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BMP is an important regulator of proepicardial identity in the chick embryo

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Abstract

The proepicardium (PE) is a transient structure formed by pericardial coelomic mesothelium at the venous pole of the embryonic heart and gives rise to several cell types of the mature heart. In order to study PE development in chick embryos, we have analyzed the expression pattern of the marker genes *Tbx18*, *Wt1*, and *Cfc*. During PE induction, the three marker genes displayed a left–right asymmetric expression pattern. In each case, expression on the right side was stronger than on the left side. The left–right asymmetric gene expression observed here is in accord with the asymmetric formation of the proepicardium in the chick embryo. While initially the marker genes were expressed in the primitive sinus horn, subsequently, expression became confined to the PE mesothelium. In order to search for signaling factors involved in PE development, we studied *Bmp2* and *Bmp4* expression. *Bmp2* was bilaterally expressed in the sinus venosus. In contrast, *Bmp4* expression was initially expressed unilaterally in the right sinus horn and subsequently in the PE. In order to assess its functional role, BMP signaling was experimentally modulated by supplying exogenous BMP2 and by inhibiting endogenous BMP signaling through the addition of Noggin. Both supplying BMP and blocking BMP signaling resulted in a loss of PE marker gene expression. Surprisingly, both experimental situations lead to cardiac myocyte formation in the PE cultures. Careful titration experiments with exogenously added BMP2 or Noggin revealed that PE-specific marker gene expression depends on a low level of BMP signaling. Implantation of BMP2-secreting cells or beads filled with Noggin protein into the right sinus horn of HH stage 11 embryos resulted in downregulation of *Tbx18* expression, corresponding to the results of the explant assay. Thus, a distinct level of BMP signaling is required for PE formation in the chick embryo.

Keywords: Wt1; Tbx18; Cfc; Bmp2; Bmp4; Popdc2; Nkx2.5; Proepicardium; Pericardium; Cardiac myocytes; Chick embryo

Introduction

The proepicardium (PE) is a transient embryonic structure that forms near to the venous pole of the developing heart (Viragh et al., 1993). By Hamburger and Hamilton (HH; Hamburger and Hamilton, 1951) stage 14/15 of chick embryonic development, there is first morphological evidence for PE formation as a unilateral outgrowth of the pericardial serosa covering the right horn of the sinus venosus (Männer, 1992; Männer et al., 2001). Between HH stages 14 and 17, multicellular villous projections extend from the PE toward the dorsal wall of the heart loop. These villi are guided to the myocardium via an extracellular matrix bridge (Nahirney et al., 2003). After reaching the myocardial surface, PE-derived mesothelial cells spread over the heart and form a simple squamous epithelium, the primitive epicardium (Hiruma and Hirakow, 1985; Männer, 1992; Viragh et al., 1993). A similar process occurs in other vertebrates including humans (Fransen and Lemanski, 1990; Hirakow, 1992; Komiyama et al., 1987; Kuhn and Liebherr, 1988; Männer et al., 2001; Perez-Pomares et al., 1997; Van den Eijnde et al., 1995; Viragh and Challice, 1981).

In the center of the PE, mesenchymal cells lie within an extensive extracellular matrix. These cells and additional mesenchymal cells that are recruited from the primitive epicardium by epithelial–mesenchymal transition (EMT) colonize the subepicardial layer of the heart (Perez-Pomares et al., 1998). Epicardial EMT is affected by FGF and TGFβ signaling (Morabito et al., 2001; Compton et al., 2006; Olivey

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et al. (2006). The PE- and epicardium-derived subepicardial mesenchymal cells become especially prominent at the sulci of the atrioventricular junction and junction between the outflow tract and the ventricular loop. Some mesenchymal cells do not reside within the subepicardium but migrate further into the myocardial and subendocardial layers including the endocardial atrioventricular cushions. Epicardium-derived cells (EPDCs) differentiate into multiple cell lineages such as fibroblasts, smooth muscle, and endothelial cells (Dettman et al., 1998; Gittenberger-de Groot et al., 1998; Mikawa and Gourdie, 1996; Männer, 1999; Vrancken Peeters et al., 1999; Wessels and Perez-Pomares, 2004). The contribution of the EPDCs to the coronary vessel endothelium is less clear, and data suggest that probably a large fraction of coronary endothelium is derived from mesenchyme within the liver anlage (Mikawa and Gourdie, 1996; Poelmann et al., 1993). Neither cell lineage tracing via a replication incompetent virus nor the quail–chick chimera technique established any material contribution by the PE to the myocardium (Mikawa and Gourdie, 1996; Gittenberger-de Groot et al., 1998; Männer, 1999). However, the embryonic epicardium plays a major role in controlling growth of the outer compact layer of ventricular myocardium (Chen et al., 2002; Männer et al., 2005; reviewed by Männer, 2006). Two autocrine signaling loops using retinoic acid and erythropoietin have been identified that maintain the ability of epicardial cells to secrete hitherto unidentified mitogens that stimulate proliferation of the compact layer myocardium (Chen et al., 2002; Stuckmann et al., 2003; Tran and Sucov, 1998). Apart from its function of secreting myotrophic factors, the epicardium may also contribute to the passive mechanics of the developing heart (Männer et al., 2005).

The myocardium, endocardium, epicardium, and pericardium all originate from the coelomic epithelium (Männer et al., 2001). Several transcription factor genes which are expressed during early myocardial development such as *Gata4*, *Tbx5*, *Tbx18*, *Tbx20*, and *Srf* are also expressed at least transiently within the PE (Haenig and Kispert, 2004; Hatcher et al., 2004; Kraus et al., 2001; Nelson et al., 2004; Tanaka and Tickle, 2004; Watt et al., 2004; Yamagishi et al., 2004). The pericardium and the epicardium also have some genes in common. For example, *Raldh2* is expressed in both the pericardium and epicardium (Niederreither et al., 1997). Little is known about the mechanisms that control PE formation and govern PE-specific gene expression. However, it was recently found that *Gata4* expression in the mouse PE is under the control of BMP4 signaling and that *Gata4* expression is essential for PE development (Rojas et al., 2005; Watt et al., 2004), suggesting a mode of gene expression regulation during PE induction that is similar to the one during heart field formation (Brand, 2003).

In this study, we analyzed the expression of the PE marker genes *Cfc*, *Tbx18*, and *Wt1* during PE formation in the chick embryo. *Cfc* (also known as Cripto; Colas and Schoenwolf, 2000; Schlange et al., 2001) belongs to the EGF-CFC gene family that encodes competence factors which are required for the binding of selected ligands of the TGFβ and Wnt families to their respective signaling receptors (Cheng et al., 2003; Gritsman et al., 1999; Shen and Schier, 2000; Tao et al., 2005). *Tbx18* is a member of the T-box family of transcription factors, and expression analysis in several vertebrates revealed an association of this gene with epicardial development, however, mutant analysis revealed no essential function in PE and epicardial development (Begemann et al., 2002; Haenig and Kispert, 2004; Kraus et al., 2001; Tanaka and Tickle, 2004). *Wt1* encodes a zinc-finger transcription factor, which is expressed during epicardial development, and mutant analysis revealed an essential function during epicardial development (Carmona et al., 2001; Kreidberg et al., 1993; Moore et al., 1999). Little is known about the signaling molecules involved in PE formation. We show here that the expression of PE marker genes is regulated by BMP signaling. Both *Bmp2*, which is expressed in the sinus venosus myocardium as well as *Bmp4*, which is present in the PE mesenchyme, may contribute to the recruitment of lateral plate mesoderm cells to the PE cell lineage. Explant cultures of PE treated with BMP2 or Noggin resulted in a loss of PE marker gene expression and instead led to the up-regulation of myocardial marker genes. Similar data were obtained after implantation of BMP2 or Noggin into the right sinus horn at HH stage 11 in vivo. We additionally show that the chicken PE is a structure, which receives left–right axis information. These data provide an entry point into the experimental analysis of PE formation in the chick.

