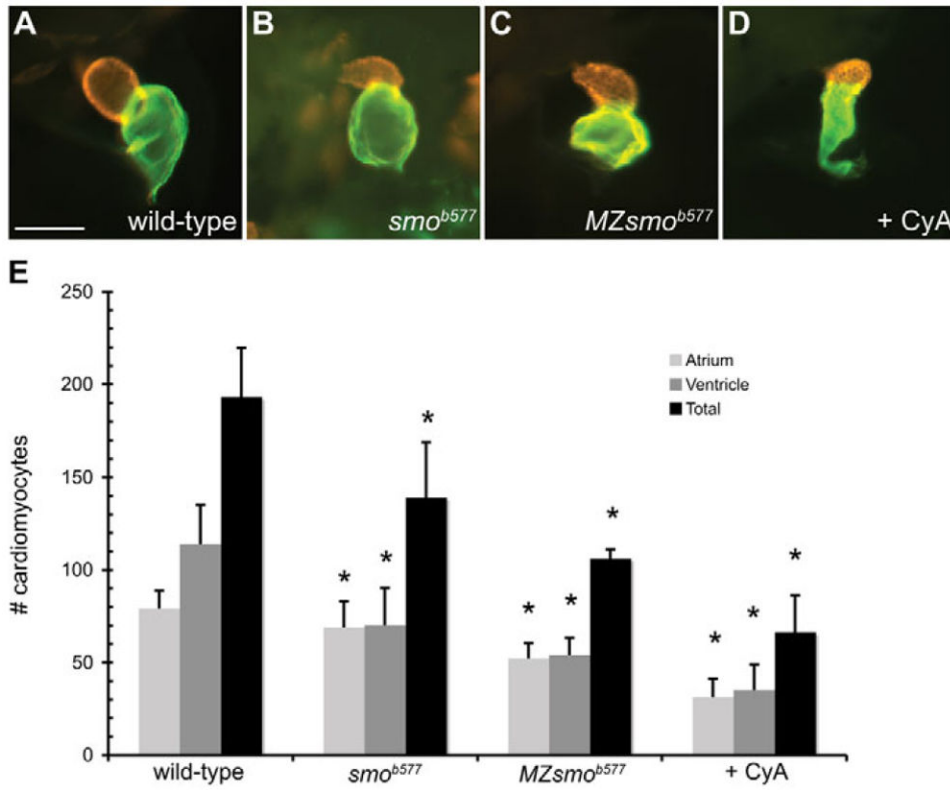


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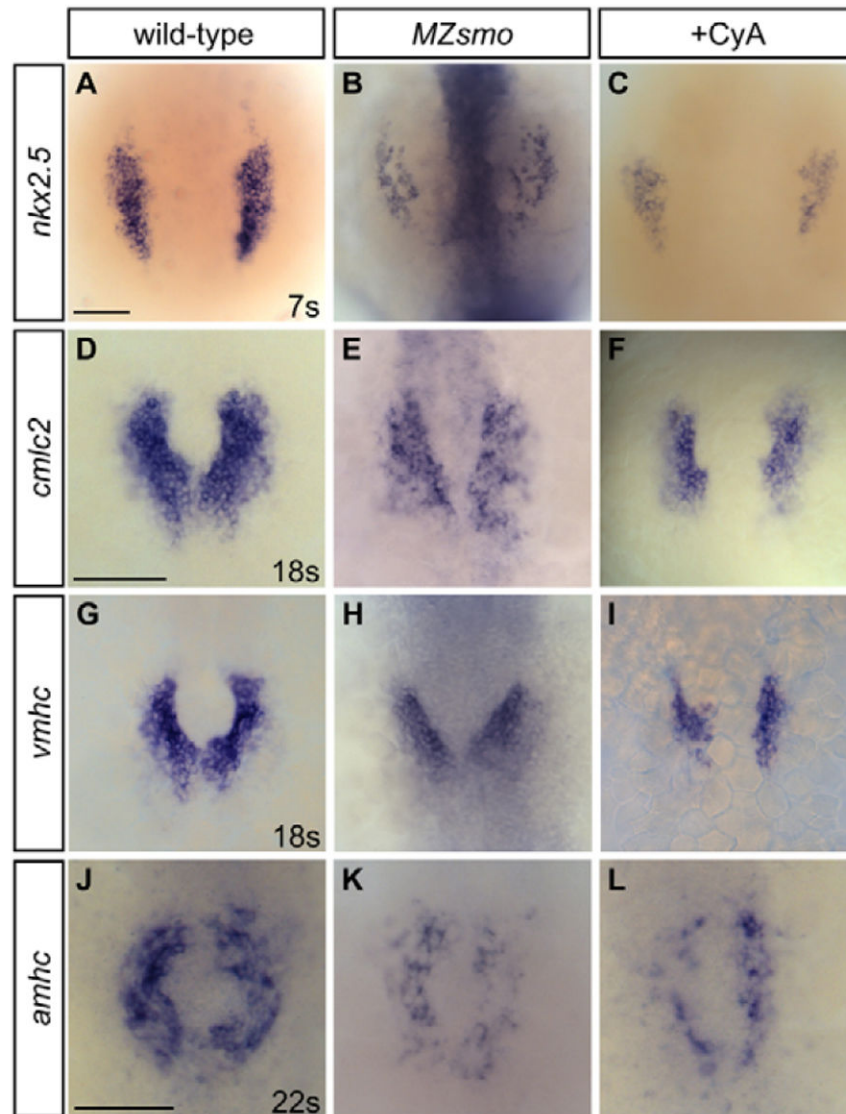
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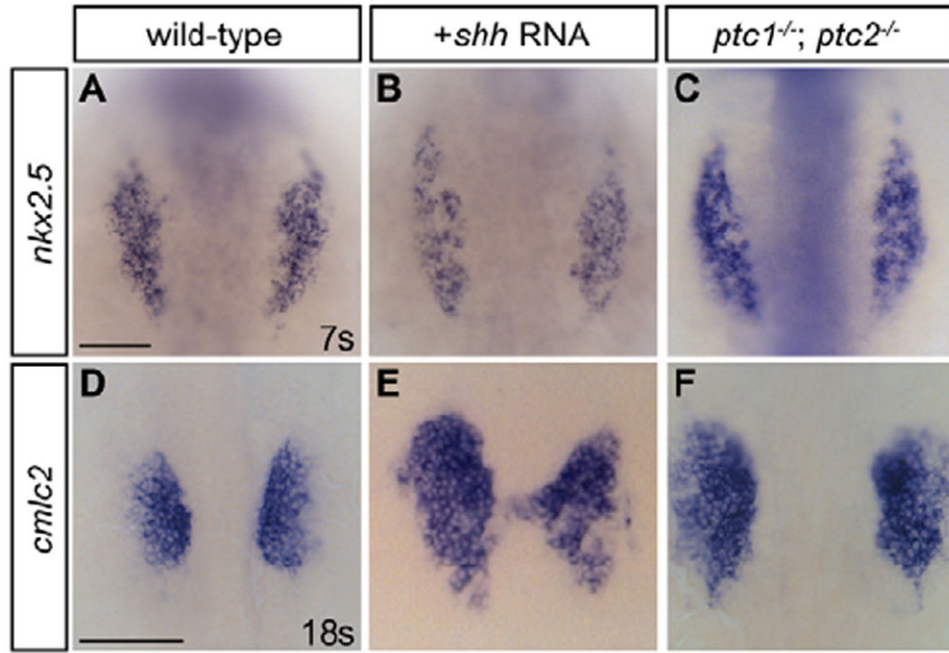
**Fig. 1. Embryos with reduced Hedgehog signaling have small cardiac chambers**  
 (A-D) Lateral views of zebrafish hearts stained with MF20 (red) and S46 (green) antibodies to visualize the ventricle and atrium at 48 hpf. MF20 marks the entire heart and S46 is atrium specific. In these superimposed images, the MF20<sup>+</sup>S46<sup>+</sup> atrial tissue appears green and the MF20<sup>+</sup>S46<sup>-</sup> ventricle is red. Scale bar: 100  $\mu$ m. (A) Wild-type heart. (B-D) Zygotic *smo*<sup>b577</sup> mutant, *MZsmo*<sup>b577</sup> mutant and CyA-treated hearts have small, misshapen ventricles and atria. (E) Quantification of cardiomyocyte number in wild-type, *smo*<sup>b577</sup>, *MZsmo*<sup>b577</sup> and CyA-treated embryos at 52 hpf. See Materials and methods for cell counting technique. Values represent the mean cell number for each category ( $\pm$ s.d.). Asterisks indicate statistically significant differences from wild type ( $P < 0.0001$  for all except the *smo*<sup>b577</sup> atrium where  $P = 0.01$ ).





