BMP and FGF regulate the differentiation of multipotent pericardial mesoderm into the myocardial or epicardial lineage

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Abstract

Proepicardial cells give rise to epicardium, coronary vasculature and cardiac fibroblasts. The proepicardium is derived from the mesodermal lining of the prospective pericardial cavity that simultaneously contributes myocardium to the venous pole of the elongating primitive heart tube. Using proepicardial explant cultures, we show that proepicardial cells have the potential to differentiate into cardiac muscle cells, reflecting the multipotency of this pericardial mesoderm. The differentiation into the myocardial or epicardial lineage is mediated by the cooperative action of BMP and FGF signaling. BMP2 is expressed in the distal IFT myocardium and stimulates cardiomyocyte formation. FGF2 is expressed in the proepicardium and stimulates differentiation into the epicardial lineage. In the base of the proepicardium, coexpression of BMP2 and FGF2 inhibits both myocardial and epicardial differentiation. We conclude that the epicardial/myocardial lineage decisions are mediated by an extrinsic, inductive mechanism, which is determined by the position of the cells in the pericardial mesoderm.

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Introduction

The embryonic heart tube is formed by fusion of the primary heart fields (Rosenquist and de Haan, 1966; Stalsberg and DeHaan, 1969) and comprises an outer myocardial layer and an inner endocardial layer. As development proceeds, myocardium is added to the posterior and anterior sides of the tube forming the inflow and outflow region, respectively (reviewed in Buckingham et al., 2005; Kelly, 2005; van den Hoff et al., 2004). The addition to the inflow is considered to be a continuous addition of myocardium from the primary heart fields, whereas the myocardium added to the outflow has been suggested to be derived from a distinct heart-forming field, referred to as the anterior or secondary heart field (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). Recent studies suggest, however, that myocardial precursor cells added to the anterior and posterior poles of the linear heart tube are derived from the same pool of precursor cells (Cai et al., 2003; Kelly, 2005; Meilhac et al., 2004), which is referred to as the second lineage and is located medially and dorsally to the cardiac crescent, which encompasses the pool of precursor cells forming the primary heart tube, i.e., the first lineage (Cai et al., 2003; Meilhac et al., 2004). Recent advances have shown that the combined action of bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs) play a crucial role in the differentiation of cardiogenic mesoderm into the myocardial lineage (reviewed in Brand, 2003). Concomitant with ongoing myocardium formation in vertebrates, including man, a subpopulation of mesothelial cells adjacent to the inflow tract (IFT) myocardium proliferates...
and forms villous outgrowths that extend into the pericardial cavity. This cluster of villous outgrowths, which contains numerous mesenchymal cells, is called the proepicardium and provides the precursors for several non-myocardial lineages within the heart including the epicardium, coronary smooth muscle cells, coronary endothelium and cardiac fibroblasts (reviewed in Munoz-Chapuli et al., 2002; Wessels and Perez-Pomares, 2004).

The molecular mechanisms underlying the formation of the proepicardium and the IFT myocardium from progenitor cells in the flanking pericardial mesoderm are largely unknown. In this study, we investigated whether the potential to form myocardium is retained in the formed proepicardium, epicardium and subepicardial mesenchyme. Secondly, we investigated the potential function of BMP and FGF in regulating the recruitment of these progenitor cells to the proepicardium and to the myocardium.

We show that cells of the chicken proepicardium are not irreversibly committed to the epicardial lineage but instead are able to spontaneously differentiate into cardiac muscle cells in vitro. This makes the proepicardial cultures a unique model to study the conditions that favor differentiation towards the myocardial or epicardial lineage. Our data suggest the existence of 3 distinct zones at the inflow tract of the heart: (1) the myocardial differentiation zone, which extends from the most distal IFT myocardium to the proepicardium. In this zone, pericardial mesodermal cells differentiate into cardiac myocytes under the control of BMP2, BMP4 and FGF8; (2) the proepicardial differentiation zone, which comprises the proepicardium proper. In this proper, pericardial mesodermal cells are recruited into the epicardial lineage under the control of FGF2 and, possibly, BMP4; (3) the transition zone, which is located between the two other zones, i.e., the pericardial mesoderm below the proepicardium and adjacent to the IFT myocardium. In this transition zone, the cells are prevented from undergoing myocardial differentiation or recruitment into the epicardial lineage by the combined action of BMP2 and FGF2.

Materials and methods

Chicken embryos

Fertilized chicken eggs were obtained from a local hatchery (Drost BV, Nieuw Loosdrecht, The Netherlands), incubated at 39°C in a moist atmosphere, and automatically turned every hour. After the appropriate incubation times, embryos were isolated in Earle’s balanced salt solution (EBBS, Life Technologies) and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). Embryos used for whole-mount immunostaining were fixed in DMSO:methanol (1:4), for in situ hybridization in methanol:acetone:water (2:2:1), or 4% PFA. Freshly prepared 4% (w/v) paraformaldehyde (PFA) in PBS, and for immunostaining were fixed in DMSO:methanol (1:4), for in situ hybridization in methanol:acetone:water (2:2:1), or 4% PFA. After isolation of HH16 (Hamburger and Hamilton, 1951). Embryos used for whole-mount immunostaining were fixed and immunofluorescently stained as described (Kruithof et al., 2003b).

Fixed whole-mount embryos were hydrated in methanol series, permeabilized with 1% Triton-X100 (v/v) and blocked with PENG (PBS, 5 mmol/l EDTA, 150 mmol/l NaCl, 0.25% (w/v) gelatin). Sections used for immuno/fluorescent staining were hydrated and incubated with TENG-T (10 mmol/l Tris, 5 mmol/l EDTA, 150 mmol/l NaCl, 0.25% (w/v) gelatin and 0.05% (v/v) Tween-20, pH 8.0). Primary antibodies were applied overnight. After extensive washing with PBS, cultures, whole mounts and sections were incubated with fluorescent secondary antibodies (Alexa conjugates; Molecular Probes).