Materials and methods

Isolation of embryonic tissue and PE explant culture

White Leghorn eggs (Lohmann, Lübeck) were routinely incubated, opened, and staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1951). For the isolation of PE, eggs were incubated until the embryos had reached HH stage 17. PE explants were excised by fine tungsten needles with careful avoidance of contaminating sinus myocardium. The tissue fragments were cultured for 3 days in serum-free M199 (Invitrogen) on fibronectin-coated culture slides (BD Biosciences) or on uncoated plastic culture dishes (Nunclon). Pericardial tissue was dissected from HH stage 22 embryos, atrial and ventricular tissue was dissected from heart loops prior to colonization with PE cells (HH stage 17 embryos) to avoid epicardial contamination.

BMP2 and Noggin treatment

PE explants were cultured for 12 h in serum-free M199. Explants were checked for the presence of contractile activity as this would indicate the presence of contaminating myocardial tissue. Explants with contractile activity were discarded. 2 ng/ml human recombinant BMP2 (#355-BM; R&D Systems) or 50 ng/ml recombinant mouse Noggin (1967-NG; R&D Systems) was added directly to the culture medium. For titration purposes, other concentrations were also used, i.e. 2, 0.2, and 0.1 ng/ml BMP2 and 5, 10, and 50 ng/ml Noggin. In another titration, experiment PE cultures were incubated with 10, 1, 0.5, 0.2, and 0.02 ng/ml BMP2 in the presence of 20 ng/ml Noggin. The PE cultures were cultured for a total period of 72 h.

BMP2 and Noggin implants in vivo

For these experiments, the embryos were explanted at HH stage 11 and cultured according to the EC culture method (Chapman et al., 2001). Q2bn cells producing BMP2 and control cells were cultured as previously

described (Andrée et al., 1998). Noggin protein was loaded on beads at a concentration of 1 μg/μl. The beads were loaded by soaking for at least 1 h at room temperature. Beads or cell aggregates were implanted into the sinus myocardium of cultured embryos. Beads loaded with PBS or Q2bn-AS cells were used as control. The embryos were subjected to whole mount in situ hybridization at the end of the culture period. Care was taken that different embryos were stained to a similar extent. The weak expression domain of Tbx18 on the left side was utilized as reference point to have the same staining intensity in each case. Moreover, staining of forming and newly formed somites was visible in each embryo.

RNA isolation, RT-PCR, and qRT-PCR

Cultured explants and dissected tissues were solubilized in lysis buffer and stored at −80°C. Subsequently, total RNA was isolated as previously described (Schlange et al., 2000). cDNA was synthesized from DNase-treated total RNA using AMV reverse transcriptase. PCR was performed using the following primer pairs: $Tbx18_{fwd}$: 5′-CATATGTGCAGACACT-3′, $Tbx18_{rev}$: 5′-CATATGTGCAGACACT-3′ (annealing temperature: 48°C, product size: 227 bp), $Wt1_{fwd}$: 5′-AGCCAGCAAGCCAT TCGC AACC-3′, $Wt1_{rev}$: 5′-TTCTCATTTTCAT ATCCTGTCC-3′ (58°C, 355 bp), Cfc_{fwd}: 5′-CTCAAGCTTTCCCGAAGGAATA-3′, Cfc_{rev}: 5′-GTTCAGCTTCA-CAACTG CC-3′ (53°C, 635 bp), $Popdc2_{fwd}$: 5′-CTGAGGGAAGGAC CGCTACATT-3′, $Popdc2_{rev}$: 5′-CAGCCTGCTGTTCCCTAGAGC-3′ (55°C, 570 bp/600 bp), $Amhc_{fwd}$: 5′-GCGGGTCCAGCTTC-TCCACTCC-3′, $Amhc_{rev}$: 5′-CCTTGACACGCCGCTC TGACTT-3′ (59°C, 412 bp), $Nkx2.5_{fwd}$: 5′-GGATCCCTCCTCGTTGC TCTCG-3′, $Nkx2.5_{rev}$: 5′-CCTTGACACGCCGCTCTGACTT-3′ (58°C, 102 bp), $Hand1_{fwd}$: 5′-CGGGGCTCAGGGGTTCAGTTC-3′, $Hand1_{rev}$: 5′-TGCCACCAG-GAGCGCGTT AT-3′ (70°C, 521 bp), $Vmhc_{fwd}$: 5′-GCTACAAACACCAAG-CAG AG-3′, $Vmhc_{rev}$: 5′-TCTTATATCTGGGAGCCAGG-3′ (60°C, 211 bp), $Bmp4_{fwd}$: 5′-TAACCGAATGCTGATGGTCA-3′, $Bmp4_{rev}$: 5′-GCTGAGGTT-GAAGACGA AGC-3′ (60°C, 420 bp), $Gapdh_{fwd}$: 5′-ACGCCATCAC-TATCTTCCAG-3′, $Gapdh_{rev}$: 5′-CAGG CCTTCACTACCCTCTTG-3′ (52°C, 578 bp). The same primer pairs were also used for quantitative real-time PCR using the MJ Research Opticon Monitor II system and the QuantiTect SYBR Green PCR Kit (Qiagen). Gene expression levels were quantified based on the threshold cycle ($C(t)$) and normalized to *Gapdh* and corrected for efficiency of each primer pair (Pfaffl, 2001). RT-PCR results were compiled from 2 to 3 different cDNA samples with PCRs performed six times.

Whole mount in situ hybridization

Whole mount in situ hybridization was carried out as described (Andrée et al., 1998). For expression analysis of *Cfc*, a 1-kb full-length cDNA was used (Schlange et al., 2000). A 1.3 kb *Wt1* cDNA probe was kindly provided by T. Kudo, Ibaraki, Japan. For *Tbx18*, a 1 kb partial cDNA clone (ChEST861E19) and for *Bmp4* a 766 bp partial cDNA clone (ChEST730M5) were identified in the ChickEST Database (Boardman et al., 2002) and obtained from the MRC geneservice. For the detection of *Bmp2*, a 460 bp fragment encoding the divergent propeptide domain of BMP2 was used (Andrée et al., 1998).

Immunohistochemistry

For immunofluorescent staining of PE explant cultures, the culture slides were washed three times with PBS, fixed with −20°C cold methanol, and air-dried. The cells were blocked for 30 min with 1% BSA in PBS and incubated for 1 h at RT with a 1:50 dilution of a WT1 antibody (Santa Cruz, sc-192) and a 1:10 dilution of the monoclonal MF20 antibody. The MF20 hybridoma developed by David Bader and Don Fishman was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. The cells were washed again three times with PBS and incubated for 1 h with TRITC-coupled anti-mouse secondary antibody and an FITC-coupled anti rabbit antibody. Nuclei were visualized by DAPI staining. Coverslips were mounted using Moviol as mounting medium and visualized using an CLSM 510-META (Carl Zeiss, Göttingen).

