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BMP signaling modulates hedgehog-induced secondary heart field proliferation

Laura A. Dyer [a], Frini A. Makadia [b], Alexandria Scott [c], Kelly Pegram [c], Mary R. Hutson [c], Margaret L. Kirby [a,c,*]

[a] Department of Cell Biology, USA
[b] Department of Chemistry, USA
[c] Department of Pediatrics (Neonatology), Duke University, Durham, NC, USA

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ABSTRACT

Sonic hedgehog signaling in the secondary heart field has a clear role in cardiac arterial pole development. In the absence of hedgehog signaling, proliferation is reduced in secondary heart field progenitors, and embryos predominantly develop pulmonary atresia. While it is expected that proliferation in the secondary heart field would be increased with elevated hedgehog signaling, this idea has never been tested. We hypothesized that up-regulating hedgehog signaling would increase secondary heart field proliferation, which would lead to arterial pole defects. In culture, secondary heart field explants proliferated up to 6-fold more in response to the hedgehog signaling agonist SAG, while myocardial differentiation and migration were unaffected. Treatment of chick embryos with SAG at HH14, just before the peak in secondary heart field proliferation, resulted unexpectedly in stenosis of both the aortic and pulmonary outlets. We examined proliferation in the secondary heart field and found that SAG-treated embryos exhibited a much milder increase in proliferation than was indicated by the in vitro experiments. To determine the source of other signaling factors that could modulate increased hedgehog signaling, we co-cultured secondary heart field explants with isolated pharyngeal endoderm or outflow tract and found that outflow tract co-cultures prevented SAG-induced proliferation. BMP2 is made and secreted by the outflow tract myocardium. To determine whether BMP signaling could prevent SAG-induced proliferation, we treated explants with SAG and BMP2 and found that BMP2 inhibited SAG-induced proliferation. In vivo, SAG-treated embryos showed up-regulated BMP2 expression and signaling. Together, these results indicate that BMP signaling from the outflow tract modulates hedgehog-induced proliferation in the secondary heart field.

Introduction

As the heart loops, the splanchnic mesoderm, located between the outflow and inflow attachments to the ventral pharynx, contributes first the myocardium and then the smooth muscle that forms the arterial pole (Waldo et al., 2005). This region of arterial pole-forming splanchnic mesoderm is called the secondary heart field. As some of the secondary heart field progenitors differentiate, others continue to proliferate to generate enough cells to provide both myocardium and smooth muscle to the arterial pole (van den Berg et al., 2009). These proliferative cells are located more caudally in the field and are adjacent to the pharyngeal endoderm, which produces Sonic hedgehog (Shh), a secreted ligand (Dyer and Kirby, 2009). The cardiac progenitors in the secondary heart field express the Shh receptor Patched2 (Ptc2). Interestingly, Ptc2 expression is more robust in the caudal secondary heart field, which is the region of highest proliferation (Dyer and Kirby, 2009).

Shh is a known mitogen and cell survival factor (reviewed in Ingham and McMahon, 2001). Among its downstream targets are cell cycle genes such as cyclins D1 and E and n-myc, which promote the G1 to S transition (Kenney and Rowitch, 2000; Oliver et al., 2003). Inhibiting Shh signaling stalls limb bud mesenchyme cells in G1, with fewer cells progressing to S phase (Zhu et al., 2008).

Recently, our lab and others have shown that Shh signaling is required for normal arterial pole development. The Shh-null mouse has pulmonary atresia (Washington Smoak et al., 2005), as do chick embryos treated with the Shh inhibitor cyclopamine (Dyer and Kirby, 2009). A Shh conditional knockout in the Nkx2.5 expression domain has a single outflow vessel (Goddeeris et al., 2007). In the chick, inhibiting hedgehog signaling at discrete stages associated with the production of secondary heart field-derived arterial pole myocardium reduced secondary heart field proliferation and resulted in pulmonary atresia/stenosis (Dyer and Kirby, 2009). However, nothing is known about what happens when the secondary heart field is exposed to excess hedgehog signaling. The function of Shh is context-dependent, and both the dose and timing of Shh exposure are critical during development (Harfe et al., 2004; Scherz et al., 2007; Yang et al., 1997; Zhu et al., 2008).

Because blocking hedgehog signaling led to pulmonary atresia/stenosis by decreasing proliferation in the secondary heart field, we

* Corresponding author. DUMC Box 103105, Durham, NC 27710, USA. Fax: +1 919 668 1599.
E-mail address: mlkirby@duke.edu (M.L. Kirby).

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hypothesized that up-regulating hedgehog signaling would increase proliferation throughout the secondary heart field, which would also lead to abnormal arterial pole development. To determine the effect of Shh signaling on secondary heart field, we treated secondary heart field explants with SAG, a hedgehog agonist. The explants showed a robust increase in proliferation in response to SAG. By contrast, when treating embryos in ovo, SAG only increased proliferation transiently and was quickly suppressed. As a result, the final arterial pole phenotype was a mild stenosis of both the aortic and pulmonary outlets after septation. To determine what interactions moderated increased hedgehog signaling, we co-cultured secondary heart field explants with isolated pharyngeal endoderm or outflow tract myocardium. Outflow tract co-cultured with secondary heart field explants suppressed SAG-induced proliferation and promoted myocardial differentiation. The outflow tract is a source of BMP2, which promotes myocardial differentiation and can also inhibit Shh-induced proliferation. Secondary heart field explants treated with SAG and BMP2 showed increased myocardial differentiation, with no increase in proliferation. In vivo, BMP2 expression was elevated at the junction of the outflow tract with the pharynx, and phosphorylated SMAD1,5,8 was up-regulated in the secondary heart field. When embryos were treated with SAG and dorsomorphin, a BMP antagonist, early embryonic lethality resulted. Together, these data show that BMP signaling from the outflow tract regulates hedgehog-induced proliferation in the secondary heart field and that a tightly synchronized rate of proliferation is required for normal arterial pole development.

Methods and Materials

Embryos

Fertilized Ross Hubert chick eggs (Gallus gallus domesticus, Pilgrim's Pride Hatchery, Siler City, NC) were incubated for 1-9 days at 37 °C and 70% humidity. Embryos were staged according to Hamburger and Hamilton (1951).

Secondary heart field explants

Secondary heart field explants were isolated at HH14 as previously described (Dyer and Kirby, 2009). Hedgehog signaling was up-regulated by applying the Smoothened agonist SAG (Calbiochem, San Diego, CA). In vitro doses were based on a previous report (Chen et al., 2002), where 100 nM showed peak signaling activity in a hedgehog reporter cell line. A range of doses from 50-200 nM was tested in vitro. Some cultures were also treated with 300 ng/ml recombinant BMP2 (R&D Systems, Minneapolis, MN).

Migration of explanted secondary heart field cells was analyzed over a 16-hour period as previously described (Dyer and Kirby, 2009). A minimum of six explants per treatment were recorded, and ANOVA and pair-wise Student's t-test were used to determine statistical significance. Proliferation in the explants was assessed using BrdU (Roche, Indianapolis, IN) incorporation as previously described (Dyer and Kirby, 2009). Four to seven explants per dose were counted, and the Kruskal-Wallis test was used to determine statistical significance.