As primary antibodies MF20 (Hybridoma bank, Iowa City, IA, USA), sarco-endoplasmatic reticulum Ca 2+ ATPase (SERCA2a; kindly provided by Dr. F. Wuytack (Eggermont et al., 1990)), myosin light chain 2v (MLC2v; kindly provided by Dr. W. Franz; Lübeck, Germany; (Katus et al., 1982), myosin heavy chain (MHC (169-II-A2); (Wessels et al., 1991), and cardiac Troponin I (cTnI, HyTest, 4T21/2, Breda, The Netherlands) were used to identify myocytes, γ-SMA (ICN; clone B4, Costa Mesa, CA, USA) to identify smooth muscle cells, pan-cytokeratin (Z0622, DAKO) to identify pericardial mesoderm and proepicardium, and anti-BrdU (anti-BrdU pure; BD Biosciences, Alphen aan den Rijn, The Netherlands) to identify incorporated BrdU. Phalloidin–Texas red conjugate (Molecular Probes) was used to visualize all cells in the cultures and propidium iodine (Sigma) or Sytox64 (Molecular Probes) to visualize the nuclei. Fluorescence in the embryos, explants or sections visualized using confocal laser scanning microscopy (Biorad MRC1024). Non-immunofluorescent MF20 staining on sections was performed as previously described (Kruithof et al., 2003b).

The total area of myocytes in the proepicardial explant culture was determined using a user-written macro in NIH-image (version 1.62). Only proepicardial explants that showed monolayer formation after the first overnight incubation were included for further analysis. In the analysis of the data, factor correction is applied to remove multiplicative between-session variation in experiments (Ruijter et al., 2006).

Fluorescent labeling of the proepicardium

Approximately 2 μl of a 1/50 dilution of the stock solution of CCFSE (5,6-carboxy-2,7 dichlorofluorescin diacetate succinimidyl ester, Molecular Probes; 6.26 mg/ml in DMSO) in Pannet-Compton saline (Stern and Holland, 1993) were injected into the pericardial cavity of chicken embryos from stages HH16–17. After a reincubation for 3 h, the proepicardia were carefully excised, extensively washed in EBSS and cultured on collagen gels in M199 with extensive villous processes but no firm attachments to the heart tube were carefully cut at their base (see Fig. 1B) to avoid inclusion of liver primordium or sinus venosus and positioned on top of a drained collagen gel. After overnight incubation to allow proper attachment to the gel, complete M199 medium (M199 medium containing penicillin/streptomycin (Stem and Holland, 1993) 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml selenium (ITS, Collaborative Research Inc.), BMP5 (R&D), FGF2, FGF4, FGF8, FGF10 (Peprotech), recombinant mouse noggin (R&D), SU4984, SU5402 (Calbiochem), or Bromo-deoxyuridin (Sigma) was added individually or in combination to the medium to a final concentration of 50 ng/ml or as indicated. All explants were cultured up to 5 days (37°C, 5% CO2) prior to fixation.

To establish epicardial cell cultures, chicken hearts of HH25 or HH36 were placed on collagen gels. During overnight incubation, epicardial monolayers formed on the surface of the collagen gels. After this overnight incubation, the hearts were removed, and complete M199 medium was added. The culture was maintained for a total of 5 days. To study epicardial formation, proepicardia were put on an agar layer (1%) against the outer myocardial side of an apex of a ventricle or a sinus venosus of an HH16 embryo and cultured in complete M199 medium for 1 day.

To enable immunohistochemical staining of non-attaching proepicardia, cardiac explants, or proepicardia directly after isolation, the explants were positioned on a gel and overlaid with a thin agarose layer (1%; Sigma). An HH16 OPT was included as positive control for the immunostaining.

The gels containing the explants were fixed and immunofluorescently stained as described (Kruithof et al., 2003b).

In vitro explant assay and immunostaining

Collagen gels were prepared according to procedures previously described (van den Hoff et al., 2001). After isolation of HH16–17 embryos, proepicardia...
complete medium as described above. After 24 h, the cultures were fixed with 4% formaldehyde. Colocalization of CCFSE-labeled cells and the MF20-antigen was performed by immunostaining using the MF20 antibody.

Non-radioactive in situ hybridization on sections

The in situ hybridization procedure and the probes used were recently described (Somi et al., 2004a,b).

RNA extraction, cDNA synthesis and PCR

RNA extraction from HH16 embryonic heart or proepicardium, cDNA synthesis and PCR were performed as previously described (Kruijthof-de Julio et al., 2005) using the following primers: fibroblast growth factor (FGF) 2 (S: 5'-GAGAGAGGAGGTTGTGTC-3' and A: 5'-CGTTTCAGTGCCACATA-3') and FGF4 (S: 5'-CAGTCTAGGAAGGAAGTG-3' and A: 5'-CCGACGAGGTGAATATCA-3').
Results

Expression patterns suggest contribution of pericardial mesoderm to the proepicardium and inflow tract myocardium

Lineage studies (Cai et al., 2003; Meilhac et al., 2004), morphological and immunohistological analyses (Kruithof et al., 2003a; Viragh and Challice, 1973) have shown the addition of myocardium to the inflow tract of the heart from the pericardial mesoderm contributing to the atrial appendage myocardium, to smooth-walled dorsal atrial myocardium, and to the caval myocardium (van den Hoff et al., 2004). To study this myocardium formation, whole-mount chicken embryos of HH stage 16 were double immunostained for cytokeratin, which is expressed by the pericardial mesoderm (Pérez-Pomares et al., 1998; Vrancken Peeters et al., 1995) and for the myocardial marker MF20 (Fig. 1). Cytokeratin staining was observed in the pericardial mesoderm, the proepicardium and in the IFT myocardium in which it tapers off (arrow in Fig. 1b). MF20 staining is present in the myocardium of the heart, in the immediate adjacent pericardial mesoderm in which it tapers off (arrow in Fig. 1b), and absent from the proepicardium proper (Fig. 1b). Immunofluorescent staining on sections showed colocalization of cytokeratin and myosin heavy chain expression in the cells of the distal IFT myocardium (Figs. 1c, d). Using the antibody directed against the myocardial marker sarcoendoplasmatic reticulum Ca\(^{2+}\) ATPase (SERCA) 2a, we observed a similar, but slightly broader expression pattern, than we observed for MF20 staining, extending into the mesodermal cells below the proepicardium (Figs. 1g, h). The distal portion of the proepicardium does not express detectable levels of SERCA2a (arrow in Fig. 1b). Double immunofluorescent staining using cytokeratin and SERCA2a also showed that the mesodermal cells adjacent to the inflow tract myocardium coexpressed both markers (Figs. 1j, k). Taken together, these observed reciprocal expression gradients of pericardial/epicardial (cytokeratin) and myocardial (MF20 and SERCA2a) markers, together with their colocalization at the cellular level, suggest the contribution of the pericardial mesoderm to the proepicardium and IFT myocardium (Fig. 1).