Results

The T-box transcription factor *Tbx18* has previously been reported to be expressed in the PE (Haenig and Kispert, 2004; Tanaka and Tickle, 2004), however, expression during the early phase of PE formation has not been addressed previously. The competence factor *Cfc* (also known as *Cripto*) has been shown to be involved in left–right (L–R) signaling within the lateral plate mesoderm (Schlange et al., 2001), however, *Cfc* has not been previously associated with epicardial development. At HH stage 10, *Tbx18* and *Cfc* were expressed in the entire tubular heart (data not shown, Schlange et al., 2001). At HH stage 11, *Tbx18* expression was present in the myocardium, being stronger in the caudal part of the tubular heart. In the inflow tract region, expression was found to be unilateral expressed on the right side (Figs. 1A, D). *Cfc* expression was found in the outflow tract and in the sinus myocardium and did not show L–R asymmetry in the heart at this stage (Figs. 1G, J). However, the right somatopleura at the caudal end of the heart field expressed *Cfc*, while the left side was unlabeled (Fig. 1J). At HH stage 13, *Tbx18* and *Cfc* were expressed in the right inflow tract (Figs. 1B, E, H, K). At HH stage 17, both genes were expressed in the PE (Figs. 1C, F, I, L). Expression of the zinc-finger transcription factor *Wt1* during epicardial development has already been described in the mouse and chick embryo (Carmona et al., 2001; Moore et al., 1999; Perez-Pomares et al., 2002), however, early PE development has not been studied. In contrast to *Tbx18* and *Cfc*, *Wt1* was not expressed in the heart at HH stage 11 but was present in the intermediate mesoderm (data not shown). At HH stage 13, prominent expression in the right sinus myocardium was observed (Figs. 1M, P). At HH stages 15 and 17, *Wt1* expression was confined to the PE mesothelium (Figs. 1N, O, Q, R).

The expression patterns of these three genes were also followed during early stages of epicardium formation. In each case, prominent expression was found to be present in the PE mesothelium and in the forming epicardium of HH stage 22 embryos (Figs. 2A, B, E, F, I, J). Sections through these hearts revealed that expression of *Tbx18*, *Cfc*, and *Wt1* was confined to the PE mesothelium and was weaker (*Wt1* and *Tbx18*) or absent (*Cfc*) from the mesenchymal core (Figs. 2C, D, G, H, K, L). A weak expression was found in the pericardial mesothelium (Figs. 2D, L), and none of the genes was expressed in myocardial cells. RT-PCR analysis revealed co-expression of *Wt1* in PE and pericardium and absence of expression in the myocardium. Expression of the other genes under study, *Cfc* and *Tbx18*, was not detected in the pericardium by RT-PCR (Fig. 2M). qRT-PCR analysis confirmed the histological findings and revealed strong expression of *Tbx18*, *Cfc*, and *Wt1* in the PE (Figs. 2N–P). While *Wt1* expression in the pericardium was significant, *Tbx18* and *Cfc* are only expressed at low levels in the pericardium (Fig. 2). None of the PE marker

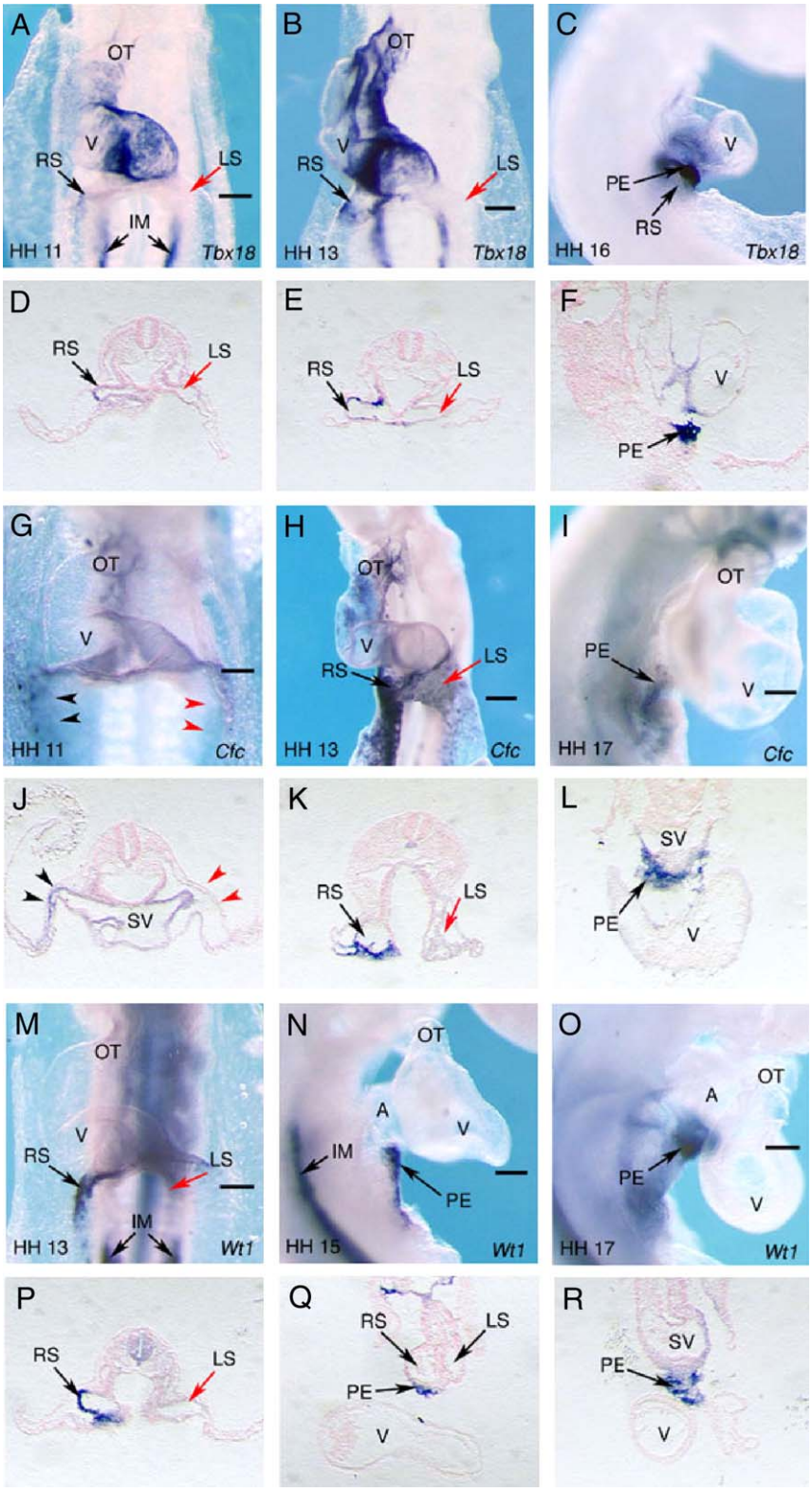

Fig. 1. Expression of PE marker genes prior to and during PE formation. Whole mount in situ hybridization of chick embryos at HH stage 11 (A, G), HH stage 13 (B, H, M), HH stage 15 (N), HH stage 16 (C) and HH stage 17 (I, O) with probes for *Tbx18* (A–C), *Cfc* (G–I), and *Wt1* (M–O). (A, B, G, H, M) Ventral views and (C, I, N, O) right-sided views. (D–F, J–L, P–R) Transversal sections through the sinus venosus area of the embryos shown in panels A–C, G–I, M–O. The plane of sectioning is indicated in the individual panel. Black arrows (or arrowheads in panels G and J) point to the right-sided expression domain of the different PE marker genes, red arrows (or arrowheads in panels G and J) point to the absence of PE marker gene expression on the left side. A—atrium; IM—intermediate mesoderm, LS—left horn of the sinus venosus; OT—outflow tract; PE—proepicardium; RS—right horn of the sinus venosus; SV—sinus venosus; V—ventricle.