To determine what effects the pharyngeal endoderm and the outflow tract have on the secondary heart field, a series of secondary heart field explants were co-cultured with pharyngeal endoderm or outflow tract explants or 2% FBS/DMEM in the presence or absence of 100 nM SAG. After 24 h, explants were fixed in cold methanol and labeled with mitotic marker pHH3 (Millipore, Billerica, MA), myocardial marker MF20 (Developmental Studies Hybridoma Bank, Iowa City, IA), and DAPI (Molecular Probes, Invitrogen). The number of pHH3- and MF20-positive cells was counted with Acapella (Perkin Elmer, Waltham, MA), and a minimum of six explants per condition were counted. The Kruskal-Wallis test was used to determine statistical significance.

Enhanced hedgehog signaling in the secondary heart field in vivo

Eggs were windowed at HH14, and 10 μl of increasing concentrations of SAG or H_2O (control) was pipetted onto the embryo; eggs were sealed with tape and incubated. Stock SAG was dissolved and diluted in distilled H_2O.

To determine the phenotypic outcome in the four-chambered heart, control and treated embryos were harvested at HH35, photographed, fixed in methacarn overnight at 4 °C, paraffin-embedded, and labeled with MF20, smooth muscle marker SM22-α (Abcam, Cambridge, MA), and DAPI. In addition, the heart weight/body weight ratio was determined at HH35. Briefly, hearts were excised, and both heart weight and total body weight were recorded. Hearts and bodies were re-weighed after dehydration by baking, and Student's t-test was used to determine statistical significance.

Arterial pole lumenal areas were quantified in transverse histological sections of control and treated hearts using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2009). Briefly, the rounded apices of the hearts were removed to provide a consistent orientation for embedding. Sections (10 μm) were photographed at 4x magnification. The luminal areas of the aortic vestibule, the pulmonary infundibulum, and the semilunar valves were quantified. To ensure that comparable regions were measured, the aortic vestibule and pulmonary infundibulum measurements were restricted to the 80 μm below the first valve leaflet. The semilunar valve measurements were only collected in regions that had all three valve leaflets present and spanned 50-140 μm in length, and there were no significant differences between the length of this region and treatment. A minimum of three embryos per treatment was measured, and Student's t-test was used to determine statistical significance.

Reverse transcription quantitative PCR (RT-qPCR)

RNA was isolated using the Qiagen RNeasy kit (Qiagen, Valencia, CA) from freshly isolated explants or after various times in culture with various treatments. RT and qPCR for Ptc1 and Ptc2 were carried out as described in Dyer and Kirby (2009). Tested genes included known Shh targets, myocardial differentiation markers, and heart field markers. All primers are listed in Supplemental Table 1, and HPRT was used as a reference gene. Data are presented as the $\log_2$-transformed expression ratios; thus, any $\log_2$-tranformed expression ratio above 1 or less than -1 represents at least a 2-fold change in expression compared to the control.

Immunohistochemical analyses

Secondary heart field proliferation was analyzed in control and SAG-treated embryos at HH15-18 as described (Dyer and Kirby, 2009). In addition, in vivo secondary heart field migration was analyzed based on Hnk1 expression at HH16 and HH18. To analyze BMP signaling, control and SAG-treated embryos were also labeled with antibodies against pSMAD1,5,8 (Cell Signaling Technology, Danvers, MA) and BMP2. All immunohistochemistry was performed as described previously (Waldo et al., 1996), with the exception that embryos labeled with pSMAD1,5,8 were fixed in ice-cold 4% paraformaledyhde in PBS containing 0.1% Tween 20.

In situ hybridization

In situ hybridization was carried out using a probe generated from a Ptc2 plasmid generously provided by Cliff Tabin (Harvard University, Cambridge, MA). For BMP2 expression, a 337-bp fragment of *Gallus gallus* BMP2 (accession number x75914) was amplified using cDNA from HH14 and HH18 chick embryonic hearts and inserted into vector pCRII. The plasmid was linearized with KpnI, and antisense

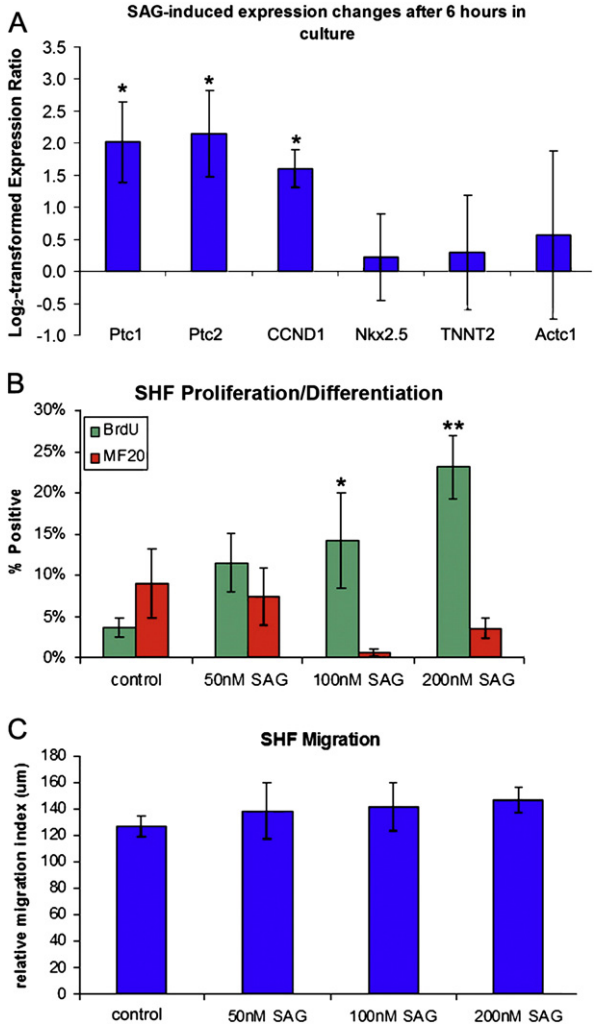

Fig. 1. Increased hedgehog signaling results in up-regulation of a cell cycle gene and proliferation within the secondary heart field. (A) qPCR was carried out on explants that were cultured for 6 hours with or without SAG. The expression ratios are compared to control explants, and all results were normalized to HPRT expression. Hedgehog receptors Ptc1 and Ptc2, as well as cell cycle gene cyclin D1 (CCND1), are significantly up-regulated after SAG treatment. Nkx2.5, troponin T (TNNT2) and cardiac α-actin (Actc1) were not significantly up-regulated by SAG treatment. All samples were run in triplicate, and the average and standard error of three independent experiments is shown. All data are $\log_2$-transformed; thus, values over 1 or less than -1 represent 2-fold differences. (B-C) Up-regulating hedgehog signaling induces proliferation in a dose-dependent manner and does not affect migration. Explants were treated with increasing doses of SAG, and proliferation, indicated by BrdU incorporation, increases up to 6-fold as compared to control embryos. No statistically significant changes were observed in myocardial differentiation as indicated by MF20 expression (B) or migration (C). A minimum of six explants per condition were analyzed; Kruskal-Wallis or ANOVA were used to determine statistical significance, followed by secondary analysis as necessary. $^{*}p<0.05$, $^{**}p<0.01$.

probe sequence was amplified using the 5'-gene specific primer and T7 as the 3' primer. The PCR product was purified with Qiagen QIAquick PCR Purification Kit and then used to generate RNA probe with the Roche DIG RNA labeling Kit (SP6/T7). The resulting DIG-labeled RNA BMP2 probe was cleaned up by LiCl/EtOH precipitation and verified by gel and dot blot.