Proepicardial cells differentiate spontaneously into cardiac muscle cells

Although cells of the proper proepicardium do not express myocardial markers in vivo (Figs. 1b′, e–h, j), explant cultures of proepicardia derived of HH16–17 chicken embryos often showed a spontaneously rhythmically contracting area after 5 days of culture, suggesting the presence of cardiomyocytes. The presence of cardiomyocytes was confirmed by immunofluorescent staining of these cultures using antisera directed against cardiac proteins (MF20 Fig. 2d′, βMHC, MLC2v or SERCA2a (not shown)).

The presence of the cardiomyocytes in these explant cultures might be due to contamination of the closely adjacent IFT myocardium or due to differentiation of the proepicardial cells during the culture. To address this issue, we fixed proepicardia directly after isolation and assessed for MF20-positive cells. In 40 out of 51 proepicardia (~80%), no MF20-positive cells were observed (Figs. 2a′,e), whereas after 5 days of culture, a large field of MF20-positive cells was present in all 143 analyzed explant cultures (Figs. 2d′, e). These findings indicate that differentiation rather than contamination attributed to the presence of myocardium in the cultured proepicardia.

BrdU had previously been shown to block myocardial differentiation but not the proliferation of already formed cardiomyocytes (Chacko and Joseph, 1974; Montgomery et al., 1994). Proepicardial cultures exposed to BrdU from the start of the culture showed the presence of BrdU in all nuclei but no formation of myocardial cells (Fig. 2k). These results support our conclusion that myocardial cells found in the explant cultures are the result of differentiation from non-myocardial cells, rather than from proliferation of contaminating cells. To minimize the probability of any potential myocardial contamination from the IFT myocardium, proepicardial cultures displaying contractions after 1 day of culture were discarded and refrained from further analyses.

The proepicardium consist of a mesothelial lining that surrounds a mesenchymal core (Pérez-Pomares et al., 1998). To investigate whether the mesothelial lining is able to differentiate into myocardium, the mesothelial lining was fluorescently labeled in ovo (Fig. 2f). Subsequently, the proepicardia were isolated and cultured for 1 day. Upon fixation and staining, a subset (about 20%) of the fluorescently labeled mesothelial cells was found to stain for MF20 as well (Fig. 2g), showing that mesothelial cells lining the proepicardium are able to differentiate into heart muscle cells in vitro.

In precardiac mesoderm explant cultures, distinct stages of myosin assembly and association of myosin-positive cells are considered a characteristic of myocardial differentiation (Eisenberg and Eisenberg, 1999; Rudy et al., 2001). To investigate whether these distinctive changes are also observed in proepicardial cultures, explants were fixed at different time points. One day after being explanted, proepicardial cultures showed small clusters of myocytes and individual myocytes with diffuse myosin staining in the cytoplasm (Figs. 2b′, h), which is characteristic of early myocardial differentiation (Han et al., 1992). After 3 days, several larger clusters of myocytes were present in these cultures (Fig. 2c′) displaying an immature myosin organization as apparent from diffuse and/or dotted myosin staining (Fig 2i). After 5 days, one large myosin-positive field of cells was generally observed (Fig. 2d′). These fields were multilayered and comprised mostly elongated and aligned myocytes in the center, which contained linearly organized myosin (Fig. 2j). Cardiomyocytes with immature myosin organization were observed around the multilayered myocardial center. These observations support the previous conclusion that proepicardial cells are able to differentiate into myocardium in vitro.
Serum-free cultures suggest active inhibition of myocardial differentiation in proepicardium

Next, we investigated whether a population of mesodermal cells in the proepicardial explants is already specified to the myocardial lineage by culturing them under serum-free conditions. In the absence of serum, most of the proepicardia failed to attach properly to the collagen gel and floated in the medium (~80%; Fig. 3a). After 3 days of culture, however, the floating proepicardial aggregates started to display rhythmic contractions, suggesting the presence of cardiac muscle cells. After 7 days of culture, the presence of cardiac muscle cells in these aggregates was confirmed by immunofluorescent staining (Fig. 3a'). If the proepicardium did attach to the collagen gel, two options appeared possible. In the first group, no mesenchymal cells were formed in the collagen gel (Fig. 3b), but cardiac muscle cells were formed, similar to the serum-containing cultures in a multilayered region of the explant, which often displayed beating activity (Fig. 3b'). In the other group, a monolayer was formed without a multilayered region (Fig. 3c), containing a few scattered non-beating MF20-positive cells (Fig. 3c'). Costaining of these cultures for the presence of BrdU showed staining in virtually all nuclei (green). Scale bar in panels a–d, f, g is 200 μm, in panel g 50 μm, and in panels h–j 400 μm.