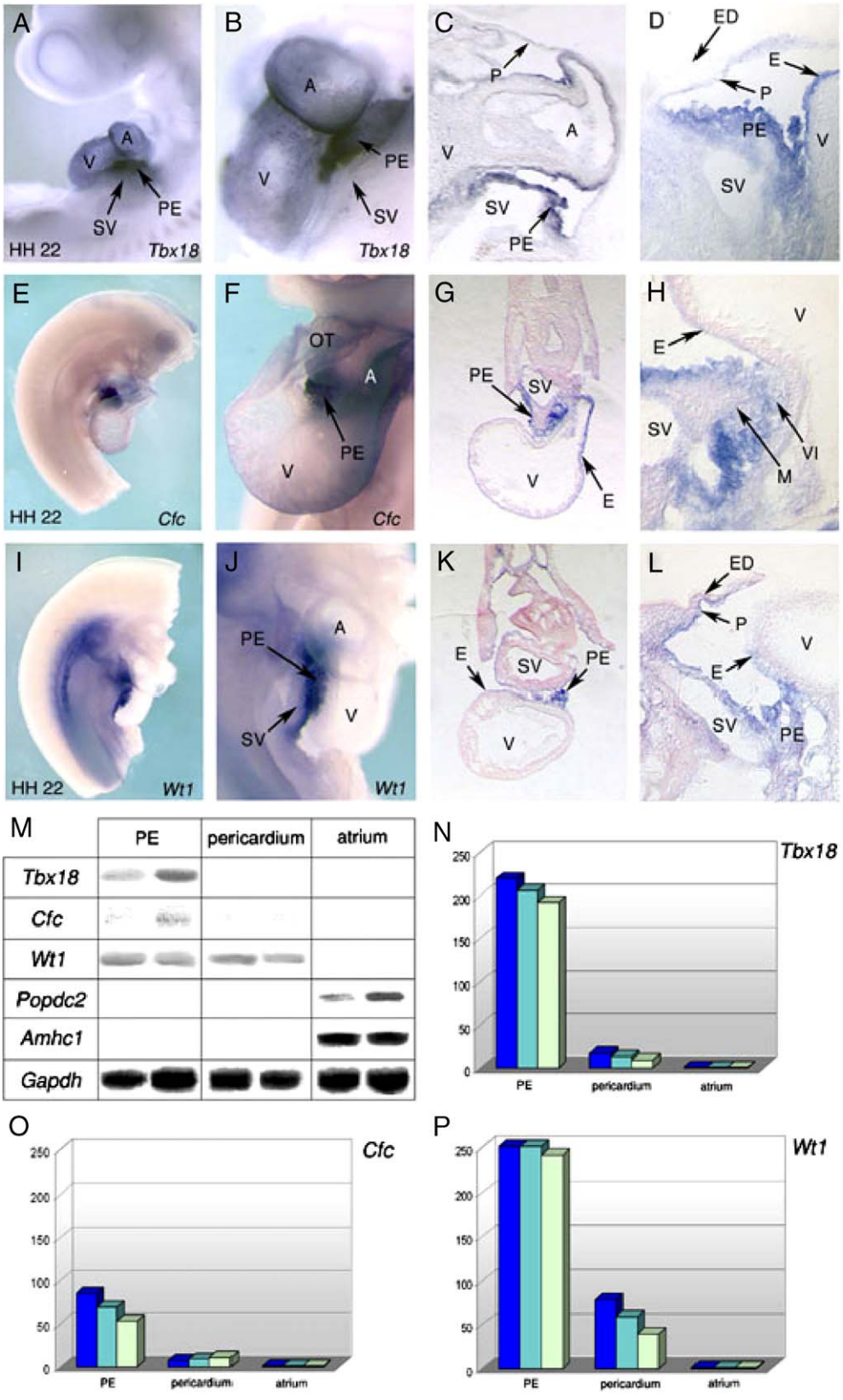

Fig. 2. PE marker genes are also expressed in the epicardial and pericardial mesothelium. Whole mount in situ hybridization of chick embryos at HH stage 22 with probes for (A, B) *Tbx18*, (E, F) *Cfc*, (I, J) *Wt1*. (A, B) left-sided views, (E, I, J) right-sided views, and (F) a ventral view showing expression of each gene in the PE. (C, D, G, H, K, L) Transversal sections through the sinus venosus area demonstrating expression in the PE, epicardium, and pericardium. (M) End-point RT-PCR for *Tbx18*, *Cfc*, *Wt1*, *Popdc2*, and *Amhc1*. For control purposes, the expression of glyceraldehyde dehydrogenase (*Gapdh*) was analyzed. (N–P) qRT-PCR analysis of (N) *Tbx18*, (O) *Cfc*, and (P) *Wt1* expression in epicardium and atrial myocardium of HH stage 17 embryos and of pericardium of HH stage 22 embryos. Different colored bars indicate independent experiments. E—epicardium; ED—ectoderm; P—pericardium; VI—villi of PE (for other abbreviations, see the legend of Fig. 1).

genes was expressed to any significant extent in the atrial myocardium of HH stage 17 embryos.

In order to identify signaling factors that might determine PE-specific gene expression, we analyzed the role of the BMP signaling factors in this context since BMPs have previously implicated into myocardium formation (Andrée et al., 1998; Brand, 2003; Schlange et al., 2000; Schultheiss et al., 1997). Analysis of *Bmp2* expression during PE formation in the chick revealed a bilaterally symmetric expression of *Bmp2* in the sinus myocardium at HH stage 11, which persisted in this location until HH stage 17 (Figs. 3A–D). *Bmp4* was first detected at HH stage 13 in the ventral layer of the right sinus horn (Figs. 3E, G). At HH stage 17, *Bmp4* was expressed in the PE (Figs. 3F, H).

Thus, BMP2 in the sinus myocardium and BMP4 in the PE epithelium are two signaling molecules that are expressed at the right place and at the right time to be candidate signaling molecules that might be involved in epicardial induction and/or maintenance of proepicardial identity. In order to analyze the role of BMPs for PE development, we made use of a PE explant assay. For this purpose, PE explants of HH stage 17 chick embryos were cultured in serum-free medium in the presence or absence of 2 ng/ml BMP2 or of the BMP antagonist Noggin at a concentration of 50 ng/ml. No significant effect on the explant outgrowth or its epithelial morphology was observed with either BMP2 or Noggin addition. However, in contrast to control cultures, a large number of BMP2 or Noggin-treated explants contained beating cardiac myocytes, which were positively stained for MyHC expression (Figs. 4A–C). RT-PCR analysis revealed a downregulation of PE-specific gene expression after BMP2 or Noggin addition (Fig. 4D). Similar data were also obtained by qRT-PCR (Figs. 4E, F). BMP2 and Noggin treatment resulted in an inhibition of PE-specific marker gene expression and simultaneously to a significant increase of myocardial marker genes (including *Popdc2*, *Nkx2.5*, *Hand1*, and *Vmhc* (Figs. 4E, F)). The extent of the loss of PE marker gene expression was more pronounced in case of Noggin treatment in comparison to BMP2. In contrast, induction of myocardial marker genes was more pronounced (with the exception of Nkx2.5) after BMP2 addition than after Noggin treatment.

In order to further analyze the phenomenon of BMP2-induced cardiac myocyte formation, we performed confocal immunohistochemical staining of PE explants using WT-1 and MF20 antibodies. In control explants, only negligible amounts of cardiac myocytes were found that were stained by the MyHC antibody but were not stained by the WT1 antibody (Figs. 5A, B). In case of the PE explants that were treated with BMP2, the MyHC antibody stained a much larger fraction of cells. However, in this case, most cardiac myocytes were co-stained by the WT1 antibody, suggesting that these cells were PE cells that had changed their differentiation pathway (Figs. 5C, D).

