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Cross talk between hedgehog and bone morphogenetic proteins occurs during cardiomyogenesis in P19 cells

Peter Junior Gianakopoulos · Ilona S. Skerjanc

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Abstract Hedgehog (Hh) signaling plays a role in heart morphogenesis and can initiate cardiomyogenesis in P19 cells. To determine if Hh signaling is essential for P19 cell cardiomyogenesis, we determined which Hh factors are expressed and the effect of Hh signal transduction inhibitors. Here, we find that the Hh gene family and their downstream mediators are expressed during cardiomyogenesis but an active Hh signaling pathway is not essential. However, loss of Hh signaling resulted in a delay of BMP-4, GATA-4, Gli2, and Meox1 expression during cardiomyogenesis. By using Noggin-overexpressing P19 cells, we determined that Hh signaling was not active during Noggin-mediated inhibition of cardiomyogenesis. Thus, there is cross talk between the Hh and BMP signaling pathways and the Hh pathway appears important for timely cardiomyogenesis.

Keywords BMP · Embryonal carcinoma · Differentiation · Gene expression · Cardiac muscle · Shh

Introduction

Heart formation requires the specification of mesoderm cells into the cardiac muscle lineage, differentiation into cardiomyocytes, and morphogenesis into the chambered heart. Signaling molecules such as sonic hedgehog (Shh) and bone morphogenetic proteins (BMPs) are involved in the commitment of cells to the cardiac muscle lineage and proper heart morphogenesis (van Wijk et al. 2007). Cardiac-destined cells express a subset of transcription factors including Nkx2–5, GATA-4, and MEF2C that in combination with each other and cofactors activate the expression of muscle specific genes (Zheng et al. 2003).

In mouse and humans, the hedgehog (Hh) gene family comprises three members termed sonic hedgehog (Shh), Indian hedgehog (Ihh) and desert hedgehog (Dhh). The zinc-finger gene family termed Gli, comprises three members (*Gli1–3*), which are transcription factors that directly regulate the genetic targets of Hh signaling. The receptor for Hh is Patched1 (Ptch1), a 12 membrane-spanning domain protein. In the absence of Shh/Ptch1 receptor complexes, Gli2/3 may function as transcriptional repressors. When Shh binds to Ptch1, this catalytically relieves the repression of another membrane protein termed Smoothened. Smoothened then positively regulates the protein activity of Gli2/3 leading to the activation of Shh-target genes, including Gli1 and Ptch1 (Ingham and McMahon 2001).

Cofactors of Gli activity include Zic1–5, which are a family of five related zinc-finger transcription factors. Zic1/2/3 and Gli1/2/3 are expressed throughout the embryonic mesoderm at E7.0 providing evidence for their possible interactions (Merzdorf 2007).

Shh also plays a role in heart morphogenesis. Mice that lack Shh or its receptor display heart defects and a delay of Nkx2–5 expression (Zhang et al. 2001). Further, Ptch1−/− mice show increased Nkx2.5 expression in the cardiac crescent (Zhang et al. 2001). Moreover, Shh or Gli2 expression in P19 cells was sufficient to induce cardiomyogenesis (Gianakopoulos and Skerjanc 2005). Recent genetic

P. J. Gianakopoulos · I. S. Skerjanc (✉)
Department of Biochemistry, Microbiology, and Immunology,
University of Ottawa,
451 Smyth Road,
Ottawa, Ontario, Canada, K1H 8M5
e-mail: iskerjan@uottawa.ca

P. J. Gianakopoulos
Department of Biochemistry, Medical Sciences Building,
University of Western Ontario,
London, Ontario, Canada, N6A 5C1

inducible fate mapping experiments showed that murine myocardial progenitors directly respond to Hh signaling as early as E7 and Hh signaling overall plays an essential early role in regulating cardiomyocyte number in zebrafish (Thomas et al. 2008).