Fig. 2. Proepicardial cells have the potential to differentiate into myocardial cells in vitro. Bright-field images of characteristic examples of a proepicardial explant directly after isolation (a) and after 1, 3, and 5 days of culture (b–d). These cultures were stained using MF20 (green; a’–d’) to identify the myocytes. Propidium iodine was used to identify the nuclei (red; a’–d’, g, h–j). Panel e shows a summary of the occurrence of myocardium in proepicardial cultures after different culture periods. Panel f shows a section through the proepicardium of an embryo in which the mesothelial cells flanking the pericardial cavity were labeled with CCFSE (green). MF20 staining (blue) after 1 day of culture of CCFSE-labeled proepicardia showed that a subset (about 20%) of the mesothelial cells (green) is able to differentiate into MF20-positive myocytes (arrow in g). The intracellular staining pattern of myosin underscores de novo differentiation of myocytes, because after 1 day of culture, the myosin staining is diffuse in the cytoplasm (h), is dotted after 3 days of culture (i), and is linearly organized after 5 days of culture (j). In line with this notion, we observed that culturing proepicardia in the presence of BrdU blocked the formation of myocyte formation completely, as assessed by MF20 staining (not shown) or cTnI staining (red; k). Costaining of these cultures for the presence of BrdU showed staining in virtually all nuclei (green). Scale bar in panels a–d, f, g is 200 μm, in panel g 50 μm, and in panels h–j 400 μm.
Epicardium and epicardium-derived cells do not differentiate spontaneously into cardiac muscle cells

In vivo experiments analyzing the fate of the proepicardial cells have never identified a contribution to the myocardial lineage (Manner, 1999), suggesting that throughout development, myocardial differentiation is actively inhibited in the derivatives of the proepicardium or that the capacity to differentiate into heart muscle cells is lost. To discriminate these two options, we established epicardial cell cultures of HH25 or HH36 chicken hearts and maintained these under similar conditions as the proepicardial cultures. After 5 days of culture, beating areas were never observed and upon immunofluorescent staining very few, if any, MF20-positive cells were observed (data not shown). These observations suggest that the ability to spontaneously differentiate into cardiomyocytes is lost in proepicardial derivatives.

γSMA expression and mesenchyme formation indicate differentiation into the epicardial lineage

To assess differentiation of proepicardial cells into the epicardial lineages in our explant cultures, we examined smooth muscle marker expression using an antiserum directed against γ-Smooth Muscle Actin (γSMA) (Landerholm et al., 1999; Wada et al., 2003) and the formation of mesenchyme in the gel using phalloidin (Dettman et al., 1998; Morabito et al., 2001). The formation of mesenchyme in the collagen gel is reminiscent of the invasion of the nascent proepicardium by mesothelium-derived mesenchymal cells (Pérez-Pomares et al., 1998) and the
invasion of epicardium-derived mesenchymal cells into the subepicardial space (Dettman et al., 1998; Morabito et al., 2001). Staining of proepicardial cultures for γSMA identified a gradient in its expression (Figs. 4a, b); with no expression in the center of the proepicardial culture (#1 in Figs. 4a, b), high levels of expression in cells that have detached from the edge of the epithelial sheet (#3 in Figs. 4a, b, and c), and low levels of expression in the remainder of the cells (#2 in Figs. 4a, b). In addition, we observed that a subset of mesenchymal cells in the collagen gel expressed low levels of γSMA (Fig. 4d).

**Members of the BMP and FGF families are expressed in the proepicardial region**

The observations that proepicardial cells are capable to differentiate into the myocardial and epicardial lineages raises
the intriguing question about the identity of the underlying molecular mechanisms that direct the differentiation into the different lineages and define the borders between the lineages. BMPs and FGFs have been implicated in the differentiation of cardiomyocytes in the heart-forming region and in the formation of epicardium-derived mesenchyme and the subsequent differentiation of coronary vessels (Lough and Sugi, 2000; Morabito et al., 2001; Schultheiss and Lassar, 1999; Wessels and Perez-Pomares, 2004). To unveil a possible involvement of BMPs and FGFs in the developmental decision of pericardial mesodermal cells to differentiate into the myocardial or epicardial lineage, we first established the expression patterns of BMPs and FGFs at the level of the proepicardium in vivo. BMP2 (Figs. 5c, e1) and BMP5 (Fig. 5f) are expressed in the most distal myocardium of the IFT (Figs. 5a, b) extending slightly into the base of the proepicardium. BMP4 is expressed in the proepicardium complementary to BMP2 and extends slightly into the distal IFT myocardium (Fig. 5d, e2). BMP6 is not expressed in this region (Fig. 5g). BMP7 (Fig. 5h) and FGF4 (Fig. 5m) are expressed throughout the entire heart in the myocardium, although FGF4 tapers off towards the distal myocardial border of the IFT (arrow in Fig.

Fig. 5. The expression patterns of BMPs and FGFs at the level of the proepicardium in HH16 chicken embryos suggest a role in the developmental decisions of the pericardial mesoderm differentiating towards the myocardial or epicardial lineage. Embryos were analyzed using non-radioactive in situ hybridization on sagittal serial sections (b–k, o, p), immunohistochemistry (a, l, m, q) or RT-PCR (n). Each expression pattern is summarized in schematic drawing. For the sake of clarity, the border of the proepicardium is outlined using a dotted line in panels l, m, o–q. Panels e1 and e2 are enlargements of the boxed area in panels c and d, respectively, to show the seemingly complementary expression patterns of BMP2 and BMP4. FGF2 and FGF4 showed typical punctuated expression (l, m, q). The arrowhead in panel l points to FGF2 expression in the extracellular matrix of the proepicardium. Panel m shows that FGF4 expression is lower in the most distal IFT myocardium than throughout the remainder of the myocardium of the heart (arrow in m). Panels o–q are neighboring sections that show the overlapping BMP2 (p) and FGF2 (q) expression in the proepicardium immediate adjacent of the IFT myocardium (o) (arrows). Comparing panels l and q shows a remarkably green fluorescence of the myocardium in panel q. As this difference might be due to the differences in fixation of the tissue, an RT-PCR analysis was performed. Panel n shows FGF2 mRNA in the proepicardium (PE), but hardly in the heart, and FGF4 mRNA in the heart but not in the PE at HH16. Therefore, the staining of myocardium found in panel q is mostly probably due to autofluorescence of the tissue, as a result of the fixation of the tissue. Abbreviations: IFT, inflow tract; PE, proepicardium. Scale bar: 100 μm.
BMP10 and FGF8 are expressed in the distal myocardium of the IFT only (Figs. 5i, j). FGF2 and FGF10 are expressed in the proepicardium (Figs. 5k, l). Because patterns of expression of FGF2 and BMP4 were immunohistochemically determined, we performed a RT-PCR analysis to confirm that FGF4 is expressed in the HH16 chicken heart, and that FGF2 is expressed in the proepicardium (Fig. 5n). The expression patterns of all analyzed BMPs and FGFs, except for BMP6, in the proepicardial region are suggestive of a role in the regulation of the differentiation of pericardial mesodermal cells towards the myocardial or the epicardial lineages.

