The myocardial potential of proepicardial cells: From development to cardiac regeneration
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Citation for published version (APA):
van Wijk, A. (2010). The myocardial potential of proepicardial cells: From development to cardiac regeneration
Epicardium and myocardium separate from a common precursor pool by crosstalk between BMP- and FGF-signaling pathways

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Circulation Research 2009;105:431-41
Abstract

Rationale
The epicardium contributes to the majority of non-myocardial cells in the adult heart. Recent studies have reported that the epicardium is derived from Nkx2.5-positive progenitors and can differentiate into cardiomyocytes. Not much is known about the relation between the myocardial and epicardial lineage during development, whereas insights into these embryonic mechanisms could facilitate the design of future regenerative strategies.

Objective
Acquiring insight into the signaling pathways involved in the lineage separation leading to the differentiation of myocardial and (pro-)epicardial cells at the inflow of the developing heart.

Methods and Results
We made 3D-reconstructions of Tbx18 gene expression patterns to give insight into the developing epicardium in relation to the developing myocardium. Next, using DiI tracing, we show that the (pro-)epicardium separates from the same precursor pool as the inflow myocardium. In vitro, we show that this lineage-separation is regulated by a crosstalk between BMP- and FGF-signaling. BMP-signaling via Smad, drives differentiation towards the myocardial lineage, which is inhibited by FGF-signaling via Mek1/2. Embryos exposed to recombinant FGF2 in vivo, show enhanced epicardium formation, while a misbalance between FGF and BMP by Mek1/2-inhibition and BMP-stimulation causes a developmental arrest of the epicardium and enhances myocardium formation at the inflow of the heart.

Conclusion
Our data show that FGF-signaling via Mek1/2 is dominant over BMP-signaling via Smad and is required to separate the epicardial lineage from pre-cardiac mesoderm. Consequently, myocardial differentiation requires BMP-signaling via Smad and inhibition of FGF-signaling at the level of Mek1/2. These findings are of clinical interest for the development of regeneration-based therapies for heart disease.
**Introduction**

In contrast to the adult heart, the embryonic heart tube is devoid of non-myocardial cells and an epicardium, consisting of an outer myocardial and an inner endocardial layer separated by cardiac jelly. The heart tube expands by recruitment of progenitor cells from the splanchnic mesoderm at both poles of the heart. The formation of the majority of non-myocardial cells starts with the development of the proepicardium from splanchnic mesodermal cells at the inflow of the heart. Its villous outgrowths extend into the pericardial cavity, attach to the atrioventricular canal and gradually envelop the entire ‘naked’ heart tube, deriving the epicardium. A subset of epicardial cells undergoes epithelial-to-mesenchymal transformation. The formed subepicardial mesenchymal cells contribute to the non-myocardial component of the heart i.e. the coronary vessels and the cardiac fibroblasts. In the adult heart, the non-myocardial component occupies approximately 25% of the myocardial volume, but comprises 60-70% of the cells.

Recently, it was reported that (pro-)epicardial cells are derived from Nkx2.5-expressing progenitors and contribute to a small proportion of the cardiomyocytes. This finding is underscored by the observation that explanted proepicardial cells spontaneously differentiate into contractile cardiomyocytes. These findings suggest that the proepicardium is derived from pre-cardiac mesoderm, rather than from the septum transversum, i.e. splanchnic mesoderm adjacent to the pre-cardiac mesoderm. The molecular mechanism that regulates the separation of pre-cardiac mesoderm into (pro-) epicardial and myocardial cells is not known. Understanding this mechanism might help the development of regenerative approaches to induce differentiation of epicardial or epicardial-derived interstitial cells into cardiomyocytes.

In this study we show that pro-epicardial cells and inflow myocardial cells are derived from a common precursor pool of cells, using Dil labeling analysis. This precursor pool is directed into the myocardial lineage by BMP-signaling via Smad or into the (pro-)epicardial lineage by FGF-signaling via Mek1/2. Mek1/2-mediated FGF-signaling is dominant over BMP-signaling. In embryos treated with BMP2 and the Mek1/2 inhibitor (U0126), epicardial development was blocked and inflow myocardium formation enhanced. Treatment with FGF2 revealed a reciprocal phenotype, showing stimulated epicardium and inhibited inflow myocardium formation. Taken together, we show that BMP-mediated myocardial differentiation is inhibited by FGF-signaling via Mek1/2 and Erk1/2 which is required to separate the epicardial lineage from a common progenitor pool.
**Materials and Methods**