Meox1 is a homeobox transcription factor that is expressed in the primitive streak and lateral plate mesoderm at E7.5–8 (Candia et al. 1992). Meox1 is a regulator of Hh signaling in vitro as it activates Gli1/2 during cardiomyogenesis (Petropoulos et al. 2004; Gianakopoulos and Skerjanc 2005). Meox factors are thought to mediate responsiveness to Hh signals in the ventral somite as Meox1−/−;Meox2−/− compound mutants at E16.5 phenocopy Shh−/− mice in sclerotomal defects, although no heart defects were detected (Mankoo et al. 2003).

BMPs are members of the transforming growth factor beta superfamily (van Wijk et al. 2007). In chick, BMP-2 and BMP-4 are expressed from tissues adjacent to precardiac mesoderm and can induce the expression of Nkx2–5 and GATA-4. Conversely, inhibition of BMP signaling by the antagonist Noggin prevented cardiac differentiation in several systems (Schultheiss et al. 1997; Shi et al. 2000; Jamali et al. 2001).

P19 are murine embryonal carcinoma cells that can differentiate into cardiomyocytes with embryoid body formation in the presence of DMSO (Skerjanc 1999). BrachyuryT is a marker of mesoderm that is transiently detected during initial aggregation steps (Vidricaire et al. 1994). To investigate the role of the Hh signaling pathway in P19 cell cardiomyogenesis, we examined the temporal expression patterns of all three Hh family gene members and their major downstream signaling components. Furthermore, we examined the ability of cardiomyogenesis to proceed in the absence of Hh or BMP signaling. Here, we show evidence for cross talk between the BMP and Hh signaling pathways, although Hh signaling was not found to be essential for cardiomyogenesis in P19 cells.

Materials and Methods

Cell culture and transfections. P19 cells were cultured as described previously (Wilton and Skerjanc 1999) in α-minimum essential media supplemented with 5% calf and 5% fetal calf serum. P19(Nog) and P19(Shh) cells were created as described previously (Jamali et al. 2001; Gianakopoulos and Skerjanc 2005). P19, P19 (Control), and P19 (Nog) cells were aggregated in the presence of 0.8% DMSO and/or 5–10 μM cyclopamine (Toronto Research Chemicals, North York, Canada, C998400) or 5 μM KAAD-cyclopamine (Toronto Research Chemicals, K171000) as described previously (Skerjanc et al. 1998). Cells were fixed for immunofluorescence on day 6 and/or harvested for RNA on the days indicated. Cyclopamine and KAAD-cyclopamine were dissolved in DMSO prior to addition as per manufacturer's instructions. KAAD-cyclopamine is a derivative of cyclopamine with ten to 20 times the potency (Taipale et al. 2000). Ten-micromolar cyclopamine and 5-μM KAAD-cyclopamine were the concentrations determined by our laboratory to be the upper limit that P19 cells could be viably cultured in.

Immunofluorescence. Myosin heavy chain (MyHC) and nuclei were detected as described previously (Ridgeway et al. 2000). Immunofluorescence was visualized with an Olympus BX50 microscope.

Northern blot, Southern blot, and reverse transcription polymerase chain reaction. Protocols for Northern blot, Southern blot, and reverse transcription polymerase chain reaction (RT-PCR); probes for Shh, Gli1/2/3, BrachyuryT, Ptch1, BMP-4, Zic1/3, Meox1, Gata-4, Nkx2.5, Tubulin, 18S, and Mef2c; primers and annealing conditions for Nkx2.5, Shh, Ihh, Dhh, and tubulin were as previously described (Takabatake et al. 1997; Petropoulos et al. 2004; Gianakopoulos and Skerjanc 2005). The probe for Dhh consisted of an approximately 1,200-bp XhoI–NdeI fragment. The probe for Ihh consisted of an approximately 1,500-bp EcoRI fragment. Quantitation via densitometry was performed with NIH Image J software. Standard error and averages were reported as indicated.