Myocardium formation at the IFT is induced by BMP2 and BMP4

To evaluate whether BMPs have a role in directing proepicardial cells into the myocardial lineage, we cultured the proepicardial explants in the presence of increasing amounts of a natural BMP inhibitor, Noggin. Noggin was found to inhibit, rather than to block, myocardium formation even at 1000 ng/ml. To better access the level of inhibition, we measured the area occupied by MF20-positive cells in the cultures. In control cultures, the myocardial area increases during the course of the experiment (Figs. 2a′–d′). It has to be considered that after 5 days of culture, these measurements are likely to be an underestimate because of the multilayered nature of the myocardial center. Cultures supplemented with increasing amounts of Noggin showed a concomitant decrease of the myocardial area (Fig. 6a). The observation that the formation of myocardium could not be blocked might be due to the experimental set up, in which Noggin was added to the cultures after allowing the explants to attach to the collagen gel during an overnight incubation. To test this possibility, we equilibrated the collagen gels with Noggin. Also using this condition, we found it impossible to prevent myocardium from being formed (Fig. 6a), suggesting that in addition to BMP, at least one other growth factor is involved in the formation of myocardium in proepicardial explant cultures or that the proepicardium comprises a subpopulation of cells that is already specified to the myocardial lineage. This latter conclusion is in line with our earlier observations on serum-free culture of proepicardia.

Because our in vivo analysis had shown that BMP4 is expressed in the proepicardium, and BMP2 and BMP5 are expressed in the base of the proepicardium, and in the IFT myocardium, we tested whether these commercially available BMPs are capable of influencing myocardium formation in vitro. The amount of myocardium was significantly less when the proepicardial explants were cultured in the presence of BMP5 (Fig. 6b). Supplementing either BMP2 or BMP4 to proepicardial explant cultures, however, increased the amount of myocardium formed (Fig. 6b). Whereas increasing the BMP2 concentration did not result in an increase in the amount of formed myocardium (data not shown), supplementing 50 ng/ml of both BMP2 and BMP4 did result in a significant increase in the amount of formed myocardium compared with 50 ng/ml of BMP2 (Figs. 3e′ and 6b), suggesting that BMP2 and BMP4 utilize different signal transduction pathways. When the gel was conditioned with BMP2, however, most of the cells were found to be cardiomyocytes (Fig. 3f′). BMP2 or BMP4 added to serum-free proepicardial cultures induced myocardium formation in the epithelial monolayer (Fig. 3d′).

The findings that BMP4 is a potent stimulator of myocardium formation in vitro and in vivo is expressed in the proepicardium proper suggest that BMP4 activity needs to be tightly controlled to prevent proepicardial cells from differentiating into cardiac muscle cells in vivo. Interestingly, when proepicardia were cultured in the presence of Noggin not only myocardium formation but also smooth muscle cell and mesenchyme formation was inhibited (Figs. 4e, o). Although BMP4 is, thus far, the only known BMP to be expressed in the proepicardium proper, culturing proepicardia in the presence of BMP4 (or BMP2) did not increase γSMA-expression or the number of mesenchymal cells in the collagen gel (Fig. 4o and not shown). In addition, BMP4 (or BMP2) did not induce mesenchyme formation in serum-free proepicardial cultures (not shown). Taken together, these observations suggest that a yet unknown BMP is required to stimulate epicardial differentiation, or that BMPs are necessary but not sufficient to promote differentiation of proepicardial cells into the epicardial lineage in vitro.

Proepicardium formation is induced by FGF2

To explore whether FGF signaling is involved in the regulation of the differentiation of proepicardium into the myocardial or epicardial lineages, we cultured proepicardia in the presence of FGF2, 4, 8, or 10. In contrast to FGF4, 8, and 10, addition of FGF2 showed a general increase in the size of the proepicardial culture (Fig. 4k), suggesting stimulation of proepicardial cell proliferation. In line with earlier studies (Dettman et al., 1998; Morabito et al., 2001), we also observed that FGF2 increased the number of mesenchymal cells in the collagen gel (Figs. 4n, o) and triggered the formation of mesenchymal cells in serum-free cultures (not shown).

Proepicardial cultures maintained in the presence of either of the FGF Receptor-1 (FGFR-1) inhibitors SU5402 or SU4984 (Calbiochem; Mohammad et al., 1997) remained small (Fig. 4j), and mesenchymal cells were not formed in the collagen gel (Fig. 3h), suggesting that FGF2 signaling through FGFR-1 stimulates proliferation and mesenchyme formation in proepicardial explants. Close examination of the mesenchymal cells that were formed in cultures treated with FGF2 showed that they were relatively small and thin and did not protrude deep into the collagen gel (Fig. 4n). Staining these explant cultures for γSMA showed that γSMA expression was decreased (Fig. 4f), suggesting that differentiation into smooth muscle cells was inhibited by FGF2.