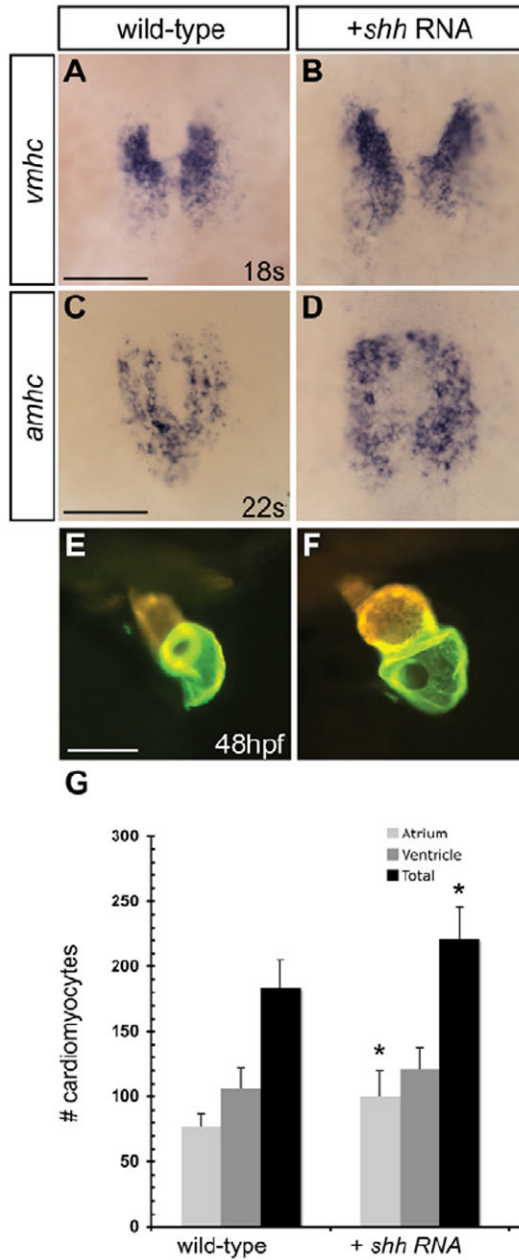
**Fig. 2. Hedgehog signaling promotes the initial establishment of cardiomyocytes**  
 (A-L) In situ hybridization detects expression of myocardial markers. Dorsal views, anterior towards the top. Scale bars: 100  $\mu$ m (bar in D applies to D-I). *MZsmo* mutant and CyA-treated embryos exhibit reduced expression of (A-C) *nkx2.5* at the seven-somite stage, (D-F) *cmlc2* at the 18-somite stage, (G-I) *vmhc* at the 18-somite stage and (J-L) *amhc* at the 22-somite stage.