Control and SAG-treated embryos were harvested at HH16 in DEPC-treated 4% paraformaldehyde. In situ hybridization was carried out as described by Wilkinson (1992).

Statistical analysis

Microsoft Excel and R (http://www.r-project.org) were used for statistical analysis. In all analyses, a p-value <0.05 was used as the mark of statistical significance.

Results

Explanted secondary heart field proliferates in response to elevated hedgehog signaling

To determine whether the hedgehog agonist SAG could up-regulate hedgehog signaling in the secondary heart field, secondary heart field mesoderm was explanted and cultured with or without SAG for 6 h. Ptc1 and Ptc2 are hedgehog receptors that are also downstream targets of hedgehog signaling. Expression of both receptors was significantly up-regulated in secondary heart field explants in response to SAG by qPCR analysis (Fig. 1A). In addition, Shh-induced cell cycle gene cyclin D1 (CCND1) was significantly up-regulated in response to SAG. However, cell cycle gene N-myc, cardiac

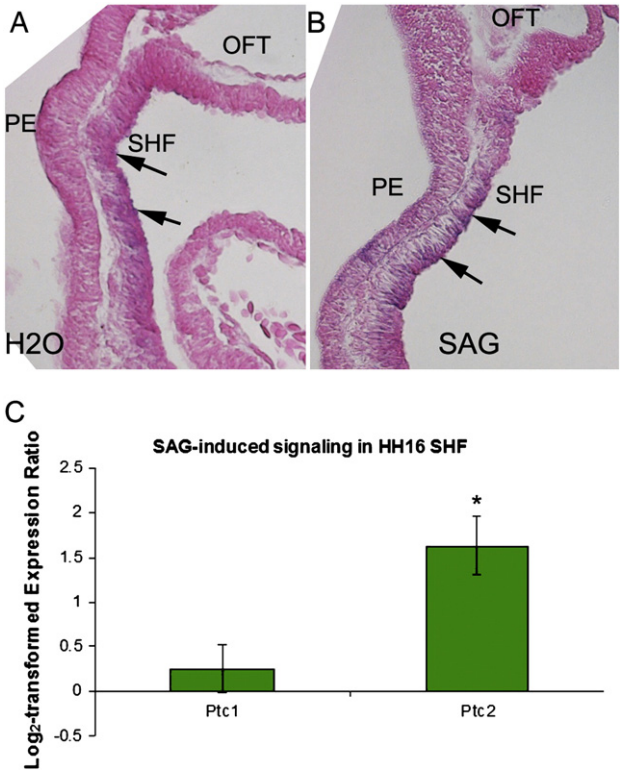

Fig. 2. Ptc2 mRNA is up-regulated in vivo in response to SAG treatment. (A, B) Ptc2 mRNA expression at HH16. Ptc2 is expressed in the secondary heart field (SHF), with strongest expression in the caudal SHF (A, arrows). After treatment with hedgehog agonist SAG at HH14, Ptc2 expression is increased (B) compared to water-treated controls (A). (C) Ptc2 up-regulation was confirmed with RT-qPCR. Embryos were treated with water or SAG at HH14, and RNA was extracted from SHF explants at HH16. Gene expression was normalized to HPRT, and data are presented as the $\log_2$-transform of the expression ratios. Thus, values greater than 1 indicate at least 2-fold changes in expression. All samples were run in triplicate, and the average and standard error of three independent experiments is shown. (PE, pharyngeal endoderm; SHF, secondary heart field; OFT, outflow tract).

Table 1
Survival and arterial pole malformations after SAG treatment at HH14.

Treatment	N	Survival (%)	Abnormal Outflow (%)
H_2O	11	10 (91%)	0
344 μM SAG	21	17 (81%)	11 (65%)
672 μM SAG	6	1 (17%)	1 (100%)
1,000 μM SAG	13	2 (15%)	1 (50%)