**In vivo assay**

After 48h of incubation, eggs were windowed and stage 11 embryos were injected with BMP2 (50ng/ml), FGF2 (50ng/ml) and/or U0126 (10μM) into the yolk-sac, taking into account the diluent volume of the egg. Control embryos were injected with growth factor solvent. After 24 hours the embryos (stage16) were isolated or re-injected, and incubated for another 24 hours (stage19-20). At least three embryos per group were used for in situ hybridization and 3D-reconstruction.12

In situ hybridization13, immunohistochemistry14, Dil-labeling15 and pro-epicardial explant cultures9, were performed as described previously. For an expanded version of the Materials and Methods: see Supplement Material.

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**Figure 1. Expression patterns of Tbx18 at the inflow of the heart**

Tbx18 mRNA expression starts in the splanchnic mesoderm covering the vitelline veins at stage 11 (a,b). At stage 16, pro-epicardial and myocardial cells of the inflow are Tbx18 positive (c,d). One day later, stage 21, the epicardium and the myocardial sleeves covering the sinus horns are Tbx18 positive (e,f). Dashed lines indicate the position of the respective sections. Abbreviations: left vitelline vein (lvv), right vitelline vein (rvv) neural tube (nt), foregut (fg), inflow tract (ift), out flow tract (oft), right atrium (ra), left atrium (la), ventricle (v), proepicardium (pe), right cardianal vein (rcv), left cardinal vein (lcv), vitelline vein (vv), Liver (li), epicardium (epi)
Results

Proepicardium and inflow myocardium develop from a Tbx18-positive population

Whole mount in situ hybridization showed that the transcription factor Tbx18 is expressed upstream of the linear heart tube and subsequently becomes confined to the proepicardium in mouse and chicken. Functional disruption of Tbx18 results in normal development of the epicardium but aberrant muscularization of the cardiac inflow. Moreover, lineage analysis showed that the Tbx18 population contributes to both the myocardial and epicardial lineages. To explore the relation of the Tbx18 expressing cells of the forming proepicardium and inflow myocardium, 3D-reconstructions were prepared of serial sections stained for Tbx18 and a myocardial marker (VMHC or cTnI). At stage 11, Tbx18 expression can be seen (Figure 1a and 1b) in mesodermal cells covering the vitelline vein. In this region all

Figure 2. Tracing the splanchnic mesoderm at the inflow of the heart

At stage 11, a Dil label is placed in the splanchnic mesoderm covering the right vitelline vein (a,a'), which is traced to cells of the proepicardium and myocardium of the inflow tract (b,b') after 24 hours of incubation (stage 20). Sections showing the presence of the label in the proepicardium at stage 16 (c,c') and the myocardium of the inflow of the heart at stage 20 (d). When the label is placed at the left vitelline vein only inflow myocardial cells are traced after 24 hours of incubation (f).
cells express Tbx18 though cardiac sarcomeric proteins and morphological characteristics of proepicardial development are absent. At stage 13, proepicardial villi start to develop within the Tbx18 positive area. Proepicardial villi at the left vitelline vein disappear whereas those at the right side expand and reach the dorsal aspect of the atrioventricular canal at stage 17. At this stage, Tbx18 is observed in the flanking inflow myocardium (Figure 1c through 1f), being more extensive at the right side. At stage 21, the entire inflow myocardium is Tbx18-postive and the epicardium now covers the entire dorsal aspect of the heart and has started to envelop the ventral side. This expression analysis suggests that the Tbx18-positive mesodermal cells covering the vitelline veins contribute to both epicardial and inflow myocardial cells.

To explore this hypothesis, we placed a Dil-label within the Tbx18-positive population covering the right vitelline vein at stage 11 (Figure 2). At stage 16 the labeled cells were found in both the proepicardium and inflow myocardium (Figure 2b through 2d). When cells of the left vitelline vein were labeled, the label was only found in the myocardium (Figure 2f). These experiments, together with the expression analysis and lineage analysis in mice, suggest that the proepicardium and inflow myocardium develop from a common progenitor pool.