Western blot analysis. P19 cells were aggregated for 4 days in the presence of 0.8% DMSO as indicated and plated onto 100-mm tissue culture dishes on day 4. P19 (Control) and P19 (Shh) cultured in monolayer were used as negative and positive controls, respectively. Extracellular media and total protein extract were harvested on day 6 using modified radio-immunoprecipitation buffer (Upstate). Thirty micrograms of protein of each sample was run on a 10% sodium dodecyl sulfate (SDS)-reducing polyacrylamide gel in 4× SDS-reducing buffer. Proteins were transferred onto immunoblot polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA). Shh protein was detected using anti-Shh antibodies (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and visualized with horseradish peroxidase-conjugated secondary antibodies (Chemicon, Temecula, CA).

Results

The temporal expression pattern of the hedgehog gene family and its mediators in differentiating P19 cells. We have shown that overexpressing Shh in P19 embryonal carcinoma cells induced cardiomyogenesis (Gianakopoulos and Skerjanc 2005). To determine which *Hh* genes are

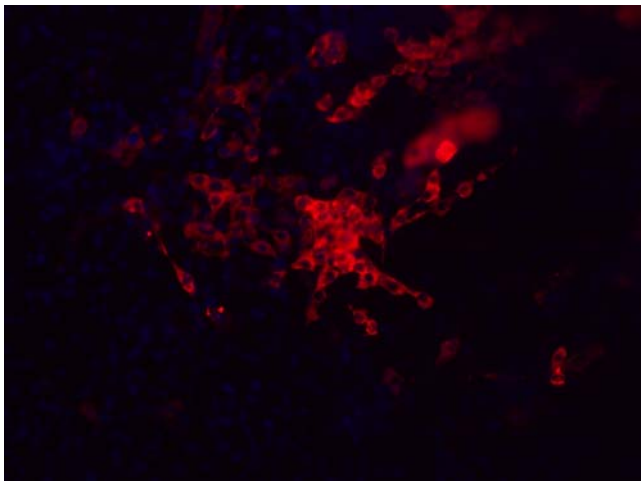


Figure 1. DMSO-induced differentiation of P19 cells yielded cardiomyocytes. P19 cells were differentiated in the presence of 0.8% DMSO. On day 6 of differentiation, cells were fixed and labeled with Hoescht dye (*blue*) to detect nuclei and anti-MyHC antibody (*red*). Immunofluorescence from both emitters was merged into one image. Magnification ×200.

activated during P19 cell differentiation, RNA levels were examined by semiquantitative RT-PCR. P19 cells were aggregated in the presence of 0.8% dimethylsulfoxide (DMSO) and RNA was harvested on each day of a 7-d time course in triplicate. On day 6, cells were fixed and stained with Hoechst dye and anti-MyHC antibodies. P19 cells were differentiated into robust levels of cardiac muscle, indicated by immunofluorescence with an anti-MyHC antibody (Fig. 1) and by enhanced levels of MEF2C and Nkx2–5 transcripts (Fig. 2*IA*, *B*). All three Hh genes were transcribed during cardiomyogenesis. Dhh and Ihh were expressed early during differentiation and all three Hh are expressed from days 4–6 (Fig. 2*IC–E*).

Northern blot analysis was performed to examine Gli1/2/3, Zic1/3, and Ptch1 gene expression, genes involved in the primary and secondary Hh signal transduction pathway. Gli3, Zic1, and Ptch1 are detectable throughout differentiation and showed no substantial changes (Fig. 2*IIJ*, *K*, *M*, respectively). In contrast, Gli1 and Gli2 are also ubiquitously expressed but showed an upregulation of transcript levels on days 4–6 (Fig. 2*IIH*, *I*, respectively). Zic3 showed an overall down-regulation of its mRNA levels from days 0 to 6 (Fig. 2*IIL*). The temporal expression pattern of GATA-4, which is involved in cardiac specification, increased during the time course with the highest expression on days 4–6 (Fig. 2*IIG*), in agreement with previous studies (Jamali et al. 2001; Gianakopoulos and Skerjanc 2005). Thus, all three *Hh* genes and their downstream signaling components are present during DMSO-induced cardiomyogenesis of P19 cells.