FGF2 prevents myocardium formation in the proepicardium

Although FGF2 stimulated growth of proepicardial cultures in general, the amount of cardiomyocytes formed was not significantly different from control cultures (Fig. 6c)
or cultures supplemented with FGF4 (Fig. 6d) or FGF10 (Fig. 6g). In cultures supplemented with FGF8, on the other hand, the amount of myocardium formed was significantly decreased (Fig. 6e). When proepicardia were cultured on collagen gels that were preconditioned with FGF4 (Fig. 6d), 8 (Fig. 6e), or 10 (Fig. 6g) the amount of myocardium
formed had decreased to a level similar using Noggin. The amount of myocardium formed was even less when the gels were preconditioned with FGF2 (Fig. 6c). Addition of either of the FGF-R-1 inhibitors did not prevent the formation of myocardium in the proepicardial culture (Fig. 3h′), indicating that signaling through the FGFR-1 is not required for myocardium formation.

**Coexpression of BMP2 and FGF2 prevents myocardial and epicardial differentiation**

During development, BMP and FGF signaling are frequently found to cooperate in lineage decisions (Alsan and Schultheiss, 2002; Kudoh et al., 2004; Niswander and Martin, 1993; Thesleff and Mikkola, 2002; Warren et al., 2003; Weaver et al., 2000). To explore whether FGF and BMP signaling also cooperate during the differentiation of pericardial mesoderm, we evaluated the effect of combining various FGFs and BMPs on myocardial and epicardial differentiation. We first analyzed the combined effect of BMP2 or BMP4 together with FGF2 or FGF10 because of their overlapping expression domains (Fig. 5). Addition of FGF10 blunted the stimulatory effect of both BMP2 and BMP4 (Fig. 6g). Addition of FGF2 to proepicardial cultures supplemented with BMP4 resulted also in a block of the stimulatory effect of BMP4 (Fig. 6c). Addition of FGF2 to proepicardial cultures supplemented with BMP2 resulted not only in neutralization of the stimulatory effect of BMP2 but in a significant lower amount of myocardium formed compared to control cultures (Fig. 6c), suggesting a synergistic inhibition of myocardium formation by FGF2 and BMP2. This effect was even more dramatic when collagen gels were preconditioned with FGF2 and BMP2, resulting in a complete block in myocardium formation (Figs. 3g′ and 6c). Also epicardial differentiation was affected by combining BMP2 and FGF2. Although the inhibitory effect of FGF2 on smooth muscle cell differentiation and the stimulating effect of FGF2 on proliferation were not altered in cultures treated with both BMP2 and FGF2 (not shown and 4l, respectively), the stimulatory effect of FGF2 on mesenchyme formation was neutralized (Fig. 4o).

**The position of the border between the myocardium and pericardial mesoderm is regulated by FGF8**

During the set-up of the proepicardial explant cultures, we had observed that myocardium formation was more pronounced when proepicardia were explanted on 300 μl (=standard) then on 500 μl collagen gels (Fig. 6a). Because we had observed that the formation of myocardium in explant cultures is dependent on BMPs, we investigated whether a differential BMP activity in the cultures on 300 μl and 500 μl collagen gels would underlie the difference in the extent of myocardium formation. For this, proepicardia were cultured on 300 μl and 500 μl collagen gels in the presence of different amounts of the BMP inhibitor Noggin. Noggin reduced the myocardial area in both cultures to approximately the same level (Fig. 6a), though a higher concentration of Noggin was required using 300 μl collagen gels. Based on these findings, we inferred that the larger amount of myocardium formed on 300 μl collagen gels is due to a higher intrinsic BMP activity.

Because we have shown that BMPs and FGFs cooperate in epicardial and myocardial formation, we evaluated whether the tested growth factors had different effect in a high (300 μl collagen gels) or low (500 μl collagen gels) intrinsic BMP activity environment. Although the amount of cardiomyocytes formed on 500 μl collagen gels was less than on 300 μl collagen gels, the effects of the tested growth factors were similar, except for FGF8. FGF8 stimulated the formation of myocardium in low intrinsic BMP activity cultures (Fig. 6f), and inhibited the formation of myocardium in high intrinsic BMP activity cultures (Fig. 6e). The finding that the effect of FGF8 on myocardium formation depends on the level of BMP activity was underscored by the observations that addition of BMP2 plus BMP4 to cultures on 500 μl collagen gels (low BMP activity), blunted the stimulatory effect of FGF8 response (Fig. 6f). In high intrinsic BMP activity cultures (300 μl collagen gel), FGF8 was found to neutralize the effects of additionally supplemented BMP2 or BMP2 plus BMP4 (Fig. 6e). Together with the observation that FGF8 is expressed in the most distal IFT myocardium, these findings point to a role of FGF8 in regulation the position of the myocardial–pericardial border at the IFT of the heart.

**BMP4 and FGF4 promote the differentiation into smooth muscle cells**

At the proepicardial stage, FGF4 is expressed throughout the entire heart and strongly decreased in the BMP2-expressing distal myocardium of the IFT (Fig. 5m). Although myocardium formation in proepicardial explants cultured on gels preconditioned with FGF4 was decreased to a level equal as observed with Noggin (Fig. 6d), FGF4 was not found to significantly influence the stimulating effect of BMP2 on myocardium formation (Fig. 6d). These observations suggest that unlike FGF8, FGF4 is not involved in the regulation of the myocardial–pericardial border at the IFT of the heart.

When FGF4 was combined with BMP4, the amount of myocardium formed was strongly reduced (Fig. 6d). The effect of FGF4 and BMP4 on myocardium formation was comparable...
to the effect of FGF2 and BMP2 on standard gels (Fig. 6c). However, when the gels were preconditioned with BMP4 and FGF4, they were not found to completely block myocardium formation like was observed for the BMP2 and FGF2 combination (Fig. 6d). Evaluating the effect of FGF4 and BMP4 on the recruitment of proepicardial cells into the epicardial lineage showed that expression of the smooth muscle marker γSMA was more extensive (Fig. 4g).