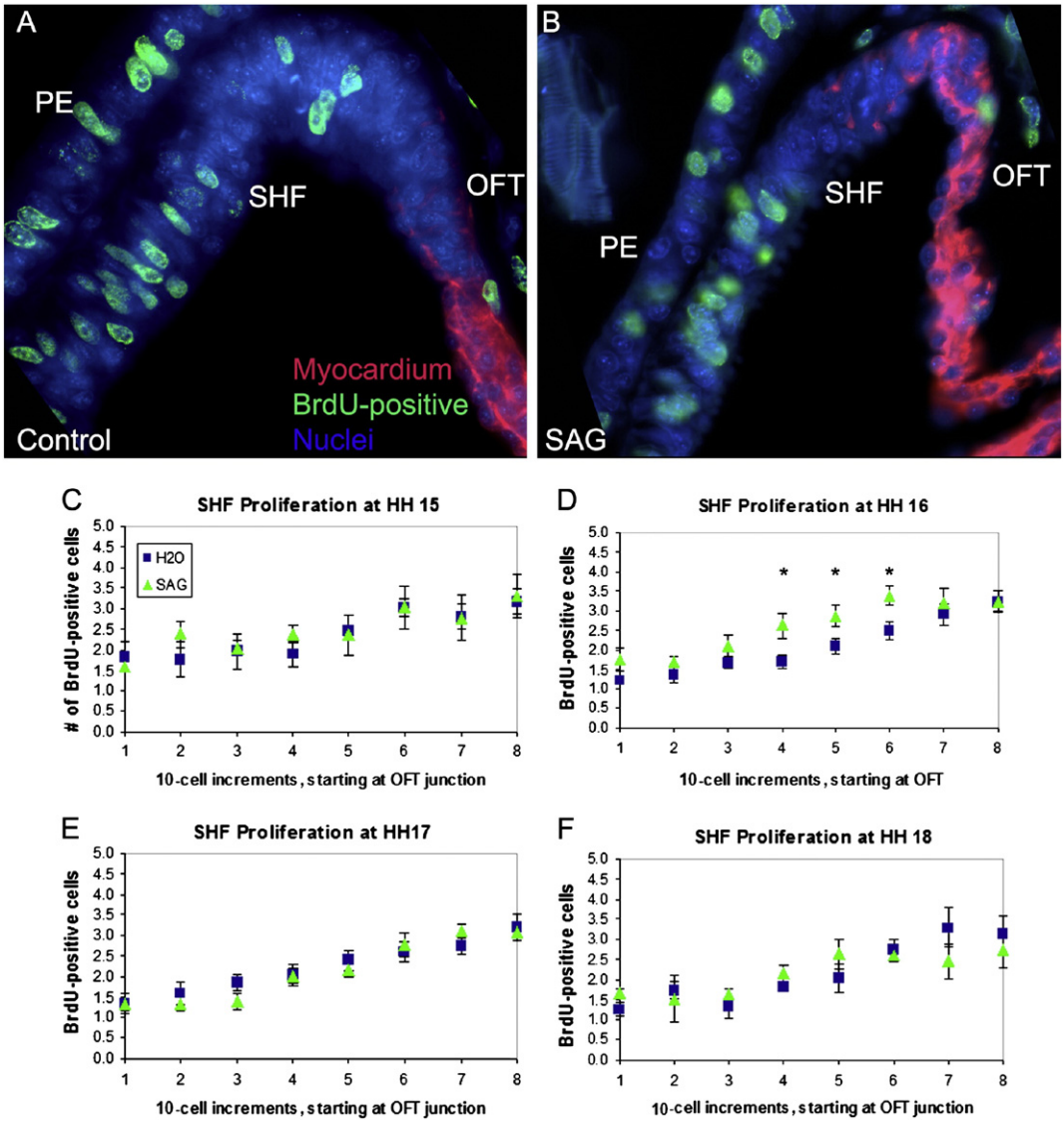

Fig. 3. Proliferation is minimally disrupted in response to increased hedgehog signaling in vivo. Proliferation is initially increased and then decreased in the caudal secondary heart field (SHF) after hedgehog up-regulation. Embryos were treated with water (Control) or SAG at HH14 and BrdU-treated at the stages indicated. (A,B) Sections were labeled with MF20 (myocardium, red), anti-BrdU (proliferating cells, green), and DAPI (nuclei, blue). Proliferating cells were counted in 10-cell increments, starting at the junction of the outflow tract (OFT) and moving caudally. No changes were observed at HH15 (C). By HH16, SAG-treated embryos showed elevated BrdU incorporation in the caudal SHF as compared to control embryos (D). This increase was no longer present by HH17 (E), and no changes were observed at HH18 (F). PE, pharyngeal endoderm. *$p<0.05$, Student's t-test.

transcription factors Nkx2.5 and Mef2c, and cardiac differentiation markers myosin heavy chain (Myh6), troponin T (TNNT2), and cardiac alpha-actin (Actc1), were not affected by SAG treatment (Fig. 1A and data not shown).

Because down-regulating hedgehog signaling results in decreased proliferation (Dyer and Kirby, 2009) and up-regulating hedgehog signaling induced cyclin D1, we examined whether proliferation was increased by SAG. Secondary heart field explants excised at HH14 were cultured with 50-200 nM SAG. Proliferation, as measured by BrdU incorporation, increased significantly in a dose-dependent manner, from 3% of cells in control explants to 22% in response to increasing doses of SAG ($p<0.01$) (Fig. 1B).

Neither myocardial differentiation (Fig. 1B, indicated by MF20 expression) nor the migration index (the area over which the explant spread normalized by the perimeter of the explant at the end of the experiment, Fig. 1C) was significantly affected by increased hedgehog signaling. Together, these data support the hypothesis that hedgehog signaling promotes proliferation in the secondary heart field.

Secondary heart field in vivo shows a weak proliferative response to elevated hedgehog signaling

To determine whether SAG up-regulates hedgehog signaling in vivo, embryos were treated at HH13-14 with SAG. The only previous report of SAG use in vivo (2.6 mM) was in pregnant mice with subsequent examination of upregulated Ptc1-lacZ in embryos (Frank-Kamenetsky et al., 2002). Because we were applying the drug directly to chick embryos, lower doses ranging from 344 to 1,000 μM were tested (Table 1 and data not shown). Doses in excess of 344 μM (0.2 mg/ml) were lethal within 12 hours; thus, 344 μM was chosen for further analysis.

At HH14, embryos were treated with either 344 μM SAG or H_2O as a control and collected at HH16 for in situ hybridization. Ptc2 expression was up-regulated in the secondary heart field of SAG-treated embryos (Fig. 2B) compared to control embryos (Fig. 2A) indicating that the secondary heart field did experience elevated hedgehog signaling. In addition, Ptc1 and Ptc2 expression was