Since both BMP2 and Noggin induced myocardial marker gene expression and resulted simultaneously in a loss-of PE-specific gene expression, we thought to analyze this result further. We first analyzed whether *Bmp4* gene expression was maintained in cultured PE explants. For this purpose, qRT-PCR analysis of *Bmp4* expression in PE explants that were freshly isolated or cultured for 3 days in serum-free medium was performed. Both freshly isolated and cultured PE explants displayed a similar level of *Bmp4* gene expression (Fig. 6A). This result suggests that, in cultured PE explants, a certain amount of BMP4 is produced which, as the Noggin data suggest (Fig. 4E), is required for maintaining PE marker gene expression. Moreover, any further increase of BMP signaling might be detrimental to PE marker gene expression. In order to analyze this into further detail, we titrated the amounts of BMP2 and Noggin that were added to the PE cultures. In this case, we confined our analysis to *Tbx18* since our previous analysis revealed that the three genes chosen in this study to monitor PE gene expression (*Tbx18*, *Wt1*, and *Cfc*) displayed co-regulation. Reducing the concentration of

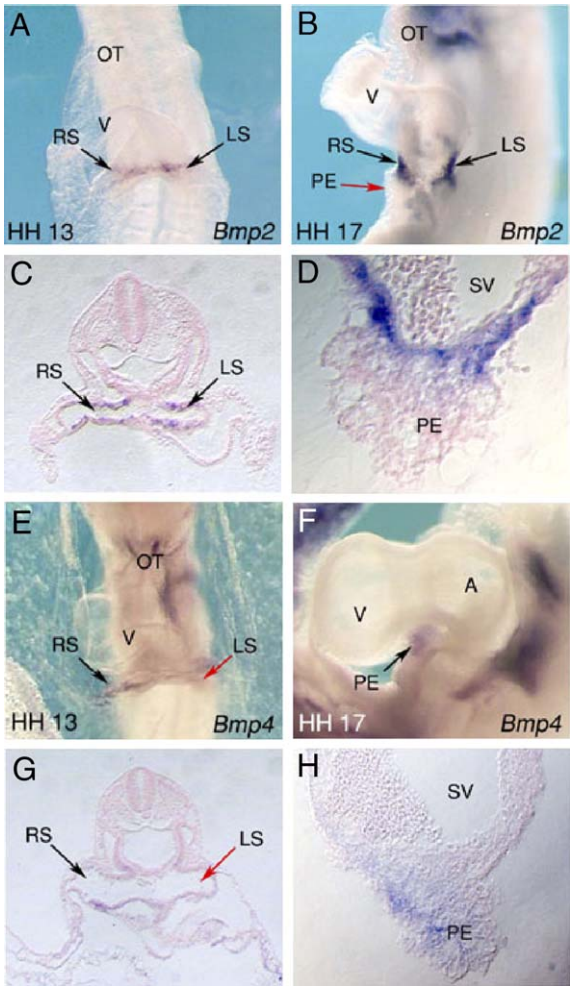

Fig. 3. Expression of *Bmp2* in the sinus venosus myocardium and of *Bmp4* in the PE. (A, B, E, F) Whole mount in situ hybridization of chick embryos at HH stage 13 (A, E) and HH stage 17 (B, F) with probes for (A, B) *Bmp2* and (E, F) *Bmp4*. All views of embryonic hearts are ventral with the exception of panel (F) which is a left lateral view; anterior is at the top, posterior is at the bottom. (C, D, G, H) Transversal sections through the sinus venosus area of the embryos shown in panels A, B, E, F. For abbreviations, see the legend of Fig. 1.

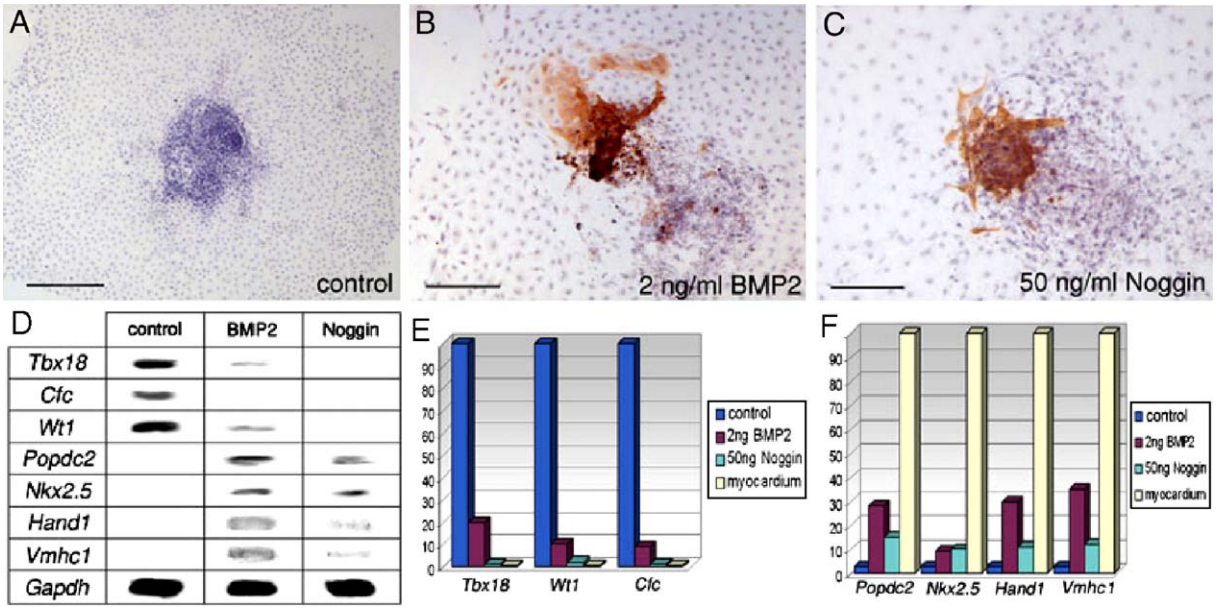

Fig. 4. BMP2 or Noggin induces myocardial differentiation in PE explants cultured in serum-free medium. (A–C) PE explants were cultured for 2 days in serum-free medium with (A) no addition, (B) 2 ng/ml BMP2, and (C) 50 ng/ml Noggin and subsequently were stained for MyHC expression (brown staining) and counterstained with hematoxylin to visualize the cells. (D) End-point RT-PCR showing downregulation of PE marker genes (*Tbx18*, *Cfc*, and *Wt1*) after BMP2 or Noggin treatment and at the same time up-regulation of myocardial marker gene expression (*Popdc2*, *Nkx2.5*, *Hand1*, and *Vmhc1*). For control purposes, the expression of glyceraldehyde dehydrogenase (*Gapdh*) was analyzed. (E, F) qRT-PCR analysis of PE and myocardial marker gene expression in (E) BMP2- and (F) Noggin-treated PE explants. RNA was isolated from PE explants and atrial myocardium. Data are represented as relative levels compared to cDNA samples of atrial myocardium in the case of cardiac marker genes and of PE explants cultured in serum-free medium in case of PE marker genes. Levels of the control samples were set arbitrarily to 100. Scale bar = 100 μm (A), 50 μm (B, C).