Co-localization of P-Erk and P-Smad in the proepicardium
Explant assays have shown that proepicardial cells can differentiate into myocardium, and that BMP2 stimulates, and FGF2 inhibits myocardium formation suggesting that these factors are regulators of the separation of the myocardial- and epicardial-lineages. Immunofluorescently marking the canonical BMP- and FGF-signaling pathways (Figure 3b) in vivo reveals the nuclear localization of P-Smad in the pro-epicardial and flanking myocardial cells (Figure 3a and 3b). In the distal part of the proepicardium P-Smad can also be observed in the cytoplasm (Figure 3b’), In these cells P-Erk could also be seen (Figure 3c and 3c’). The cytoplasmatic localization of P-Smad in the presence of P-Erk and the nuclear localization of P-Smad in the absence of P-Erk are indicative for an intracellular interaction between these pathways. Such an interaction has been shown in Xenopus, in which P-Erk phosphorylates P-Smad, resulting in abrogation of BMP-signaling due to cytoplasmic P-Smad accumulation and degradation (Figure 3d). A comparable interaction may also be operational in the separation of the progenitor population into the pro-epicardial and myocardial lineages.

Mek-mediated FGF-signaling inhibits Smad-mediated BMP-signaling
To evaluate whether FGF-signaling via Mek and Erk effects Smad phosphorylation, rat cardiomyocyte-like-cells (H10-cells) were treated with U0126 in the absence or presence of BMP2 and/or FGF2. Western blot analysis revealed that P-Erk could be detected in the absence or presence of FGF2 (Figure 3e), indicating that FGF-signaling is endogenously active in H10-cells. Phosphorylation of Erk is strongly inhibited by U0126 even in the presence of FGF2. Stimulation of H10-cells with BMP2 induced low levels of P-Smad after 15 minutes, becoming
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Figure 3. **BMP- and FGF-signaling at the inflow of the heart**

Immunofluorescent images of the proepicardium and flanking inflow myocardium showing, cardiac Troponin I (a), P-Smad (b) and P-Erk1/2 (c). The panels a’-c’ show and overlay the respective patterns with the nuclei. Panel d schematically shows the interaction of BMP-signaling via Smad (1) and FGF-signaling via Erk1/2 (2). Erk1/2 phosphorylation of P-Smad results in abrogation of BMP-signaling due to cytoplasmic P-Smad accumulation and degradation.\(^{18,19}\)

H10-cells have elevated levels of P-Erk, which can be inhibited by the Mek1/2 inhibitor U0126 (e). Phosphorylation of Smad upon BMP2 stimulation is increased when Mek1/2 is inhibited (f). Pretreatment with FGF2 inhibits the phosphorylation of Smad upon BMP2 stimulation (g). This interaction was also shown in freshly isolated pro-epicardial cells, the dashed line indicates the position of the section (h). BMP2 stimulated Smad phosphorylation which is inhibited by simultaneous addition of FGF2. When Mek1/2 was inhibited, Erk1/2 was not phosphorylated upon FGF stimulation. The inhibiting effect of FGF2 on Smad phosphorylation was uncoupled by addition of U0126 in combination with BMP2+ FGF2 (i).
**Graphs**

Effect of U0126 on myo-area and cell number

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**Number of cells per cell type**

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**References**

BMP2 FGF2

BMP2+FGF2

control

- U0126

+ U0126
Figure 4. The effect of Mek1/2 inhibition on myocardium formation in vitro

Representative examples of control (a), BMP2- (b), FGF2- (c) and BMP2+FGF2-treated (d) pro-epicardial cultures in the absence (a-d) or presence of U0126 (a'-d') after 5 days of culture in which myocardium is visualized in red (cTnI) and nuclei in green. e: The graph shows the changes in the area of myocardium (y-axis) and the number of nuclei (x-axis) in each of the conditions (mean +/- SEM). Inhibition of Mek1/2 (grey arrows) decreases the number of cells without affecting the myocardial area in control and BMP2-treated cultures, whereas in FGF2- and BMP2+FGF2-treated cultures the myocardial area is increased without affecting the total cell number. f: The bar graph shows the effect of the treatments on the number of myocardial- and non-myocardial cells. Parameters that are not significantly different are represented at the level of their geometric-mean-value. In control and BMP2-treated cultures, U0126 affects the number of non-myocardial cells without affecting the number of myocardial cells, while U0126 increases the number of myocardial cells in FGF2 and BMP2+FGF2-treated cultures. g: Cultured pro-epicardia were analyzed by qRT-PCR and compared to myocardial (SV: sinus venosus; A: atrium, and V: ventricle) and epicardial cells. Panel h shows the expression of Tbx18, Nkx2.5, and cTnI in the proepicardium and flanking sinus venosus.