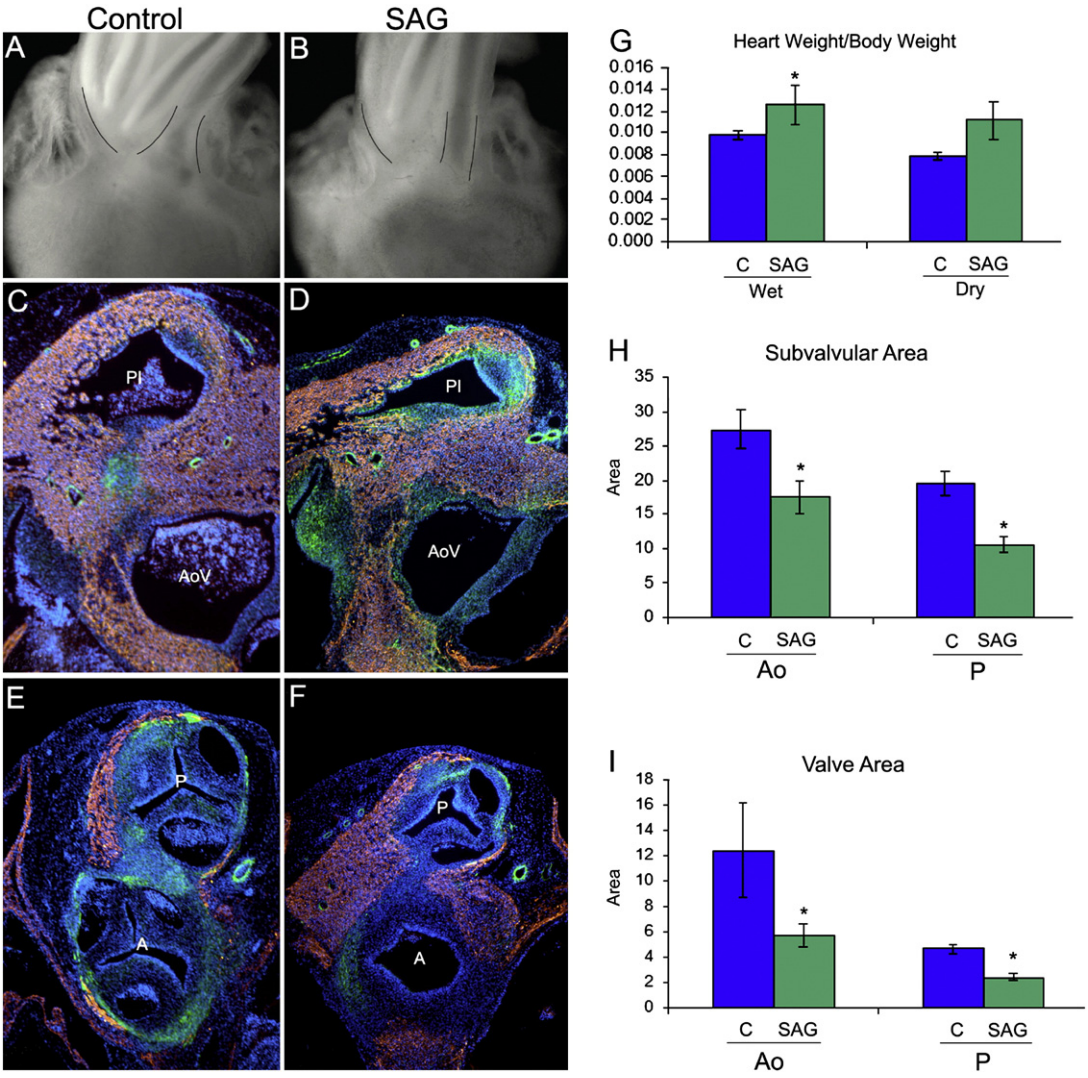

Fig. 4. Up-regulated hedgehog signaling causes arterial pole stenosis. Embryos were treated with water (Control, A, C, E) or SAG (B, D, F) at HH14 and developed to HH35. Treated embryos appeared to have a narrower arterial pole (compare B and A), despite having a higher heart weight/body weight ratio (G). Stenosis was confirmed in histological sections. Sections were labeled with MF20 (myocardium, red), α-SM22 (smooth muscle, green), and DAPI (nuclei, blue). Below the outflow tract valves, both the pulmonary infundibulum (PI) and aortic vestibule (AoV) were significantly smaller in SAG-treated embryos (D) than in control embryos (C). At the level of the outflow tract valves, both the pulmonary artery (P) and aorta (Ao) were significantly smaller in treated embryos (F) as compared to control embryos (E). (H,I) Area measurement is 10^4 um^2. N = 4 controls and 9 SAG-treated embryos. Student's t-test was used to calculate statistical significance; *$p<0.05$.

quantified in secondary heart fields dissected from comparably treated embryos using RT-qPCR. Ptc2 expression was significantly up-regulated at HH16 ($p<0.05$, Fig. 2C) in response to SAG treatment, while Ptc1 was unchanged at this stage.

We examined proliferation in the secondary heart field after embryos were treated with SAG or H_2O at HH14. Embryos were labeled with BrdU and collected at HH15-18. The embryos were sectioned sagittally to allow the cells in the secondary heart field to be counted. This mesoderm is a continuous layer extending from the splanchnic mesoderm into the outflow myocardium. The more caudal region of the secondary heart field mesoderm is pseudostratified, and the cells reorganize to a stratified cell layer as they turn into the outflow tract and begin to differentiate as myocardium (Fig. 3A). This organization was subtly disrupted in SAG-treated embryos.

Normally, proliferation is greater in the caudal secondary heart field, adjacent to the Shh-producing pharyngeal endoderm, compared to secondary heart field cells that are closer to the outflow tract (Fig. 3A and Dyer and Kirby, 2009). After SAG treatment, the highly proliferative region appeared largely comparable to control embryos (Fig. 3B).

To quantify these observations, the number of proliferating cells was counted in 10-cell increments, starting at the junction of the secondary heart field with the outflow tract. At HH15, no changes were observed between control and treated embryos (Fig. 3C). However, proliferation was increased in a discrete region of secondary heart field by HH16 after treatment with SAG, as compared to control embryos (Fig. 3C). Interestingly, this increased level of proliferation was limited to this one region at a single time point. SAG-treated embryos showed normal proliferation compared to their control counterparts at HH17 and 18 (Fig. 3C). This unexpected weak response to elevated hedgehog signaling in vivo suggests that hedgehog signaling is counter-balanced by other factors in the regulation of secondary heart field proliferation.

Up-regulated hedgehog signaling results in aortic and pulmonary outflow stenosis

Previous studies have shown that changes in secondary heart field proliferation result in arterial pole defects (Waldo et al., 2005; Xu et al., 2004; Hutson et al., 2006). Even though up-regulated hedgehog

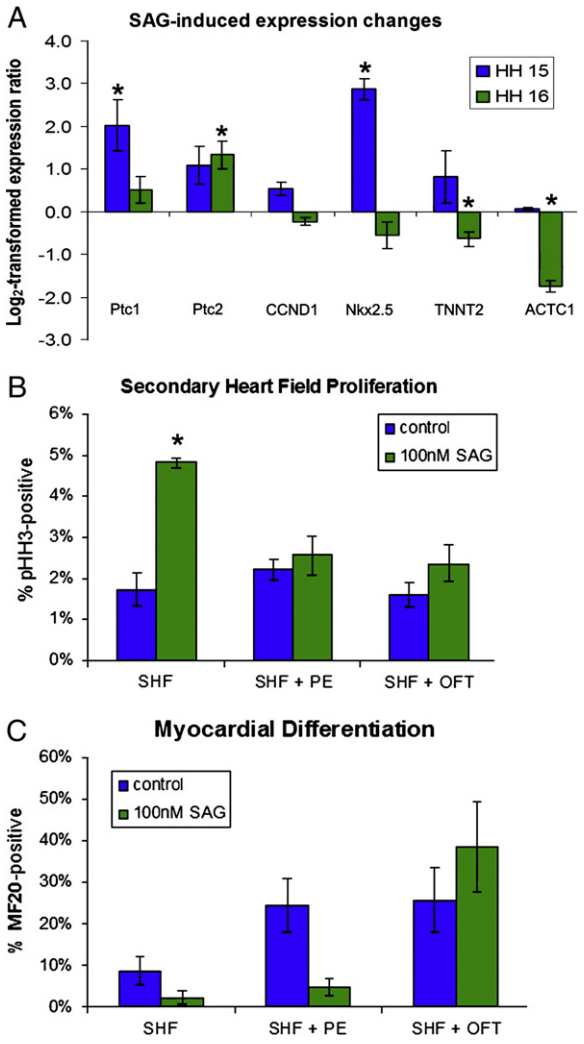

Fig. 5. Other tissues modulate Shh signaling. (A) Embryos were treated with H_2O (control) or SAG (SAG) at HH14. At HH15, secondary heart field mesoderm was excised for RT-qPCR. Results are presented as the log_2-transformed expression ratio compared to control HH15 secondary heart fields, and all values greater than 1 represent at least a 2-fold increase. Unlike the in vitro results after 6 hours of isolated culture, explants from SAG-treated HH15 embryos showed significant increases in Ptc1 and heart field marker Nkx2.5 compared to explants from control embryos while explants from HH16 embryos showed an increase in Ptc2 and significant decreases in myocardial markers troponin T (TNNT2) and alpha cardiac actin (ACTC1). All genes were normalized to HPRT expression. (B, C) To determine how in vivo environment affects the secondary heart field, explants were co-cultured with pharyngeal endoderm or outflow tract in the presence or absence of 100 nM SAG. When secondary heart field mesoderm was cultured by itself, SAG induced an up-regulation in proliferation (B) but not myocardial differentiation (C). If explants were cultured with pharyngeal endoderm (PE) or outflow tract (OFT), SAG could not up-regulate proliferation (B). Explants cultured with pharyngeal endoderm showed increased myocardial differentiation, which was inhibited by SAG, and explants cultured with OFT showed increased myocardial differentiation regardless of whether SAG was present. *$p<0.05$.