**Sinus venosus restrains the formation of epicardium**

Our in vitro analysis, thus far, unveiled the cardiomyogenic potential of proepicardial cells, and furthermore, that IFT myocardium produces factors that stimulate differentiation of proepicardial cells into myocardial cells. The combination of these two properties might underlie why the epicardium is initially formed at the dorsal side of the heart and subsequently envelopes the heart in anterior and posterior direction, rather than forming from posterior to anterior, i.e., from IFT to OFT. Phrased in another way, the primitive epicardial cells need to avoid the cardiogenic signals of the IFT until they are irreversibly committed to the epicardial lineage. To test this hypothesis, we isolated the sinus venosus and the apex of the ventricle of HH16 chicken embryo and positioned an isolated proepicardium against these two different “naked” sources of myocardium (Figs. 7a, d). Inspecting these cocultures by light microscopy, we observed that the proepicardium adhered to ventricular myocardium more efficiently than to sinus venosus myocardium. Immunofluorescent staining of the formed aggregates after 1 day of culture showed that an epithelial cytokeratin-positive sheet of cells had formed over the initially ‘naked’ ventricular

![Fig. 7. Intrinsic differences between myocardial compartments modulate epicardium formation. Panels a, b, d, and e show bright field micrographs of a proepicardial explant (PE) recombined with a ventricular explant (VE) (a and b) or a sinus venosus (SV) (d and e) at the start of the culture (a and d) and after 1 day of culture (b and e). Staining these cultures for cytokeratin (red) and MF20 (green) showed that a cytokeratin-positive epithelial sheet had formed over the ventricle (arrow in c″), whereas only individual cytokeratin-positive cells had populated the sinus venosus myocardium (arrow in f″). Arrowheads point to the absence (c′) or to the presence (f′) of myocardium in the proepicardial portion of the aggregates. Scale bar: 100 μm.](image-url)
myocardium (Figs. 7b, c), whereas no or some individual cytokeratin-positive cells were found on the sinus venosus myocardium (Figs. 7e, f). In addition, MF20-positive cells were observed in the proepicardial part when attached to the sinus venosus (arrow in Fig. 7f) but not when attached to the ventricle (arrow in Fig. 7c'). These data suggest an intrinsic difference between sinus venosus and ventricular myocardium, preventing or allowing epicardium, respectively.

**Discussion**

The proepicardium is a transient structure formed from the mesothelial lining of the pericardial mesoderm adjacent to the IFT myocardium and gives rise to the epicardium, the coronary vasculature and cardiac fibroblasts, which we refer to as the epicardial lineage for the sake of clarity. Culture of a proepicardium on a thick collagen gel revealed the formation of myocardium besides differentiation along the epicardial lineage. This myocardium might be the result of de novo differentiation of proepicardial cells into cardiomyocytes or the result of proliferation of a contamination of the closely adjacent IFT myocardium during the isolation of the proepicardium. To discriminate these two options, we analyzed 51 proepicardia for the presence of cardiomyocytes directly after isolation and found that 80% did not contain any cardiomyocyte, whereas all 143 proepicardia cultured for 5 days contained a large field of cardiomyocytes, indicating that these cardiomyocytes are formed by differentiation. This notion is further substantiated by the observation that BrdU blocked the formation of myocardium in cultures of proepicardia, which is comparable to the block in differentiation of cardiac progenitor cells from HH5 by BrdU (Montgomery et al., 1994). Secondly, we showed that the mesothelial lining of the proepicardium is able to differentiate into myocardial cells, and thirdly, that the intracellular myosin-staining patterns found in the proepicardial cultures are characteristic for myocardial differentiation. These findings seem to be unexpected because quail–chicken chimera and retroviral labeling experiments had never identified a contribution of proepicardial cells to the myocardium in vivo. Nevertheless, our findings are consistent with fate-mapping experiments in *Xenopus*, chicken, and zebrafish that suggested that the region with myocardial potency, the heart field, extends beyond the cells actually contributing to the formed heart (Cohen-Gould and Mikawa, 1996; Lee et al., 1994; Raffin et al., 2000; Serbedzija et al., 1998). Overexpression of the cardiac-enriched transcription factors Nkx2.5 and Nkx2.3 in *Xenopus* (Cleaver et al., 1996) and zebrafish (Chen and Fishman, 1996) resulted in slightly larger hearts but not in the development of ectopic ones, also suggesting that a region of the embryo near the heart has properties that predispose it to a myocardial fate.

In addition, we show γSMA-positive cells and mesenchyme in the proepicardial explant cultures, which are indicative for differentiation into the epicardial lineage. High γSMA expression, however, is not found in mesenchyme that has formed in the gel but rather in scattered epithelial cells at the periphery of the culture. These scattered epithelial cells do not seem to migrate into the collagen gel possibly indicating that this is not a true epithelium-to-mesenchyme transformation. In the mesenchymal cells in the collagen gel, a low level of γSMA expression is found that may reflect the relatively undifferentiated state of the newly formed (pro)epicardial-derived mesenchymal cells in vivo (Pérez-Pomares et al., 2002).

Our explant assays show that the proepicardium, which does not express myocardial (this study) or smooth muscle markers in vivo (Landerholm et al., 1999), has the potential to differentiate into cells of the myocardial and epicardial lineages in vitro, indicating that the proepicardium is composed of precursor pools of cells that are restricted to either the epicardial or myocardial lineage, or one multipotential precursor pool of cells, which has the capacity to differentiate into both the myocardial and epicardial lineage. Data, thus far, are in favor of the latter possibility because of the following observations. Analysis of the expression pattern of myocardial markers (MF20 and SERCA2a) in combination with a pericardial/epicardial marker (cytokeratin) showed coexpression in the pericardial mesoderm adjacent to both the IFT myocardium and the proepicardium proper. The far majority of all cells in proepicardial explants differentiate into cardiomyocytes, when the collagen gels are preconditioned with BMP2 (Fig. 3f'), whereas Landerholm and colleagues had shown that the majority (if not all) of proepicardial cells can differentiate into smooth muscle cells (Landerholm et al., 1999). Classical fate map studies have shown colocalization of the precursors of myocardial, endocardial, and epicardial cells in early mouse and chicken embryos (Garcia-Martínez et al., 1993; Tam et al., 1997). And, in addition, fate map studies using the Cre/Lox system have also revealed a common origin of the myocardium, endocardium and epicardium (Sage et al., 2000; Stanley et al., 2002).