Hedgehog signaling is not essential for cardiomyogenesis in P19 cells. Since Shh is sufficient to induce cardiomyogenesis (Gianakopoulos and Skerjanc 2005), we examined whether Hh signaling is essential. P19 cells were aggregated in the presence of 0.8% DMSO (P19(DMSO)) alone or with 5–10 μM of cyclopamine/KAAD-cyclopamine (P19

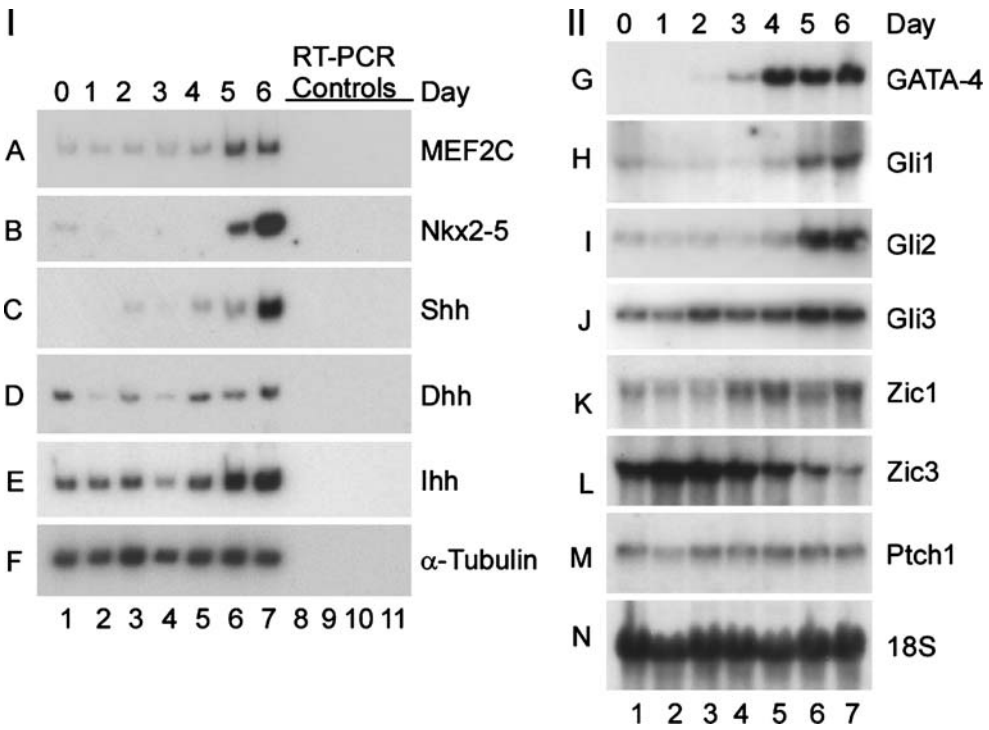


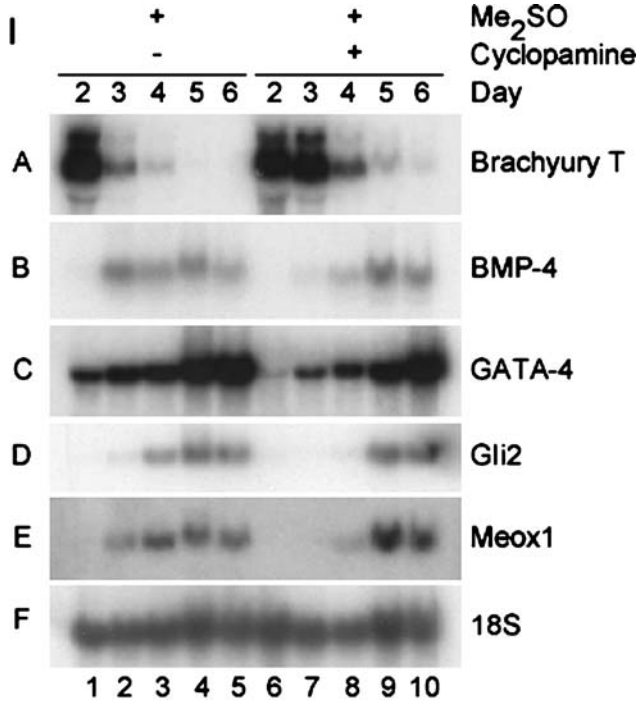
Figure 2. The hedgehog gene family is expressed in DMSO-induced differentiation of P19 cells. P19 cells were aggregated in the presence of 0.8% DMSO, and total RNA was harvested on a daily basis for 6 d. *I* RT-PCR was carried out, subjected to Southern blot analysis, and probed with the factors indicated on the *right*. RT-PCR controls include no reverse transcriptase (*lane 8*), control RNA (*lane 9*), no RNA (*lane 10*), and PCR-H_20 (*lane 11*). The loading control was α-tubulin (*IF*). *II* Northern blot analysis was performed on 12 μg of RNA from each time point, probed with labeled cDNA fragments corresponding to the factors indicated on the *right*. *Lanes* are indicated at the *bottom*. The results shown are representative of three independent experiments.