signaling had a mild effect on secondary heart field proliferation in vivo, we examined whether the embryos might still have arterial pole defects. Embryos were treated with 344 μM SAG or H_2O at HH13-14 and were allowed to develop until HH35. Of the SAG-treated embryos, approximately 80% ($n=17$) survived to HH35. Analysis of the whole mounts showed that the brachiocephalic arteries, which branch off the aorta, were straighter in a significant percentage of the treated embryos (47% vs. 0% in controls, $p=0.01$, Fig. 4B). In addition, the SAG-exposed hearts appeared to have narrower pulmonary and systemic outlets compared to control embryos (compare Figs. 4B and A, $n=10$). However, the wet heart weight/body weight ratio was significantly higher in SAG-treated embryos than in control embryos, suggesting that the SAG-treated hearts were actually larger than their control counterparts (Fig. 4C). The increased ratio seen in SAG-treated embryos was not caused by edema, as indicated by the fact that the heart weight/body weight ratio tended towards being higher in the SAG-treated embryos as compared to control embryos when hearts and embryos were dehydrated. This increase in heart size potentially represents a hypertrophic response to the narrowed outlets.

To quantify the smaller arterial outlets, hearts were transversely sectioned and labeled with MF20 and smooth muscle marker SM22. Because the secondary heart field contributes both myocardium below the semilunar valves and smooth muscle distal to the valves, we focused on the size of the pulmonary infundibulum, the aortic vestibule, and the outlets at the level of the valve leaflets. In SAG-treated embryos, the aortic vestibule was reduced in size by 35% ($p<0.05$), and the pulmonary infundibulum was reduced in size by 45% ($p<0.05$) compared to the control embryos (Figs. 4C, D, and H). At the level of the valve leaflets, both SAG-treated outlets were reduced by approximately 50% ($p<0.05$) as compared to control embryos (Figs. 4E, F, and I).

In vivo context moderates secondary heart field behavior

Based on the in vitro proliferation studies, the in vivo proliferation response and the resulting phenotypes were unexpected. To understand the response of the secondary heart field more clearly, we subjected dissected secondary heart fields from HH15 (6 h post-treatment) and HH16 (12 h post-treatment) embryos that had been treated with H_2O or SAG at HH14 to qPCR. Ptc1 was significantly up-regulated at HH15 in response to SAG treatment, while Ptc2 was up-regulated at HH16 (Fig. 5A), suggesting that the two genes have different kinetic responses to hedgehog signaling. While the cell cycle gene CCND1 was increased in culture (Fig. 2B), it was not increased in vivo at either HH15 or 16 (Fig. 5). However, unlike the in vitro experiment, the cardiac transcription factors Nkx2.5 and Mef2c were significantly up-regulated after the in vivo SAG treatment.

These data combined with the proliferation data suggested that the in vivo proliferative response may be mitigated by signaling from adjacent tissues. In order to identify adjacent tissues that have the potential to dampen the in vivo response, secondary heart field explants were co-cultured with pharyngeal endoderm or outflow tract explants in the presence or absence of SAG. Culturing the secondary heart field with SAG again induced a proliferative response, which was blocked by co-culturing with pharyngeal endoderm or outflow tract (Fig. 5B). Because increasing doses of SAG beyond a certain dose result in reduced hedgehog signaling (Chen et al., 2002), the lack of proliferation induced by SAG and endoderm, the endogenous Shh source, is not surprising. Interestingly, outflow tract co-cultures tended to promote myocardial differentiation in the secondary heart field explants even in the presence of SAG (Fig. 5C). These data show that the outflow tract in particular alters proliferation and possibly differentiation in secondary heart field explants in the presence of SAG.

BMP2 is secreted by the outflow tract and has been shown to inhibit Shh-induced proliferation in cerebellar granule cells (Alvarez-Rodriguez et al., 2007), and both Nkx2.5 and Mef2c are downstream targets of BMP signaling (Monzen et al., 1999; Prall et al., 2007; Qi et al., 2007; Schultheiss et al., 1997; Shi et al., 2000). In mouse, BMP4 is expressed throughout the outflow tract and into the splanchnic mesoderm (Ilagan et al., 2006). In chick, BMP2 mRNA is expressed in the outflow tract and the contiguous splanchnic mesoderm at HH14; however, this expression does not extend throughout the secondary heart field (Waldo et al., 2001). We examined BMP2 protein expression in control embryos at HH14-16. Similar to the mRNA expression studies, BMP2 was expressed in the myocardium, the

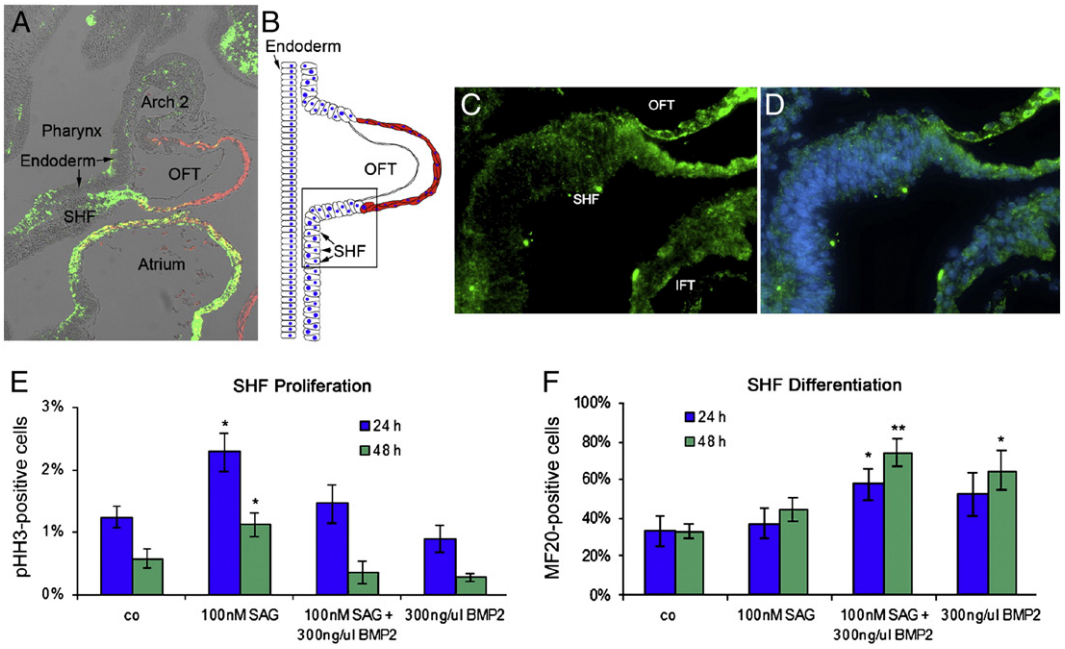

Fig. 6. BMP signaling modulates hedgehog signaling. (A) Mid-sagittal section through the foregut showing the junction of the outflow myocardium (red) with the ventral pharyngeal splanchnic mesoderm. The secondary heart field (SHF) is located caudal to the outflow tract (OFT). Cells can be seen migrating from the SHF (green) to the outflow tract. (B) Schematic showing the location of the SHF with respect to the OFT and pharynx. Box illustrates area shown in C and D. (C,D) BMP2 immunohistochemistry indicates that BMP2 is expressed by the myocardium and SHF near the myocardial OFT. (E) SAG up-regulates proliferation in explanted SHF mesoderm while BMP2 downregulates proliferation in explants treated with SAG. (F) SAG has no effect on myocardial differentiation while BMP2 promotes differentiation regardless of SAG treatment. *$p<0.05$ and **$p<0.01$ vs. control (co). IFT, inflow tract.

endocardium, and the cranial-most secondary heart field at HH16 (Figs. 6A-D).