Mek inhibition prevents the dominant effect of FGF-signaling on BMP-induced myocardial differentiation

To evaluate the interaction between BMP- and FGF-signaling on the differentiation of pro-epicardial cells, the myocardial area, the number of myocardial and non-myocardial cells was determined in cultured proepicardia (Figure 4a through 4d, Online Figure I and Online Table I). In control, BMP2- or FGF2-treated explants the total number of cells was not significantly different after 5 days of culture. In BMP2+FGF2-treated cultures the total number of cells was significantly (p<0.05) larger from day 2 onwards. Cardiomyocytes were virtually absent from FGF2- and BMP2+FGF2-treated cultures. In controls and BMP2-treated cultures

more prominent after 30 minutes (Figure 3f). H10-cells treated with U0126+BMP2, reveals high levels of P-Smad after 15 minutes (Figure 3f), showing an inhibiting effect of FGF-signaling via Mek on Smad-mediated BMP-signaling. To further substantiate the inhibition of FGF-signaling on Smad-phosphorylation, H10-cells were pre-incubated with FGF2 and subsequently stimulated with BMP2. Pre-incubation with FGF2 reduced the level of P-Smad in BMP2- and in BMP2+FGF2-treated cells compared to cells that were not pre-treated with FGF2 (Figure 3g).

To establish whether this interaction is operational in pro-epicardia, freshly isolated pro-epicardia were stimulated with BMP2, resulting in phosphorylation of Smad (Figure 3h and 3i). Reduced Smad-phosphorylation was found when proepicardia were simultaneously stimulated with FGF2. Stimulating with FGF2 alone did not effect Smad-phosphorylation. The inhibiting effect of FGF-signaling on Smad-phosphorylation was abolished when U0126 was added, indicating that also in proepicardia FGF-signaling via Mek and Erk mediates an inhibitory effect on BMP-induced Smad-phosphorylation.

Taken together, these results suggest that Smad-mediated BMP-signaling recruits progenitor cells into the myocardial lineage and Erk-mediated FGF-signaling inhibits Smad-mediated BMP-signaling, preventing differentiation of the proepicardium into the myocardial lineage.
cardiomyocytes were observed from 2 days onwards. At 5 days of culture the myocardial area was 2.1-fold larger in BMP2-treated cultures compared to controls. Calculating the average cardiomyocyte size showed a similar size in all conditions (279 ± 80μm²), indicating that the changes in myocardial area are the result of de novo differentiation rather than of hypertrophy. These observations suggest that the inhibition of myocardial differentiation by FGF2 is dominant over the stimulatory effect of BMP2. To further substantiate this hypothesis, proepicardia were cultured in the presence of U0126 (Figure 4a’ through d’). In control and BMP2-treated cultures, U0126 caused a significant reduction of 64% and 40%, respectively, in the total number of cells without significantly altering the myocardial area, number or size of cardiomyocytes.

However, in FGF2-treated cultures, U0126 addition resulted in a 23% increase in total cell number with myocardial formation returning to control levels. In BMP2+FGF2-treated cultures U0126 did not significantly influence the total cell number but like FGF-treated cultures, myocardial area returned to control levels (Figure 4e and f and Online Table II).

The relative contribution of myocardial and non-myocardial cells under the different conditions was calculated and plotted (Figure 4f), showing that in control and BMP2-treated explants U0126 reduces the number of non-cardiomyocytes. FGF2-stimulation results in an increase in the non-cardiomyocyte population. Upon FGF2- or BMP2+FGF2-treatment virtually no cardiomyocytes are formed. Under these conditions co-treatment with U0126 reverses the inhibitory effect on cardiomyocyte formation to control levels, without inhibiting the stimulatory effect on non-cardiomyocyte formation.

Taken together these data show that BMP-mediated myocardial differentiation is inhibited by FGF-signaling via Mek1/2 and Erk1/2. Shifting the balance in favor of BMP2-signaling by adding BMP2 and simultaneously inhibiting Mek1/2-mediated FGF-signaling results in an almost complete differentiation of pro-epicardial cells into cardiomyocytes.