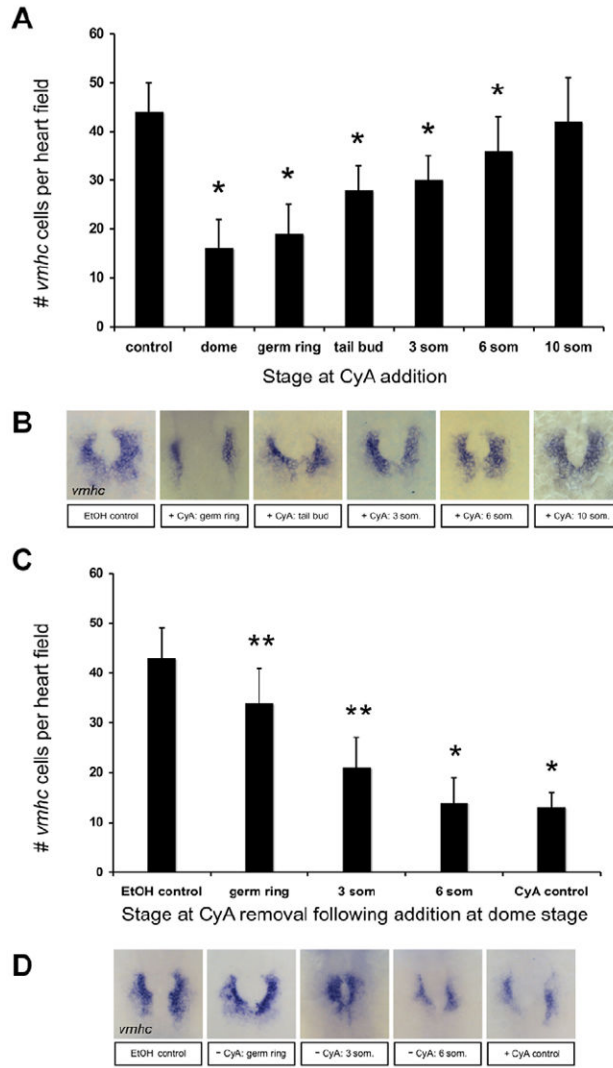
**Fig. 3. Increased Hedgehog signaling expands cardiomyocyte numbers**

(A-F) Dorsal views, anterior towards the top. Scale bars: 100  $\mu\text{m}$ . (A-C) *nkx2.5* expression at the seven-somite stage is subtly expanded in *shh*-injected and *ptc1;ptc2* double mutant embryos. The observed extent of anterior-posterior enlargement was variable. (D-F) *cmlc2* expression at the 18-somite stage is expanded in *shh*-injected and *ptc1;ptc2* double mutant embryos. The degree of expansion was variable and frequently coupled with disorganized morphology of migrating cardiomyocyte populations, as in E.



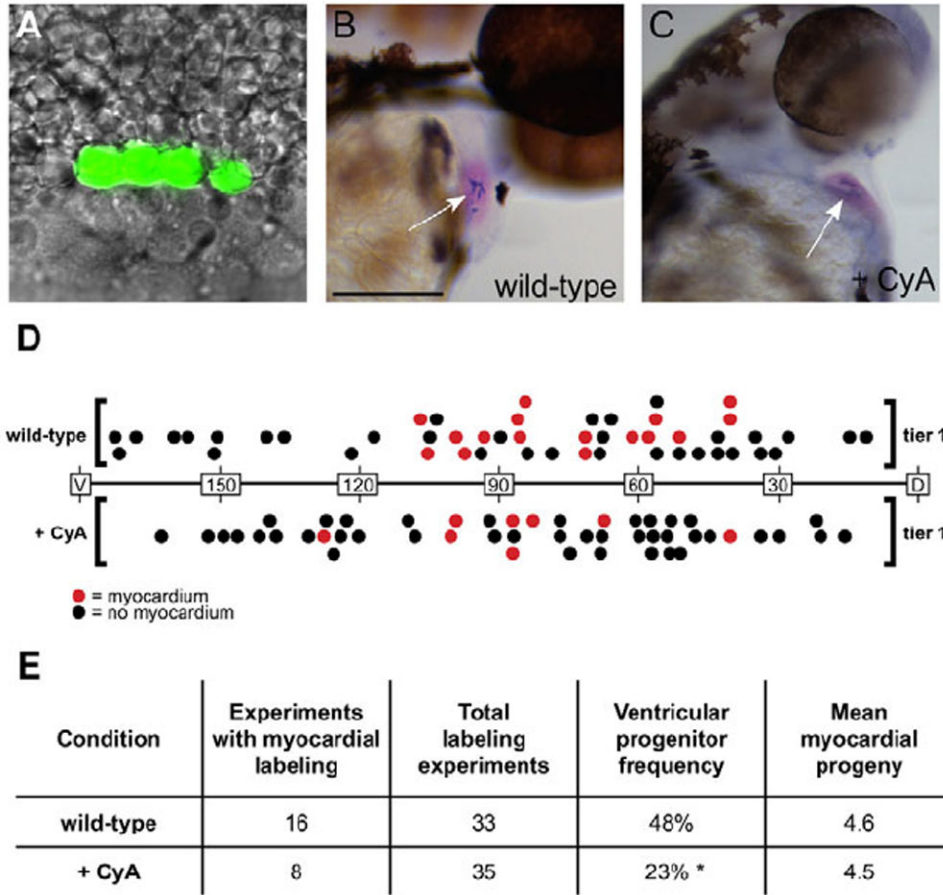
**Fig. 4. Increased Hh signaling expands ventricular and atrial populations**