To determine if BMP2 could dampen the SAG-induced proliferation, secondary heart field explants were treated simultaneously with SAG and BMP2. SAG treatment alone significantly increased proliferation, as described above, and BMP2 treatment alone resulted in a slight but non-significant reduction in proliferation after either 24 or 48 h of culture (Fig. 6E). Instead, BMP2 treatment induced myocardial differentiation, as reported previously (Waldo et al., 2001; Hutson et al., 2010). Explants that were treated with both SAG and BMP2 showed the same proliferative response as controls, suggesting that BMP counteracts hedgehog-induced proliferation. In addition, SAG treatment alone did not promote myocardial differentiation, whereas the presence of SAG and BMP2 significantly increased myocardial differentiation (Fig. 6F). Together, these data indicate that, in the presence of up-regulated hedgehog and BMP signaling, the secondary heart field responds primarily to the BMP signal.

To evaluate whether BMP2 expression is upregulated in the distal outflow tract by in vivo SAG treatment at HH14, we performed in situ hybridization and qPCR. By HH16 BMP2 was dramatically elevated in the distal outflow tract and the adjacent secondary heart field of SAG-treated embryos (Figs. 7E-H) compared to control embryos (Figs. 7A-D). qPCR confirmed that BMP2 was elevated 2.49 times in HH14/15 outflow tracts exposed to SAG compared with water-treated controls (data not shown).

To determine whether SAG-induced proliferation in the secondary heart field could be dampened in vivo by BMP signaling, pSMAD1,5,8, which is indicative of BMP activity, was analyzed immunohistochemically (Figs. 7I-N). At HH15, pSMAD was expressed in the outflow tract myocardium and endocardium of the control embryos; pSMAD expression was elevated in the secondary heart field adjacent to the outflow tract, where proliferation is normally decreased, as compared to the more highly proliferative caudal secondary heart field. After SAG treatment at HH14, pSMAD expression at HH15 was greatly reduced in the outflow tract and the secondary heart field compared to the control embryo, indicating that BMP signaling was dampened in these cells. However, at HH16 pSMAD expression was elevated in the secondary heart field of SAG-treated embryos compared with control embryos. Thus, after SAG treatment at HH13-14, BMP mRNA expression was elevated at HH14-16, and BMP signaling increased by HH16. This timing coincides with the later stages of myocardial addition by the secondary heart field.

Further, increased BMP signaling in the secondary heart field was accompanied by the appearance of prematurely or ectopically differentiating myocardium in the secondary heart field of most SAG-treated embryos at HH16-18 (Table 2 and data not shown). While some control embryos also had some ectopic myocardial cells in the secondary heart field, the SAG-treated embryos had significantly more ectopic MF20-positive cells within the secondary heart field (2.8 ± 1.2 in HH16 controls vs. 9.6 ± 5.1 in HH16 SAG-treated embryos; $p<0.05$). The up-regulation of pSMAD expression in the secondary heart field coupled with the ectopic myocardial differentiation suggests that BMP signaling via pSMAD dampens the proliferative response to SAG and initiates premature myocardial differentiation in the secondary heart field.

To determine whether proliferation could be upregulated by SAG in vivo by suppressing BMP signaling, we co-treated embryos with SAG and the BMP inhibitor dorsomorphin. Unfortunately, these embryos underwent early embryonic lethality (data not shown) prior to a time when we could assess whether inhibiting BMP signaling allowed SAG to affect proliferation of the secondary heart field progenitors.

Discussion

Our data show that up-regulated hedgehog signaling causes increased proliferation of secondary heart field progenitors if the signal is unopposed by other signaling pathways. However, the complex signaling feedback network that is present between various tissues in vivo is not observed in the much simpler situation that can be created in vitro. Thus, our in vivo results suggest that up-regulation of hedgehog signaling leads to elevated BMP signaling by the outflow tract and that this elevation in turn reduces the proliferative effect of hedgehog signaling on the secondary heart field progenitors. First, elevated hedgehog signaling expands the proliferative niche within