(Cyclo)). On day 6 of differentiation, cells were fixed and stained with Hoechst dye to detect nuclei and anti-MyHC antibodies. Results showed via immunofluorescence that there was no evident change in the amount or morphology of cardiomyocytes in the presence of cyclopamine (data not shown).

Although there was no loss of cardiomyogenesis in the presence of Hh inhibitors, Northern blot analysis was performed on a time course of differentiation to determine if cardiomyogenesis was delayed. BrachyuryT mRNA expression was prolonged in cells treated with cyclopamine, compared to control cells (Fig. 3*IA*). Quantification via densitometry indicated a 200±45% (standard error; n=3) increase of BrachyuryT on day 4 with cyclopamine (Fig. 3*II*), suggesting a delay in the transition from mesoderm to cardiomyoblast. Furthermore, BMP-4, GATA-4, Gli2, and Meox1 all showed a reduction and/or delay in their transcript levels in the presence of cyclopamine on days 2–4 (Fig. 3*IB–E*, *II*). However, by day 6, when cardiomyocytes were observed (data not shown), the levels of these factors were equivalent in the presence or absence of cyclopamine. Loss of Gli1 expression was observed (Fig. 3*II*), indicating that the Hh pathway was inhibited. Taken together, these data indicate that although Hh signaling is not essential for cardiomyogenesis, differentiation is impeded, as evidenced by the delayed/decreased expression of GATA-4, Meox1, Gli2, BMP-4, and the prolongation of BrachyuryT.

Noggin inhibits the Shh signaling pathway. Noggin inhibits cardiomyogenesis in P19 and P19CL6 cells, with the simultaneous loss of GATA-4 and Nkx2.5 mRNA expression (Monzen et al. 1999; Jamali et al. 2001). To determine the effect of Noggin on the Hh signaling pathway, P19 cells overexpressing Noggin (P19(Nog)) and control cells were aggregated in the presence of 0.8% DMSO. Cells were fixed on day 6 and stained with Hoechst dye and anti-MyHC antibodies. As in previous studies (Jamali et al. 2001), there was a total absence of detectable cardiomyocyte formation in differentiations with P19 (Nog) cells while P19 (Control) cells gave robust levels of cardiomyocytes (Fig. 4).

Total RNA was harvested during a time course of differentiation. Noggin transcripts were detected at high levels in P19 (Nog) cell lines and not in P19 (Control) cells (Fig. 5*IA*). As expected, GATA-4 transcripts were not detected in P19 (Nog) cell lines, indicating the inhibition of cardiomyogenesis (Fig. 5*IB*). Investigation into the expression of Hh signaling components revealed that Gli1 showed a 92% loss of transcripts in P19 (Nog) compared to P19 (Control) cells (Fig. 5*IC*, *II*). Furthermore, Gli2 and Ptch1 transcript levels were downregulated, 49% and 56%, respectively, on day 4 in P19 (Nog) compared to P19 (Control) cells (Fig. 5*ID*, *F*, *II*). In contrast, Gli3 showed no major changes in its mRNA levels or temporal expression pattern (Fig. 5*IE*). Hence, Noggin-mediated inhibition of cardiomyogenesis results in the loss of Gli1 transcripts and the downregulation of Gli2 and Ptch1 mRNA.