**Phenotypic characterization of the explants**

The expression levels of genes characteristic for the myocardial and epicardial lineages were determined using qRT-PCR and compared between HH16 proepicardium, sinus venosus, atrium, ventricle, and HH24 epicardium (Figure 4g). In control and U0126+BMP2-treated cultures, the expression levels of the myocardial genes, AMHC, VMHC, and BNF, closely resembled the expression observed in atria. Upon FGF2- or BMP2+FGF2-treatment the expression levels of myocardial genes were lower. In control explants the expression levels of Cx43, a marker for working myocardium, was approximately 2-fold higher than in atrial samples and more than 3-fold higher than in BMP2+FGF2-treated explants. In FGF2- or BMP2+FGF2-treated explants the expression was at a similar low level as in proepicardium. In situ hybridization analysis showed that Tbx18-expression tapers off and Nkx2.5-expression gradually increases in the sinus venosus myocardium (Figure 4h). Taken together, these analyses indicate that proepicardial explants differentiate into working myocardium during the culture and BMP2+U0126 further promotes this differentiation.
Analysis of the epicardial marker genes showed that, in line with previous reports, Tbx18 and Raldh2 are down-regulated during culture and are hardly effected by the different culture conditions. Expression of γ smooth muscle actin (SMA), a marker for coronary smooth muscle cells, increased in control explants, was not effected by BMP2+FGF2- or BMP2+U0126-treatment and was highest when epicardial differentiation is promoted by FGF2. Flk1, a marker for endothelial/endocardial cells, decreased during culture compared to proepicardium. Based on Flk1 and γ SMA expression, proepicardial cells differentiate in coronary smooth muscle cells rather than into endothelial cells. When treated with BMP2+FGF2, proepicardial cells showed an almost 2-fold higher expression level of Flk1 compared to 5 days cultured controls. Taken together, the analyses of the epicardial marker genes suggest that proepicardial cells differentiate along the epicardial lineage. When myocardial differentiation is inhibited by FGF2 or BMP2+FGF2, differentiation into the smooth muscle cell lineage or endothelial/endocardial lineage, respectively, is promoted.

**Altering BMP- and FGF-signaling in vivo**

To uncouple FGF- and BMP-signaling in vivo, we injected eggs prior to pro-epicardial induction (stage 11) with BMP2, FGF2 or growth factor solvent in combination with or without U0126. After 24 hours of re-incubation the embryos (stage 16 to 17) were isolated and none of the embryos showed gross morphological abnormalities. To visualize all myocardium (VMHC or cTnl) and the proepicardium and inflow myocardium (Tbx18) in situ hybridization was used. In all (n=10) embryos treated with U0126+BMP2 a small sac-like proepicardium without villous projections had developed and epicardium consistently failed to develop (Figure 5a and 5b). Within the rudimentary proepicardium myocardial strands were present. The efficacy of this treatment was assessed by immunohistochemical staining for P-Smad and P-Erk (Supplemental Figure II). In the FGF2-treated embryos (n=3), epicardium formation was enhanced compared to control embryos (n=3) (Figure 5a and 5c). The epicardium had almost completely enveloped the atrioventricular canal, which is normally observed at stage 18. In BMP2-treated (n=3) or U0126-treated (n=3) embryos the proepicardium was similar to controls (Supplemental Figure III).

After 48 hours of re-incubation all isolated embryos showed morphological characteristics that qualified them as stage 20 embryos (Supplemental Figure IV). Treatment with either U0126 (n=3) or BMP2 (n=3) revealed no abnormalities (Supplemental Figure III). FGF2 and U0126+BMP2-treated embryos displayed abnormalities in epicardium and inflow myocardium. To identify abnormalities in the Tbx18- and cTnl-expression domains 3D-reconstructions were made. In control embryos the epicardium covered the dorsal aspect of the ventricle and the entire atrioventricular canal (Figure 6a). In U0126+BMP2-treated embryos (n=8), epicardium failed to develop; the proepicardium remained a small sac-like structure without villous protrusions (Figure 6b). The myocardium of the ventricles was thin, showed no compact layer and hardly any trabeculae. This abnormality is most probably secondary to the absence of an epicardium. Compared to the controls, extensive
myocardial sleeves covering the sinus horns were present (Figure 6b and 6e). In FGF2-stimulated embryos (n=2), on the other hand, (pro)epicardial cells had formed a bridge over the entire length of the systemic inflow that was attached to the atrioventricular canal, while inflow myocardium formation was diminished (Figure 6c and 6f, Online 3D-PDF).

Taken together, these data show that the interaction between FGF- and BMP-signaling at the level of Mek1/2 is important in the regulation of differentiation of progenitor cells at the inflow of the heart. Simultaneous inhibition of Mek-mediated FGF-signaling and stimulation of BMP-signaling results in preferential differentiation of progenitor cells into myocardial cells at the expense of epicardial cells, while stimulation of FGF-signaling leads to a reciprocal phenotype (Figure 7a).