The multipotency of the proepicardial cells raises the intriguing question about the identity of the molecular mechanisms that direct the pericardial mesodermal cells into the myocardial or epicardial lineages and define the border between the two developing lineages. The differentiation into the myocardial or epicardial lineage seems to be mediated by an intrinsic, inductive mechanism, which is determined by the position of the precursor cells in the pericardial mesoderm. We show that BMP2, 4, 5, 7, and 10, FGF2, 4, 8, and 10, are expressed in the region of the IFT of the heart during the development of the proepicardium, suggesting a possible involvement of these BMPs and FGFs in this process. Since proepicardial cells still possess the multipotential characteristics of the pericardial lining, i.e., differentiation towards the myocardial or epicardial lineages, our explant assay provides a unique model to study the involvement of these factors in the molecular mechanisms controlling the developmental switch of the mesodermal cells lining the pericardial cavity into the epicardial or myocardial lineage. We determined the effect of the growth factors on myocardium formation in the in vitro assay by measuring the myosin-positive area. Although the initial appearance of cardiac muscle cells in the proepicardial cultures is established by differentiation, we do not know whether the subsequent increase in myocardial tissue is the result of differentiation or proliferation. The presence of individual cells with immature myosin organization around
the myocardial center suggests that these cardiac muscle cells are the result of differentiation. In the myocardial center, which is comprised of mostly elongated and aligned myocytes containing linearly organized myosin, proliferation is likely to contribute to the increase of the myocardial area after the initial cardiac muscle cells have formed by differentiation. BrdU staining indeed shows that proliferation contributes to the increase of the myocardial center (data not shown). Compared with the myocardial center, the individual immature myocardial cells surrounding the myocardial center, however, shows far less BrdU staining, suggesting that indeed differentiation rather than proliferation accounts for the formation of these myocardial cells. Addition of BMP2 results in an increase in the myocardial area (Fig. 3f). The amount of individual myocardial cells in these cultures has greatly increased, suggesting that BMP2-stimulated differentiation accounts for the increase in myocardial area, rather than stimulation of proliferation. The overall number of cells remains similar in these cultures. On the other hand, addition of FGF2 results in a decrease in the myocardial area. The amount of individual myocardial cells in these cultures has diminished, whereas the overall number of cells has increased, suggesting that FGF2 inhibits the differentiation of myocardial cells and not the proliferation. We are, however, not able to determine the exact contribution of differentiation or proliferation to the amount of formed myocardium. Together, these observations suggest an opposite role for FGF2 and the FGFR-1 in cardiomyocyte formation at the IFT of the heart then in the heart-forming regions (Barron et al., 2000; Lough et al., 1996; Zhu et al., 1999).

Based on the in vivo expression patterns and the effects of the respective growth factors on myocardial and epicardial cell formation in the in vitro explant assay, the region adjacent to the IFT myocardium can be divided into three zones (Fig. 6h): (1) the myocardial differentiation zone expressing BMP2, 4, 5, 7 and 10 and FGF4 and 8, (2) the epicardial differentiation zone expressing BMP4, FGF2 and 10 and (3) the transition zone or precursor zone expressing BMP2, 4, and 5, FGF2 and 10 (Fig. 5). We would like to propose the following developmental mechanism to underlie the elongation of the forming heart tube at its posterior side and the concomitant formation of the proepicardium (Fig. 6h). In the transition zone, pericardial mesodermal cells are exposed to myocardial differentiation-stimulating signals BMP2 and BMP4 and start the cardiac program, as evident from the low but significant expression of myosins and SERCA2a. However, due to the combination of FGF2 and BMP2, further differentiation into the myocardial or epicardial lineage is strongly suppressed.

In the myocardial differentiation zone, i.e., in the absence of FGF2, BMP2 and BMP4 drive the pericardial mesodermal cells into the myocardial lineage which is evidenced by the expression of myosins and SERCA2a. A negative feedback loop in this region is provided by FGF8, of which its action depends on the level of local BMP activity, thereby regulating the positioning of the myocardial border. A role for FGF8 has also been proposed in cardiomyocyte formation from precardiac mesoderm in the heart forming region (Alsan and Schultheiss, 2002) and from the secondary heart field (Waldo et al., 2001).

In the epicardial differentiation zone, i.e., in the absence of BMP2, FGF2 stimulates recruitment of pericardial mesodermal cells into epicardial-lineage, although subsequent smooth muscle cell differentiation is inhibited. A comparable role for FGF2 has been described in hematopoiesis, where FGF2 sustains the proliferation of hematopoietic progenitor cells while maintaining their primitive phenotype (Kashiwakura and Takahashi, 2005). BMP4, on the other hand, is not sufficient but might be necessary for epicardial differentiation. Both FGF2 and FGF10 prevent formation of myocardium. Due to the enlargement of the proepicardium and its inability to attach to the sinus venosus myocardium, it becomes in contact with the myocardium of the dorsal side of the heart. Due to this direct contact between the proepicardium and the myocardium of the heart, the BMP4 and FGF2-expressing proepicardial cells become exposed to FGF4, which further stimulates its differentiation along the epicardial lineage, as well as inhibits the option to form cardiomyocytes (Fig. 4h).

Currently, we are testing whether the proposed molecular mechanism is also operational during development in vivo by implanting growth factor loaded beads, and secondly, we are establishing the molecular change during the transition from proepicardium to epicardium and its derivatives. Progressively restricted expression of either of the receptors or of a component of downstream signalling molecules required for myocardial differentiation throughout development may play a role in the developmental restriction of the cardiomyogenic ability when proepicardial cells differentiate towards the epicardial lineage. If we map out the intersecting molecular genetic pathways that regulate the differentiation of proepicardial cells into epicardial cells and are, therefore, responsible for the loss of the potential to differentiate into cardiomyocytes, we may ultimately find epicardium-derived cells, i.e., the cardiac fibroblasts, a rich source of progenitor cells to be clinically manipulated in order to regenerate a wide array of impaired structures and functions in the ailing heart, including the formation of myocardium.

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