II

	Days	% Change
Brachyury T	4	200 +/- 45, n=3 ↑
BMP-4	4	60 +/- 7, n=4 ↓
GATA-4	2	68 +/- 6, n=3 ↓
	3	72 +/- 8, n=4 ↓
Gli2	4	67 +/- 15, n=2 ↓
Meox1	4	59 +/- 11, n=3 ↓
Gli1	6	53 +/- 10, n=3 ↓

Figure 3. In the presence of cyclopamine, cardiomyogenesis was delayed but not inhibited. P19 cells were aggregated in the presence of 0.8% DMSO (*lanes 1–10*) and in the absence (*lanes 1–5*) or presence of 10 μM of cyclopamine (*lanes 6–10*). Total RNA was harvested on days 2 through 6. *I* Northern blot analysis with 12 μg of RNA from each time point were hybridized with labeled cDNA fragments corresponding to the factors indicated on the *right*. The loading control was 18S rRNA. *Lanes* are indicated at the *bottom*. *II* Northern blots were quantified using densitometry via Image J software (NIH). *Arrows* indicate if the percent change shows increased (*arrow up*) or decreased (*arrow down*) mRNA expression levels of treated versus untreated cells.

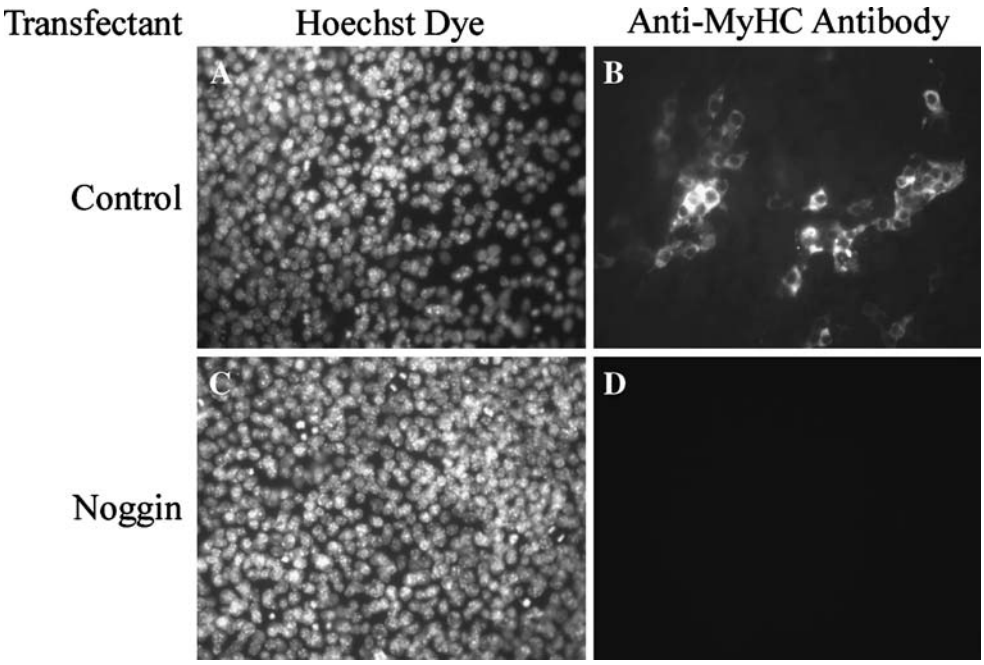
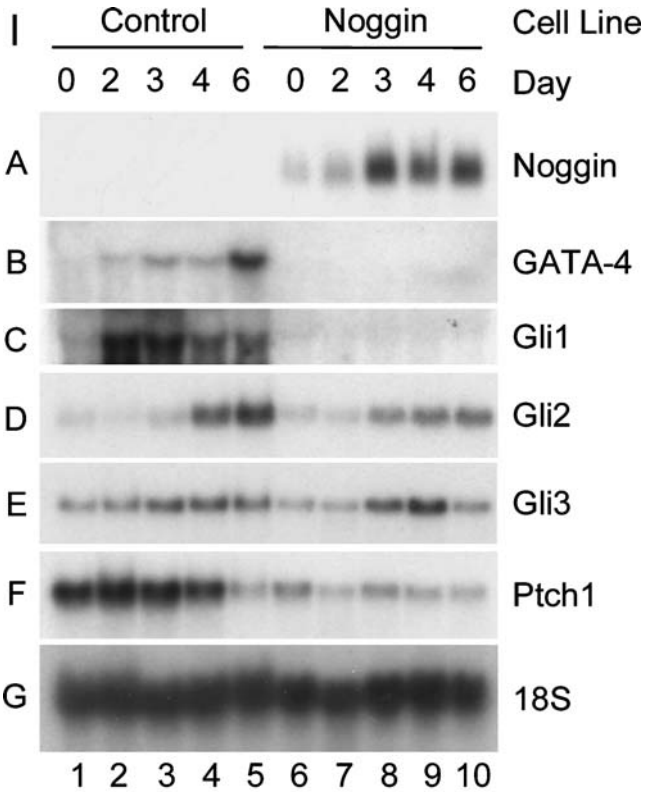