(A-D) Dorsal views, anterior towards the top. (A,B) *vmhc* expression at the 18-somite stage and (C,D) *amhc* expression at the 22-somite stage are expanded in *shh*-injected embryos. (E,F) At 48 hpf, *shh*-injected embryos often exhibit enlarged hearts, as indicated by MF20/S46 immunofluorescence. Scale bars: 100  $\mu$ m. (G) Quantification of cardiomyocyte number in wild-type and *shh* RNA-injected embryos at 52 hpf. Values represent the mean cell number for each category ( $\pm$ s.d.). Asterisks indicate statistically significant differences from wild type ( $P$  0.0005).



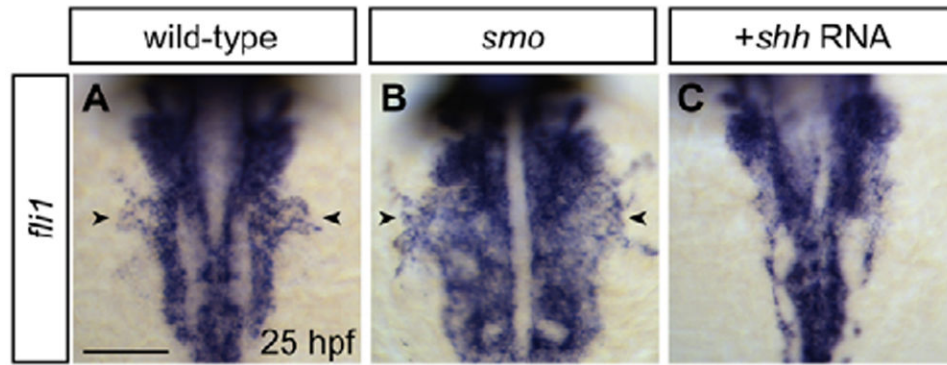
**Fig. 5. Hedgehog signaling activity is required early for the formation of the appropriate number of cardiomyocytes**

(A) Quantification of the number of *vmhc*-expressing cells per bilateral heart field at the 18-somite stage, comparing control embryos with embryos treated with CyA beginning at the indicated stages. Values represent the mean cell number for each category ( $\pm$ s.d.). Asterisks indicate statistically significant differences from controls ( $P < 0.004$ ). (B) Representative images of *vmhc* expression at the 18-somite stage in control embryos and embryos exposed to CyA beginning at the indicated stages. (C) Quantification of the impact of inhibiting Hh signaling during defined time windows on the number of *vmhc*-expressing cells. CyA was added at dome stage and removed at indicated stages. CyA control embryos were exposed continually between the dome and 18-somite stages. Asterisks indicate statistically significant differences from ethanol controls ( $P < 0.0024$ ). Double asterisks indicate that a treatment also results in a statistically significant difference from CyA controls ( $P < 0.001$ ). (D) Representative images of *vmhc* expression in embryos treated as indicated.