**Inhibition of epicardium formation is not the result of changes in apoptosis or proliferation**

During normal development proepicardial differentiation is induced at both vitelline veins. At the left side the proepicardial villi disappear while the right-sided proepicardial villi expand.16 To establish the role of apoptosis or proliferation in the inhibited proepicardia and increased inflow myocardium formation in U0126+BMP2-treated embryos, we performed TUNEL staining and analyzed BrdU incorporation. In control and U0126+BMP2-treated embryos, no TUNEL-positive cells could be detected in the inflow region while in other regions of the embryo TUNEL-positive cells were present as expected (Figure 5e and 5g).20 No differences in proliferation index between (pro)epicardial and inflow myocardial cells and between control and U0126+BMP2-treated embryos were found (2-way ANOVA p=0.116 and p=0.701, respectively). These findings further support the idea that the observed changes in (pro-)epicardium and inflow myocardium formation are due to a shift in the separation of precursor cells into the myocardial and (pro)epicardial lineages.

**Figure 5. 24h treatment of developing embryos** (previous page)

In embryos treated with U0126+BMP2 for 24h before sacrifice, the proepicardium is a small sac-like structure (b) compared to controls (a) (arrow heads), and in the base of the rudimentary proepicardium myocardial strands are present (red arrow) (b). In FGF2-treated embryos an epicardium has already started to develop (black arrow) (c). In control and U0126+BMP2-treated embryos, proliferation is similar as assessed by BrdU incorporation (red) (d,f). TUNEL assays showed no apoptotic cells in the proepicardium of control (e) or U0126+BMP2-treated embryos (g).
**Figure 6. 48h treatment of developing embryos** (p. 108 & 109)

In embryos treated with U0126+BMP2 for 48 hours before sacrifice, the epicardium is absent (b') while in control embryos almost the entire myocardium is covered with epicardium (a'). In FGF-treated embryos proepicardium formation takes place in a larger area of the venous pole of the heart, which coincides with smaller myocardial sleeves covering the sinus horns (c,f). 3D-reconstructions of the heart of a control (d) and U0126+BMP2-treated (e) shows that the epicardium is absent and the inflow myocardium is more extensive in U0126+BMP2-treated embryos. At the outflow pole the myocardial border is located closer to the aortic arch arteries (arrow head). The dashed lines in the 3D-reconstructions indicate the position of the sections shown in panels a-c. (See also Online. 3D-PDF).
Figure 7. Model of separation of epicardial and myocardial cells from progenitors by BMP2 and FGF2

Balanced BMP2+FGF2-signaling drives proliferation of progenitors. When the balance shifts in favor of FGF-signaling via Erk1/2, the progenitors differentiate into epicardial cells. Shifting the balance in favor of BMP-signaling via Smad results in myocardial differentiation. BMP-signaling is inhibited by FGF-signaling via Erk, which leads to cytoplasmic accumulation and degradation of P-Smad (a). A similar interaction of BMP- and FGF-signaling might still be operational in epicardium-derived cells in the formed heart (e) because cytoplasmic P-Smad (c) en P-Erk (d) are present in the subepicardial mesenchyme. The dashed line highlights the boundary between myocardium and epicardium based on cTnI staining (b-b'). Panels b'-d' show an overlay of all nuclei. Panel e shows the position of the enlargements (b-d) in the heart.
**Discussion**

**Myocardial differentiation of Tbx18-positive splanchnic mesoderm upon Smad-mediated BMP-signaling is inhibited by Mek-mediated FGF-signaling**

The early heart tube increases in length by recruitment of myocardial cells from flanking mesoderm and becomes ensheathed by an epicardial layer. Using gene expression analysis (Figure 1) and Dil-labeling (Figure 2), we show that caudal of the inflow of the heart a Tbx18-positive progenitor population contributes to both inflow myocardium and epicardium. Regarding the Dil-labeling experiment we are aware that this experiment cannot exclude the possibility that two different progenitor pools are already present at stage 11. Based on the Dil-labeling experiment and gene expression analyses we conclude that a small homogenously Tbx18-expressing group of cells gives rise to myocardial and pro-epicardial cells. In situ hybridization analyses showed that the mesoderm covering the vitelline veins as well as the proepicardium and adjacent inflow myocardium stain homogeneously for Tbx18 (Figure 1). No morphological signs of epicardial differentiation are visible, and no signs of myocardial differentiation are evident, suggesting that progenitors have not yet made a lineage split at stage 11. Moreover, the pro-epicardial cells still possess the capacity to differentiate into cardiomyocytes in vitro (Supplemental Figure I), also suggestive of a common progenitor pool.