Figure 4. The overexpression of Noggin in aggregated P19 cells inhibited the formation of cardiomyocytes. P19 (Control) cells (*A*, *B*) and P19 (Nog) cells (*C*, *D*) were differentiated in the presence of 0.8% DMSO. On day 6 of the differentiation, cells were fixed and stained with Hoechst dye to detect nuclei (*A*, *C*) and anti-MyHC antibody (*B*, *D*). Magnification ×200.



II

	Days	% Decrease
Gli1	0-6	92 +/-5, n=4
Gli2	4	49 +/- 2, n=2
Ptch1	3	62 +/- 13, n=2
	4	56 +/- 18, n=2

Discussion

We have shown that all three Hh gene members are expressed in P19 cell cardiomyogenesis. However, only Shh is activated during aggregation while Dhh and Ihh are also expressed in monolayer culture. In agreement with results in the embryo (Goodrich et al. 1997), Hh signaling was not essential for cardiomyogenesis, although there was a decrease and/or delay of expression of BMP-4, Gli1/2, Meox1, and GATA-4. Finally, Noggin regulated Hh signaling by blocking the expression of Gli1/2 and Ptch1 during Noggin-mediated inhibition of cardiomyogenesis. In summary, Shh signaling may be required for timely cardiomyogenesis and may work, in part, by activating BMP signaling pathways. In contrast, BMP signaling, which is essential for cardiac muscle specification, can regulate the expression of Hh signaling components.

Our findings indicate that Hh signaling is dispensable for cardiomyogenesis. This agrees with in vivo data that *Smo*$^{-/-}$, *Shh*$^{-/-}$;*Ihh*$^{-/-}$, and *Gli2*$^{-/-}$;*Gli3*$^{-/+}$ mice formed normal cardiomyocytes but displayed defective heart looping (Tsukui et al. 1999; Zhang et al. 2001). However, Hh signaling appeared to facilitate the timely differentiation of cardiomyocytes, because P19[Cyclo] cells exhibited a delayed loss of BrachyuryT and upregulation of cardiomyo-

◀ **Figure 5.** The overexpression of Noggin inhibits Gli1 transcription and downregulates Ptch1 and Gli2 expression. P19 (Noggin) and P19 (Control) cells were aggregated in the presence of DMSO for 6 d. *I* Total RNA was harvested in a time course of differentiation and Northern blots containing 12 μg of total RNA were probed as indicated to the *right*. *Lanes* are indicated at the *bottom*. *II* Northern blots in *I* were quantified using densitometry via Image J software (NIH).

cyte factors (Fig. 3). In *Smo*$^{-/-}$ mice, there was a delay of Nkx2.5 expression in the bilateral heart fields and an impediment in the formation of the linear heart tube (Zhang et al. 2001). A delay in GATA-4 but not Nkx2.5 expression was detected in P19 cells (data not shown; Fig. 2). While the discrepancy of Nkx2.5 expression is not clear, the delay in cardiomyogenesis in P19 cells is consistent with the reduced numbers of cardiomyocytes in hearts lacking Hh signaling (Zhang et al. 2001; Thomas et al. 2008).