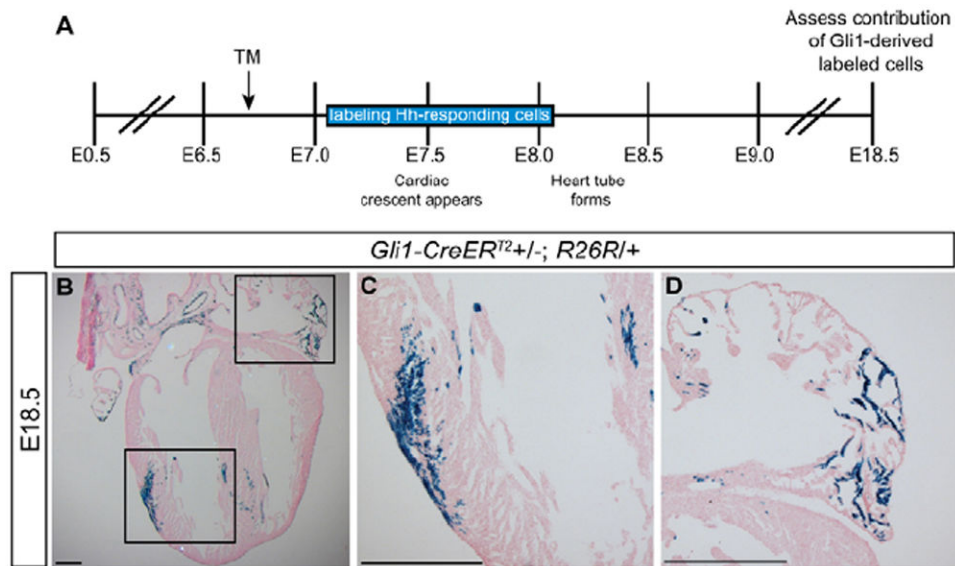
**Fig. 6. Embryos with reduced Hedgehog signaling contain fewer myocardial progenitors**  
**(A)** Example of a 40% epiboly embryo in which four adjacent blastomeres in the first row above the embryonic margin (tier 1) were labeled by photoactivation of caged fluoresceindextran. Fluorescein is pseudocolored in green and superimposed on a DIC image. **(B,C)** 48 hpf embryos with labeled contributions to ventricular myocardium. **(B)** Six labeled cardiomyocytes found within a wild-type ventricle. Scale bar: 100  $\mu$ m. **(C)** Five labeled cardiomyocytes found within a CyA-treated ventricle. Myocardium is visualized via in situ hybridization for *cmlc2* (pink) and labeled cells are marked in blue (arrows). **(D)** 40% epiboly myocardial fate maps of tier 1 blastomeres in wild-type and CyA-treated embryos. Wild-type fate map is shown above the horizontal axis and CyA-treated fate map is shown below the axis. Longitude coordinates of the margin are depicted with dorsal as the origin ( $0^\circ$ ) and ventral as  $180^\circ$ . Each circle represents an individual labeling experiment. Red circles represent experiments in which the labeled blastomeres contributed to ventricular myocardium, and black circles represent embryos in which the labeled cells did not become cardiomyocytes. The position of each circle indicates the median longitude value of the labeled blastomeres. Experiments from the left and right sides are merged for convenience of presentation. **(E)** Results from fate-mapping experiments conducted  $50^\circ$ - $125^\circ$  from dorsal, the region within tier 1 where ventricular myocardial progenitors are found. A ratio of the number of experiments yielding labeled ventricular myocardium to the total number of experiments was used to determine the ventricular progenitor frequency. Asterisk

indicates a statistically significant difference from wild-type ( $P=0.0418$ , Fisher's exact test). The mean number of myocardial progeny per embryo was determined by counting labeled cardiomyocytes in relevant cases (see Table S5 in the supplementary material).