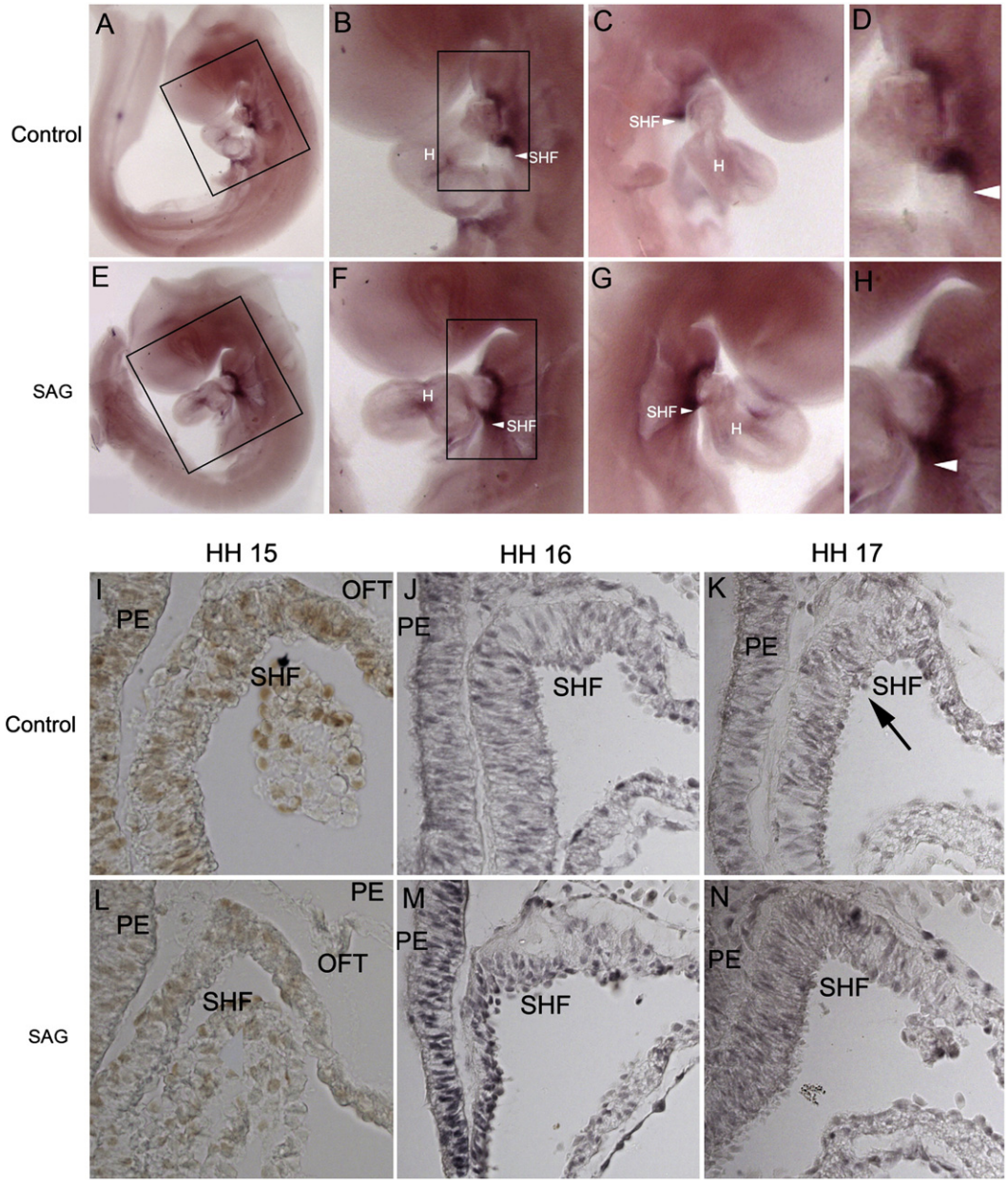

Fig. 7. BMP signaling is elevated by SAG treatment. (A-H) In situ hybridization of BMP2 at HH16 in controls treated with water (A-D) and after SAG treatment (E-H) at HH14 shows elevated expression around the junction of the distal outflow tract (OFT) with the pharynx. (I-N) BMP signaling in the secondary heart field (SHF), as indicated by pSMAD1,5,8 expression at HH15, 16 and 17. Embryos were treated with water (Control, I, J, K) or SAG (L, M, N) at HH14, and pSMAD (brown/black) was analyzed at HH15-17. Control embryos show robust pSMAD expression in the OFT myocardium and decreased expression throughout the SHF at HH15. After SAG treatment, HH15 embryos show decreased pSMAD expression. pSMAD appears upregulated in the SHF and endoderm at HH16. By HH17, pSMAD expression appears the same in both control and SAG-treated embryos. IFT, inflow tract.

the secondary heart field, thus reducing the population of cells that can contribute to the myocardium at that stage. Then, elevated BMP signaling represses the hedgehog-induced increase in proliferation and leads to premature differentiation of the secondary heart field-derived myocardium. The end result to heart development is stenosis of the systemic and pulmonary outlets possibly caused by abnormal patterning of the outflow tract. This defect is in stark contrast with the consequences of down-regulating hedgehog signaling, which results in arterial pole defects limited to the pulmonary outlet including pulmonary atresia/stenosis (Chiang et al., 1996; Dyer and Kirby, 2009; Goddeeris et al., 2007; Washington Smoak et al., 2005). These results show how Shh works in concert with other signaling pathways to induce both proliferation and differentiation. Among the pathways that are known to interact with Shh, we examined BMP because this signaling pathway is important throughout heart development, from the initial induction of the cardiogenic mesoderm (Schultheiss et al., 1997) to outflow tract cushion formation (Kim et al., 2001). In mouse, BMP4 is expressed by the outflow tract myocardium, where it

Table 2
Number of embryos with ectopic myocardium in the secondary heart field after in controls (C) and after SAG treatment at HH14.

Stage	C	SAG
16	6/15	5/7*
17	0/9	5/10*
18	1/5	3/5*

*($p<0.05$, Fisher exact test).

promotes myocardial differentiation by inducing Nkx2.5, Gata4, and ventricular myosin heavy chain (Monzen et al., 1999; Schultheiss et al., 1997; Shi et al., 2000). Because BMP4 is involved in so many aspects of development, the BMP4-null mouse dies by E6.5 (Winnier et al., 1995). Conditionally knocking out BMP4 under the Nkx2.5 promoter yields mice with an unseptated outflow tract (Liu et al., 2004). The BMP2-null mouse is also embryonic lethal, with mice dying at E8.5 due to cardiac developmental delays (Zhang and Evans, 1996) and impaired neural crest migration (Correia et al., 2007). In chick, both BMP4 and BMP2 are present in the outflow tract (Somi et al., 2004). Secondary heart field explants treated with BMP2 induce myocardial differentiation and decrease proliferation (Waldo et al., 2001; Hutson et al., 2010).

Interactions between the Shh and BMP signaling pathways have been previously observed during development. Cerebellar granular neuron precursors (CGNPs) that are co-cultured with both Shh and BMP2 show decreased proliferation in comparison with CGNPs that are treated with Shh alone. Specifically, BMP2 down-regulates Shh target n-myc, and this decrease is seen before changes are seen in other Shh targets, such as Gli1 (Alvarez-Rodriguez et al., 2007). This ability to inhibit proliferation is independent of BMP2's role in promoting differentiation. Furthermore, application of BMP2 to either chick feather germs or limb buds down-regulates Shh expression, and application of the BMP2 inhibitor noggin up-regulates Shh expression (Bastida et al., 2009; Harris et al., 2002). Shh can also affect the BMP pathway. In the limb mesenchyme, BMP2 signaling is up-regulated in response to exogenous Shh (Yang et al., 1997). Shh can also down-regulate BMP4 in the limb mesenchyme, and this process is thought to occur through Tbx3 (Tumpel et al., 2002). Recent evidence also suggests that Shh induces BMP repressor Gremlin in the developing limb bud (Benazet et al., 2009).

Based on these studies, we questioned whether BMP signaling was altered after SAG treatment and how the secondary heart field would behave in response to alterations in both pathways. In the presence of SAG alone, secondary heart field explants proliferated more than control explants, as expected. When treated with both SAG and BMP2, explants behaved as BMP2-treated explants; proliferation was slightly but not significantly decreased as compared to controls, and myocardial differentiation increased. Other studies in our lab have shown a significant depression of secondary heart field proliferation by BMP2 (Hutson et al., 2010), and it is unclear why this was not the case in this study. The secondary heart field serves as a progenitor pool that must generate sufficient myocardium and smooth muscle to form the arterial pole. The BMP2 differentiation signal balances the Shh-induced proliferation, ensuring the gradual addition of secondary heart field-derived myocardial cells to the outflow tract thus ensuring normal arterial pole development.

We have shown that BMP2 and Shh exert opposing effects to maintain a specific region of proliferation within the secondary heart field and coordinate proliferation with differentiation and outflow patterning. When hedgehog signaling is up-regulated, BMP is up-regulated and inhibits hedgehog-induced proliferation. The BMP overrides other signaling that maintains the progenitor pool and induces ectopic myocardium.

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