BMP2 that was added to the PE explants from 2 ng/ml to 0.1 ng/ml resulted in a step-wise increase in Tbx18 expression (Fig. 6B). Likewise reducing the Noggin concentration from 50 ng/ml to 5 ng/ml resulted in an increase in *Tbx18* expression, which at 5 ng reached almost control level (Fig. 6B). In another titration experiment, the Noggin concentration was kept constant at 20 ng/ml and then increasing amounts of BMP2 (0.02–10 ng/ml) were added. In PE explants that were cultured in the presence of 20 ng/ml Noggin and BMP2 at 0.02 and 0.2 ng/ml, *Tbx18* was not expressed (Fig. 6C). At 0.5 ng/ml of BMP2, *Tbx18* was expressed close to the level found in control cultures. A further increase of BMP2 resulted in a step-wise reduction of Tbx18 expression. These data indicate that BMP signaling is required for PE marker gene expression, however, only low levels of BMP are compatible with a PE-specific gene program. We also analyzed myocardial marker gene expression and its dependence on BMP signaling. We found that, in the presence of 20 ng/ml Noggin, the addition of BMP at 0.5 and 2 ng/ml resulted in significant levels of *Vmhc* gene expression (Fig. 6D). A further decrease to 0.2 and 0.02 ng/ml was not compatible with *Vmhc* gene expression.

In order to exclude the possibility that the observed gene regulatory mechanisms represent artifacts of in vitro culture experiments, we also employed implantation experiments into cultured embryos. Implantation of beads filled with Noggin into the right sinus horn at HH stage 11 (Figs. 7A–D) and expression analyzed at HH stage 14 revealed an inhibition of *Tbx18* expression in the PE (20/23, 87%). Likewise, implantation of BMP2-expressing cell aggregates into the right sinus horn (Figs. 7E–H) resulted in a loss of *Tbx18* expression (12/19, 63%). These data correspond to the results obtained in the explant culture experiments.

Discussion

We have studied the expression of *Tbx18*, *Wt1*, and *Cfc* during early stages of PE development. Our data show that PE development in the chick embryo is already underway as early as HH stage 11. It starts with unilateral expression of *Tbx18* within the right sinus horn. By HH stage 13, all three genes under study are asymmetrically expressed in the right sinus horn. The finding that PE marker gene expression is L–R asymmetric is in accordance with the asymmetric morphogenesis of the PE in the chick and stands in contrast to the situation in mice, where a left and right PE are formed. Subsequently, the two anlagen move towards the midline where they merge to form a single PE (Männer et al., 2001; Schulte et al., in preparation). These genes are also expressed in the pericardial mesothelium, which like the PE and myocardium is a derivative of the lateral plate mesoderm. We found that BMP signaling is an important regulator of PE development. Using an explant assay, we show that the addition of BMP2 reduced PE-specific gene expression in a concentration-dependent manner. Low levels of exogenous BMP2 are compatible with PE marker gene expression. However, increasing concentrations of BMP2 as well as addition of Noggin interfered with PE-specific marker gene expression. Experimental results after implantation of BMP2-expressing

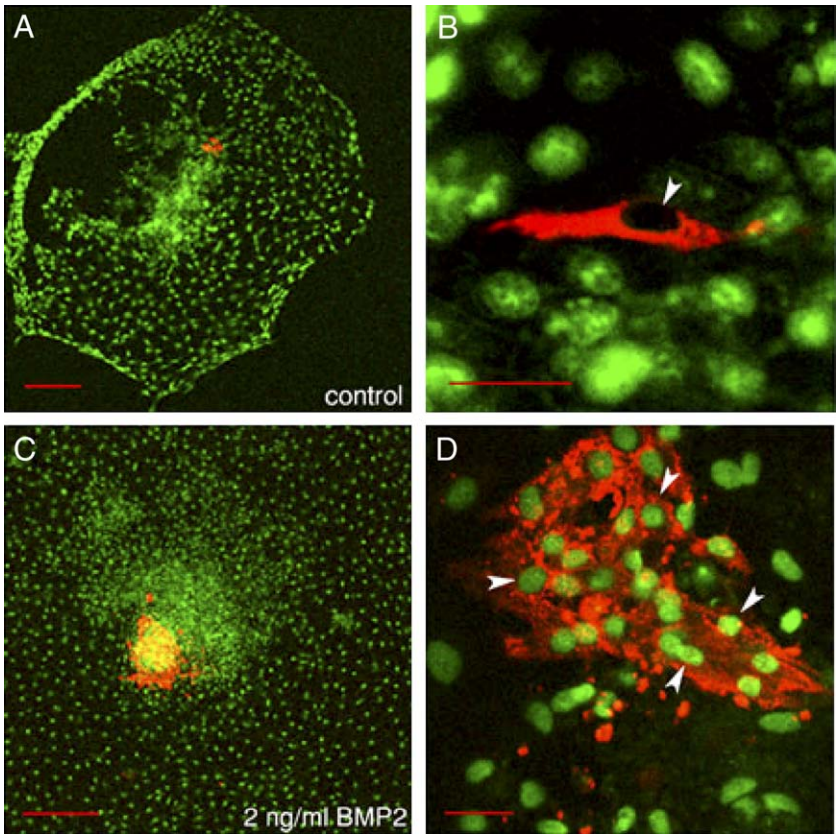

Fig. 5. Induction of myocardial differentiation occurs in PE cells after BMP2 treatment. Confocal microscopy of immunofluorescent staining of PE explants using WT1 (green label) and MyHC (red label) antibodies. (A, C) Low-power views of PE explants cultured in (A) serum-free medium or (C) after the addition of 2 ng/ml BMP2. (B) High-power view of a single cardiac myocyte and surrounding MyHC negative epicardial cells in a control culture. (D) Cluster of MyHC positive cardiac myocytes also labeled by the WT1 antibody. Arrowheads in panels (B, D) point to cardiac myocytes. Scale bar = 100 μm (A, C), 10 μm (B, D).

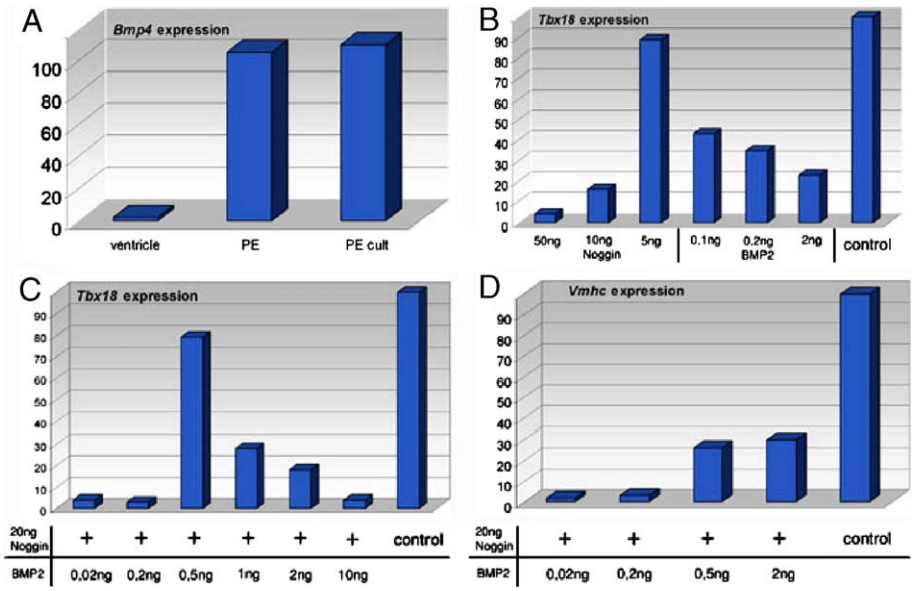

Fig. 6. Low levels of BMP signaling are required for maintenance of proepicardial gene expression. (A) qRT-PCR analysis of *Bmp4* expression in proepicardial explants directly after isolation (PE) and after 3 days of culture (cult.). (B) qRT-PCR of *Tbx18* expression in PE explants that were cultured in serum-free medium with the addition of different concentrations of Noggin (50, 10, and 5 ng/ml) and of BMP2 (0.1, 0.2, and 2 ng/ml). (C) qRT-PCR of *Tbx18* expression in PE explants that were cultured in serum-free medium in the presence of 20 ng/ml Noggin and increasing concentrations of BMP2 (0.02, 0.2, 0.5, 1 and 2 and 10 ng/ml). (D) qRT-PCR of *Vmhc1* expression in PE explants that were cultured in serum-free medium in the presence of 20 ng/ml Noggin and increasing concentrations of BMP2 (0.02, 0.2, 0.5, and 2 ng/ml). In each panel, data are represented as relative levels compared to the level of cDNA samples of untreated control cultures (B, C) or to freshly excised PE explants (A) or compared to cDNA samples of atrial myocardium (D). Levels of the control samples were set arbitrarily to 100.