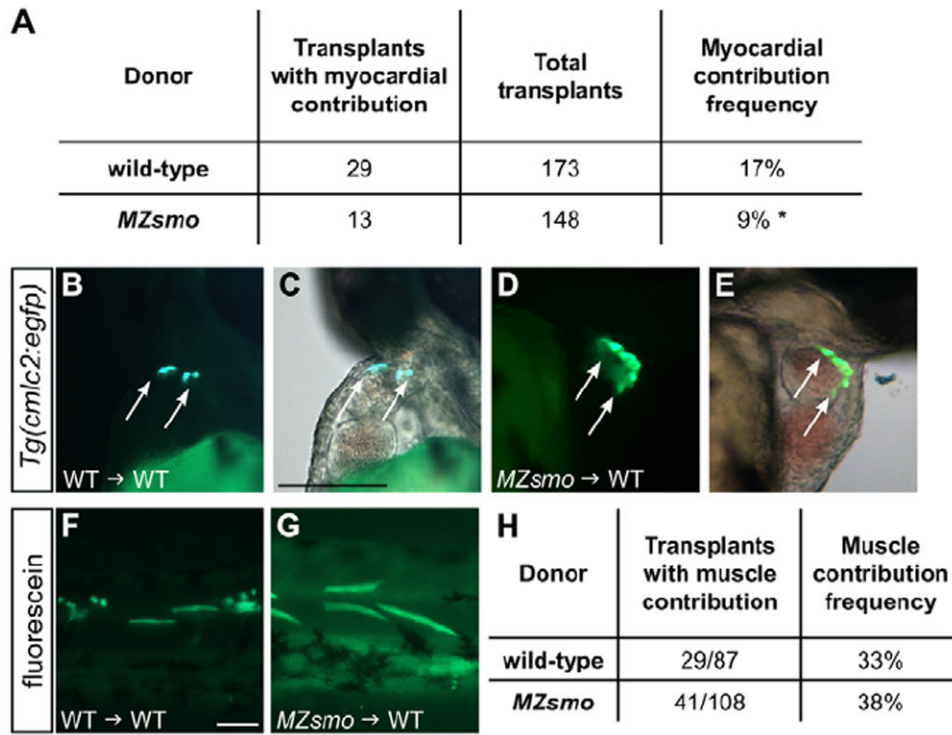
**Fig. 7. Altered Hedgehog signaling affects development of the common cardinal vein**  
 (A-C) In situ hybridization detects expression of the endothelial gene *fli1* at 25 hpf. Dorsal views, anterior towards the top. Scale bar: 100  $\mu$ m. (A) Formation of the common cardinal vein (CCV, arrowheads) is evident bilaterally in wild-type embryos. (B) CCV (arrowheads) is expanded in *smo* mutants. (C) CCV is absent in *shh*-injected embryos.





**Fig. 8. Cardiac progenitors directly respond to Hedgehog signals in mouse**

(A) Depiction of GIFM strategy and corresponding stages of heart development. Tamoxifen was given at 5 pm on E6.5 to mark Hh-responsive cells from ~E7.0-E8.0. (B-D) Transverse sections of a *Gli1-CreER<sup>T2</sup>/+; R26R/+* mouse heart at E18.5. (C,D) High-magnification views of the section depicted in B. Scale bars: 500  $\mu$ m. Cardiac progenitors that directly responded to Hh signals contributed to labeled cells (blue) in both ventricular myocardium (C) and atrial myocardium (D).



**Fig. 9. Hedgehog signaling is required cell autonomously for effective contribution to myocardium**

(A) Transplantation results. Expression of *Tg(cmlc2:egfp)* in host embryos at 48 hpf indicated myocardial contribution. A ratio of hosts with donor-derived cardiomyocytes to all host embryos screened was used to determine myocardial contribution frequency. Asterisk indicates a statistically significant difference from wild type ( $P=0.0346$ , normal approximation of the  $\chi^2$  test). (B) Wild-type donor-derived cells expressing *Tg(cmlc2:egfp)* in wild-type host myocardium at 48 hpf. (C) Superimposed bright-field and fluorescent images. Scale bar: 100  $\mu$ m. (D) *MZsmo* donor-derived cardiomyocytes expressing *Tg(cmlc2:egfp)* in wild-type host myocardium. (E) Superimposed bright-field and fluorescent images. (F,G) Wild-type and *MZsmo* donor-derived skeletal fast muscle cells marked with fluorescein lineage tracer in wild-type host. Scale bar: 100  $\mu$ m. (H) Donor cell incorporation into skeletal fast muscle lineage, scored at 48 hpf.