Initial proepicardial explant analyses have shown that BMPs and FGFs influenced myocardium and/or epicardium formation. Gene expression analyses showed that BMP2 is expressed in the proepicardium and inflow myocardium and FGF2 in the stroma of the proepicardium. In this study we focused on the signaling pathways conveying BMP2 and FGF2 signals and their interaction, BMP2 being the strongest stimulator of myocardial formation and FGF2 preventing spontaneous and BMP-induced myocardial formation in vitro (Supplemental Figure I).

During development FGF-signaling is transduced by three major pathways, PI3Kinase, PLCg, and Erk1/2. Testing the effect of inhibitors on the various pathways showed that the inhibiting effect on myocardium formation of FGF2-signaling is transduced via Mek and Erk (Figure 4 and data not shown). This finding was further supported by Western blotting and immunofluorescence, showing P-Erk expression in the proepicardium. BMP-signaling is transduced by two major pathways, Smad and p38. Inhibition of the p38 pathway did not effect myocardium formation (data not shown). Smad inhibitors are not commercially available, but western blotting and immunofluorescence showed different expression of P-Smad in the proepicardium and inflow myocardium. Interestingly, in the region of the proepicardium that expresses P-Erk, P-Smad is located in the cytoplasm. In Xenopus, phosphorylation of the linker in Smad by P-Erk results in a disruption of BMP-mediated Smad-signaling. In line with this finding we found that stimulating explants with BMP2+FGF2 prevents myocardium formation and inhibiting FGF-signaling, using U0126, in the presence of BMP2 strongly stimulated myocardium formation; on average 75% of the
cells in the explant cultures differentiated into cardiomyocytes. (Figure 4) Moreover, directly challenging pro-epicardia with these substances showed the expected changes in P-Erk and P-Smad on Western blots (Figure 3i). This finding probably also underlies the spontaneous differentiation into cardiomyocytes upon explanting, the FGF concentration becomes diluted thus diminishing the FGF-inhibition of BMP-mediated myocardial differentiation.

To evaluate whether this interaction between FGF- and BMP-signaling is also operational in vivo, embryos were treated in ovo. In line with our in vitro observations the combination of U0126+BMP2 resulted in enhanced myocardial differentiation at both poles of the heart (Figure 6a and 6b), blocked expansion of the proepicardium, and consequently, the epicardium did not develop. FGF2 alone resulted in reduced inflow myocardium formation and enhanced epicardium formation. A potential pitfall of the in vivo model is that the entire embryo is exposed, which, as a consequence, might influence other organs as well. Approaches for local administration failed to provide a continuously effective concentration of the applied factors; most probably due to dilution in the egg. However, upon visual inspection of forming limbs, somites, etc, the development of the treated embryos appeared normal. Liver development has been reported to be inhibited by U0126.24 Only in U0126+BMP2-treated embryos we could observe a mild inhibition of hepatogenesis (data not shown). The difference in effect of U0126 on the liver is probably due to a dose difference, 10 versus 50μM. An indirect effect of U0126+BMP2 via the liver on the progenitor pool is unlikely because of the presence of a developing liver, the presence of Tbx18 which is induced by signals from the liver,25 and the consistence between the in vitro and in vivo results. Finally, in the FGF2-treated embryos no ectopic induction of Tbx18 was observed, indicating that FGF2 is most likely not the liver-derived inductor of Tbx18 in this progenitor population.25

Epicardium and regeneration
The finding that inflow myocardial and epicardial cells originate from a common Tbx18-expressing progenitor pool, is underscored by several knockout phenotypes showing inflow and/or epicardium defects. Tbx18 knockout mice show delayed formation of the myocardial sleeves covering the sinus horns, though epicardial development appears normal.17 Podoplanin, a coelomic and myocardial marker, knockout mice have both epicardial and inflow myocardial defects.26 When the mammalian homolog of Caenorhabditis elegans polarity proteins, PAR3 (PARD3 – Mouse Genome Informatics), is deficient, epicardial development is defective.27