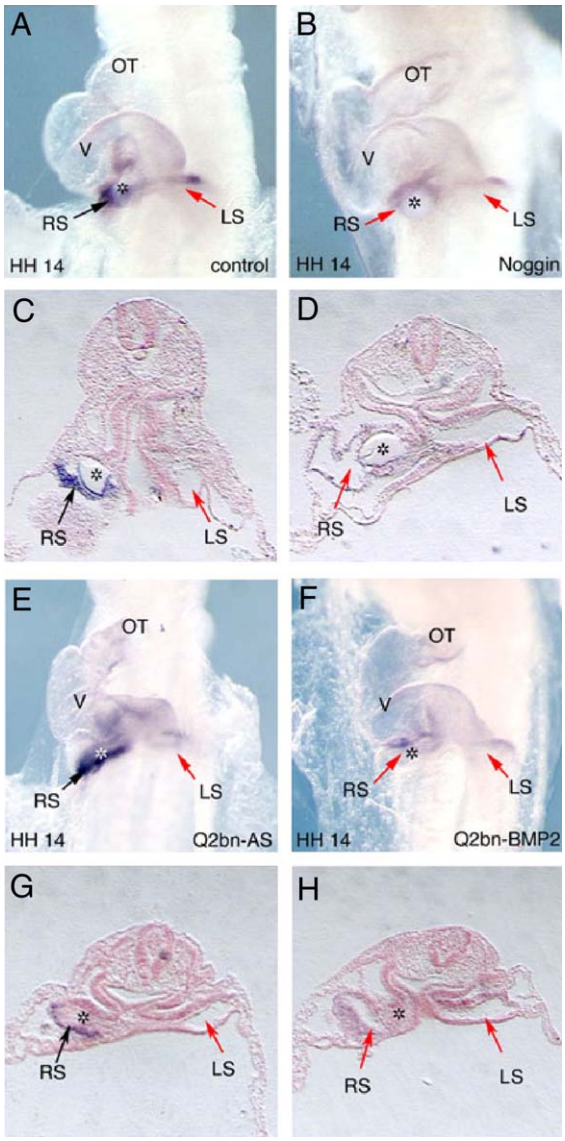

Fig. 7. Implantation of BMP2-producing cells or Noggin-loaded beads into the right sinus venosus horn of cultured HH stage 11 chick embryos interfered with Tbx18 expression. All views of embryonic hearts are ventral; anterior is at the top, posterior is at the bottom. (A, B, E, F) Whole mount in situ hybridization with a Tbx18 cRNA probe of chick embryos at HH stage 14 that were implanted at HH stage 11 with: (A) a control bead, (B) a bead loaded with Noggin, (E) a control cell implant (Q2bn-AS), or (F) an implant of BMP2 secreting cells. (C, D, G, H) Transversal sections through the sinus venosus area of the embryos shown in panels A, B, E, F, respectively. Asterisks in each panel indicate the position of the bead or cell implant. For abbreviations, see the legend of Fig. 1.

cells and Noggin-filled beads into the right sinus horn of cultured chick embryos confirmed the in vitro findings and resulted in a loss of *Tbx18* expression. These data indicate that probably a low level of BMP signaling is required for inducing and/or maintaining PE-specific marker gene expression.

PE-specific gene expression requires a precise control of BMP signaling

Both the implantation of BMP2 and Noggin into the sinus venosus area at HH stage 11 as well as treatment of cultured PE explants isolated from of HH stage 17 embryos resulted in a loss-of-PE-specific marker gene expression. This indicates that a low level of BMP signaling is required to promote PE formation. Sources of BMP signaling are the myocardial expression domain of *Bmp2* in the sinus venosus and *Bmp4*, which is expressed in the PE mesothelium itself. It will be important in the future to measure the actual BMP protein concentration within the PE. This might be accomplished by co-culturing the PE with a BMP reporter cell line. In addition, an analysis of nuclear phospho-Smad1 in the PE cells in comparison to myocardial and pericardial cells would provide important additional information with regard to the actual presence of high and low levels of BMP signaling in the sinus venosus and PE, respectively.

Interestingly, there are several genes that are co-expressed in the intermediate mesoderm and in the PE. Among them are *Wt1*, *Tbx18*, and epicardin. Recent data by the group of Thomas Schultheiss suggests that intermediate mesoderm is specified by low levels of BMP (James and Schultheiss, 2005). It is therefore possible that the gene regulatory elements that govern *Tbx18* expression in the intermediate mesoderm and in the PE share a similar mode of regulation.

The titration experiments have provided evidence for a precise measurement of the BMP concentration by the PE cells. It is well documented that cells within a developing field are able to exactly measure the morphogen concentration. For example, amphibian cells during gastrulation are able to measure the exact amount of occupied activin receptors and that even slight changes (i.e. a 3-fold change) affect the type of genes that are transcribed (Dyson and Gurdon, 1998). Morphogens typically have three to four thresholds (Ashe and Briscoe, 2006). Since increasing concentrations of BMP lead to gradual downregulation of Tbx18 expression while several myocardial marker genes were upregulated in the presence of BMP, we would suggest that in the inflow tract region probably high to intermediate levels of BMP promote cardiac myocyte formation, while proepicardial cell identity requires low amounts of BMP signals. Since in vivo PE cells do not contribute to the myocardial cell lineage (Männer, 1999, Männer et al., 2001), there are probably antagonists present that prevent myocardial cell recruitment of PE cells. BMP is probably only one of the determinants that is active in the inflow tract to allocate cells to the PE and myocardial cell lineages, and further work is required to precisely analyze the inductive interactions within the inflow tract.

The presence of BMP gene expression close to the PE anlage seems to be evolutionary conserved since, in the mouse, *Bmp4* is strongly expressed in the sinus myocardium and septum transversum mesenchyme at day 8.5 and expression is maintained in the septum transversum by day 9.5 (Rossi et al., 2001). At day 10.5, expression is confined to the pericardium and absent from the epicardium (Stottmann et al., 2004). In the chick, *Bmp4* mRNA is observed in the PE and subsequently in the epicardium and subepicardial mesenchyme (Somi et al., 2004). Another tissue that develops in close neighborhood to the PE is the

liver. Interestingly, both the heart and the septum transversum are required for liver formation and recent analysis implicates BMP4 for liver induction (Willier and Rawles, 1931; Rossi et al., 2001; Zhang et al., 2004). Moreover, it was recently found that *Gata4* expression in the mouse PE is under the control of BMP4 signaling and that *Gata4* expression is essential for PE development (Rojas et al., 2005; Watt et al., 2004), suggesting the presence of a regulatory network of gene expression during PE induction that has some similarities with that during heart field formation (Brand, 2003). Most likely aside from BMP, there are probably other signaling inputs that are required for PE formation. FGF1, FGF2, and FGF8 have been shown to be present in the heart and functions as inducers of liver and lung in a dose-dependent manner (Jung et al., 1999; Serls et al., 2005). Whether BMP acts together with FGF to promote PE development requires further studies. Other signaling molecules such as Wnt, Shh, or Notch have not been studied with regard to PE induction, however, it is likely that multiple signaling molecules converge to induce PE formation.