Cyclopamine delayed the expression of BMP-4 mRNA (Fig. 3), consistent with findings in several systems that Shh/Ihh signaling regulates BMP-2/4 signals (Bitgood and McMahon 1995; Bhardwaj et al. 2001). Furthermore, Gli factors directly regulate *BMP-2/4* promoters (Kawai and Sugiura 2001; Zhao et al. 2006). Finally, in mouse embryos treated with cyclopamine at E8.0, the linear heart tube did not form and there was decreased BMP-4 expression in the lateral plate mesoderm (Nagase et al. 2006). BMP-4 was still activated in P19[Cyclo] cells (Fig. 3), although its expression was delayed. Altogether, there is compelling evidence that BMPs are regulated by Shh/Ihh during cardiomyogenesis.

The efficiency of abrogation of the Hh signaling pathway by cyclopamine was shown by the downregulation of the expression of *Gli1*, a genetic target of Hh (Platt et al. 1997; Fig. 3*II*). However, the degree of downregulation of Gli1 was much less than in mouse embryo studies utilizing 20-μM cyclopamine (Nagase et al. 2005). The discrepancy of our results with those in vivo is unclear but there may be a Hh-independent mechanism of activating Gli1. For example, BMPs were essential to the wild-type expression of Gli1 in P19 cells (Fig. 5). In zebrafish, embryos cultured in 100-μM cyclopamine or *Smo*$^{-/-}$ embryos still show sustained Gli1 activation (Karlstrom et al. 2003), indicating that a Hh-independent mechanism may also be operating in in vivo systems. Using 5 μM of the more potent derivative of cyclopamine, KAAD-cyclopamine (Taipale et al. 2000) did not result in a furthered downregulation of Gli1 transcription in P19 cells (data not shown).

Analogous to Ptch1, Gli3 transcript levels appeared unchanged in P19 cell cardiomyogenesis (Fig. 2*IIJ*, *M*). Gli3 may be activated by the Wnt class of secreted factors in development (Borycki and Emerson 2000; Petropoulos and Skerjanc 2002) and Gli3 was not affected in P19 [Cyclo] cells (data not shown) in agreement with these data.

Hh signaling can regulate proliferation in a wide variety of wild-type systems and in several types of cancers (Berman et al. 2002). We observed a decrease in proliferation in cyclopamine-treated aggregates (data not shown), further indicating that cyclopamine was active and blocking Hh signaling.

Meox1 is sufficient but not essential for cardiomyogenesis (Mankoo et al. 2003; Petropoulos et al. 2004; Gianakopoulos and Skerjanc 2005). The delay of Meox1 activation in P19[Cyclo] cells (Fig. 3) further supports a role for Meox1 in cardiomyogenesis. Meox1 can induce Gli1/2 expression (Gianakopoulos and Skerjanc 2005) and is expressed prior to Gli2 (Fig. 3*ID, E*), consistent with a role for Meox1 as a regulator of the Hh pathway leading to cardiomyogenesis.

Noggin inhibited the expression of Gli1 and Gli2 (Fig. 5*IC, D, II*) during cardiomyogenesis, suggesting that BMP signaling may regulate Hh signaling. It is possible that inhibition of Gli1/2 via Noggin may be due to an inhibitory effect of Wnt-mediated activation of Gli2/3 (Borycki and Emerson 2000; Borycki et al. 2000; Petropoulos and Skerjanc 2002).

Conclusions

Our results indicate cross talk between Hh and BMP signaling pathways during cardiomyogenesis. Hh signaling regulates BMP expression while Noggin, an inhibitor of BMP, can inhibit the expression of Hh target genes. Moreover, Hh signaling is not essential for cardiomyogenesis, indicating that there are alternative means by which BMPs are mobilized and cardiac precursor cells can be specified/committed to the cardiomyogenic lineage.

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