BMP2 and Noggin induce cardiac myocyte differentiation in PE explants

We observed that the addition of both Noggin and BMP2 resulted in an induction of cardiac myocyte formation. At first sight, this is a confusing result given that cardiac myogenesis is known to depend on a BMP signaling input (Andrée et al., 1998; Brand, 2003; Schlange et al., 2000; Schultheiss et al., 1997). However, recent data in ES cells show that both the addition of BMP2 and BMP inhibition by Noggin resulted in enhanced formation of cardiac myocytes (Kawai et al., 2004; Yuasa et al., 2005). Likewise, a two-step model of BMP2-mediated inhibition and induction of cardiac myogenesis has been proposed for the chick embryo (Ladd et al., 1998; Yatskievych et al., 1997). Thus, it appears that heart formation in mammals and birds occurs by multiple steps, first, BMP inhibition promotes cardiac mesoderm formation and subsequently BMP2 becomes an essential factor for heart formation in the embryo (Brand, 2005). Whether such a two-step model also applies to myocyte formation in the epicardial explant culture is an open question.

The fact that BMP2 and Noggin are both able to induce cardiac myocyte formation is most easily understood if one assumes that different cell populations are responding to high BMP concentrations or to the absence of BMP signaling. The PE is a heterogeneous cell population, and the complexity is at present poorly understood. Clonal analysis of differentiation capacity and fate mapping experiments will shed more light on this issue. BMP2 and Noggin induced cardiac myocytes in cells that were in close contact to the mesenchymal core, and induction of cardiac myocytes did not occur in epithelial cells in the periphery of the PE explant. Thus, it is only a subfraction of the PE cells that is able to change its cell fate in response to alterations in the levels of BMP signaling. That the PE is a heterogeneous cell population is for example demonstrated by the fact that *Wt1*, *Cfc*, and *Tbx18* expression is confined to the mesothelium of the PE and these genes were absent from the mesenchymal core of the PE. Potentially, the mesenchyme or alternatively cells in the direct neighborhood are responsive to changes in BMP signaling. Most likely, these cells are not fully committed to the PE cell lineage and either can become (1) cardiac myocytes, or (2) PE mesothelial cells, or (3) go on to form mesenchymal cells that will later become subepicardial mesenchyme. We show that the cells that undergo myocardial differentiation in response to BMP2 treatment were co-expressing WT1. This finding makes it unlikely that the BMP-responsive cells are mesenchymal cells since we have shown that at least in vivo mesenchymal cells do not express WT1 mRNA. Whether this is true for PE cells in culture is not properly studied at present. We also observed that at the mRNA level *Wt1* gets dramatically downregulated, yet the WT1 protein is readily detectable in BMP2-treated PE cultures. The protein product might have a longer half-life time than the mRNA, and thus BMP2-induced downregulation may not be easily visualized by immunofluorescent staining.

Cell transplantation studies using the quail–chick chimera technique as well as cell fate analysis using retroviral cell labeling have revealed that PE-derived cells will not contribute to the myocardial cell lineage in vivo in the chick (Mikawa and Gourdie, 1996; Gittenberger-de Groot et al., 1998; Männer, 1999). Likewise, cell fate analysis in the mouse revealed no evidence for a contribution of PE-derived cells to the myocardial cell lineage (Merki et al., 2005). Thus, despite their ability to undergo cardiac myocyte formation in vitro, PE-derived cells probably do not undergo cardiac myocyte differentiation in vivo.

Interestingly, among the four myocardial marker genes that have been studied here, *Popdc2*, *Hand1*, and *Vmhc1* were all similarly induced by BMP2 and Noggin treatment, however, *Nkx2.5* only responded weakly to both treatments (Fig. 4F). However, there might exist cardiac myocyte formation that does not involve *Nkx2.5*. There is evidence that in mice sinus myocardium develops by an *Nkx2.5*-independent mechanism (V. Christoffels, personal communication). It will be interesting to get further insight into the mechanism of cardiac myocyte formation in the PE explants.

PE development in the chick displays L–R asymmetry

PE development in the chick embryo is subject to left–right (L–R) asymmetry since only on the right side a fully developed PE is formed (Männer et al., 2001). *Cfc* and *Tbx18* are symmetrically expressed in the inflow tract during tubular heart formation at HH stage 10 (Haenig and Kispert, 2004; Schlange et al., 2001). At HH stage 11, *Tbx18* displayed L–R asymmetry being expressed on the right sinus horn and absent on the left side. This asymmetric expression pattern was more pronounced at HH stage 13, and at this stage also *Cfc* and *Wt1* were asymmetrically expressed. Interestingly, using scanning electron microscopy, at HH stage 14/15, bleb-like protrusions of the pericardial mesothelium are found bilaterally at the ventral wall of the right and left horns

of the sinus venosus (Männer et al., 2001). Subsequent to this stage, however, the left PE anlage ceases development and only the right anlage develops into a cauliflower-like accumulation of mesothelial villi. Thus, asymmetric gene expression precedes morphological asymmetry by about two to three stages. Although during the last decade we have learned a lot about how the L–R axis is initially established (Raya and Belmonte, 2004), we lack an understanding of how the heart or any other organ actually utilizes this L–R axis information to transduce it into asymmetric morphogenesis (Männer, 2004). The chick PE might be a good model to get further insight into this process. It appears that this L–R asymmetry is specific to avian embryos since there is evidence that the PE outgrowth in mammals might be present on both sinus horns (Männer et al., 2001). A candidate repressor of PE development on the left side is *Pitx2*, which from HH stage 8 onwards demarcates the left side of the heart tube and is expressed in the left-sided sinus horn and absent from the right (Campione et al., 2001). However, whether *Pitx2* is indeed repressing, PE development on the left side or whether this involves a different transcription factor is presently unknown. The fact however that *Pitx2* is similarly expressed on the left side of the inflow tract in the mouse, which seems to form a PE on the left and right sinus horns, makes it unlikely that *Pitx2* is involved in determining sidedness of PE development in avian embryos.

Possible functions of Cfc in mesothelial cells

The member of the EGF-CFC gene family *Cfc* was found in this study to be specifically expressed in the mesothelium of the PE, the epicardium, and pericardium. At present, it is not clear what function this competence factor might have in these cells. Two possible scenarios can be envisioned. On the one hand, *Cfc* might function as a competence factor for a Nodal-related signaling factor, which might be important for maintaining a mesothelial cell identity (Yan et al., 2002). On the other hand, EGF-CFC factors have been also implicated into a Nodal-independent function. In zebrafish embryos, evidence has accumulated which suggests that, aside from a function as a competence factor, members of the EGF-CFC family might control cell migration (Warga and Kane, 2003). Likewise, it is a common finding in human transformed cells that the *CFC2* gene is over-expressed and tumor invasiveness correlates with a Nodal-independent signaling function, which might be acting through Glypican1 and c-Src (Bianco et al., 2003). Moreover, recent work in *Xenopus* indicates that FRL1, the frog homologue of CFC, might also act as a competence factor for WNT11 (Tao et al., 2005). Loss-of-function experiments are required to further define the function of *Cfc* during PE development.

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