The common origin of epicardial and myocardial cells led to the idea that epicardially-derived cells might play a role in cardiac regeneration and serve as a source for cardiomyocytes. Initial observations in zebrafish support this idea. Upon ventricular amputation the myocardium regenerates. During regeneration embryonic genes, like Tbx18 and Raldh2, are reactivated in the epicardium, and new cardiomyocytes are formed from progenitor cells.28 The origin of these progenitors is unclear, but the epicardium is a potential candidate.
Whether adult mammalian epicardium is able to behave similarly upon myocardial injury, remains unclear. Nevertheless, a role of the epicardium during regeneration is plausible based on the following findings; (1) c-Kit-positive cells are present in the subepicardial mesenchyme,\textsuperscript{29,30} (2) Wt1 expression is induced in coronary vessels at an infarct's border zone,\textsuperscript{31} (3) Subepicardial mesenchyme starts to proliferate after myocardial infarction.\textsuperscript{32} (4) A subset of adult cardiac stem cells can differentiate into coronary vessels when injected in infarcted hearts.\textsuperscript{33} In this respect it is of relevance to note that the coronary arteries have a (pro)epicardial origin.

Altogether, these observations suggest that the (pro)epicardium and epicardial-derived cells contribute to the cardiac stem cells population observed in several studies.\textsuperscript{34,35} In this respect, the presence of both P-Smad and P-Erk in stage 33 epicardium indicates that at later stages a balance between FGF and BMP-signaling might be operational (Figure 7b through 7d). Differentiation of these progenitors into cardiomyocytes may also require a shift in the balance between BMP- and FGF-signaling in favor of BMP by Mek1/2-uncoupling, offering promising inroads to cardiac regeneration from (pro)epicardial-derived cells.

**Acknowledgements**

We thank Mr J Hagoort for preparing the 3D-PDF.

**Sources and Funding**

This work was supported by the Netherlands Heart Foundation 1996M002 (B.v.W., G.v.d.B., M.J.B.v.d.H. and A.F.M.M.); European Community’s Sixth Framework Program contract (‘HeartRepair’) LSHM-CT-2005-018630 (M.J.B.v.d.H, S.v.d.V., A.F.M.M.); Deutsche Forschungsgemeinschaft (M.S.) and a Rosalind Franklin Fellowship of the University of Groningen (M.S.) and the Duke Brumley NPRI Children’s Heart Foundation (to M.L.K.).

**Disclosures**

None
References


8. Saga Y, Kitajima S, Miyagawa-Tomita S. Mesp1 expression is the earliest sign of cardiovascular development. TCM. 2000;10:345-352


23. van Wijk B, Moorman AFM, van den Hoff MJB. Role of bone morphogenetic proteins in cardiac differentiation. Cardiovasc Res. 2007;74:244-255


32. Flink IL. Cell cycle reentry of ventricular and atrial cardiomyocytes and cells within the epicardium following amputation of the ventricular apex in the axolotl, Ambystoma mexicanum: confocal microscopic immunofluorescent image analysis of bromodeoxyuridine-labeled nuclei. Anat Embryol. 2002;205:235-244


35. Wessels A, Perez-Pomares JM. The epicardium and epicardially-derived cells (EPDCs) as cardiac stem cells. Anat Rec. 2004;276A:43-57
Supplemental Material and Methods

In situ hybridization and immuno-histochemistry
Fertilized chicken eggs were obtained from a local hatchery (Drost BV, Nieuw Loosdrecht, The Netherlands) and incubated at 39°C. After incubation, embryos were isolated and staged according to Hamburger and Hamilton.1

Isolated embryos were fixed in 4% (w/v) paraformaldehyde (PFA) in PBS, embedded in paraplast and sectioned at 7μm for immunohistochemistry or 12μm for in situ hybridization. Sections were deparaffinized and rehydrated in a graded alcohol series. In situ hybridization was performed as previously described.2 Cardiac Troponin I (cTnI)3, VHMC4 and Tbx18 (ChestID 519c15) specific probes were used. 3D-reconstruction of patterns of gene expression determined by in situ hybridization was carried out as described previously.5

Immunohistochemistry was performed as previously described.6 The following primary antibodies were used: anti-cardiac Troponin I (cTnI) (HyTest Ltd), anti-BrdU (Becton Dickinson) anti-Smad1/5/8 (Santa Cruz), anti-P-Smad1/5/8, anti-P-Erk1/2, anti-Erk1/2 (Cell Signaling Technology) and anti-Tubulin (Santa Cruz). Alexa647 or Alexa568 conjugated goat-anti-rabbit and goat-anti-mouse antibodies (Molecular Probes) were used as secondary antibodies. Nuclei were visualized using SytoxGreen (Molecular Probes).

To detect apoptosis a TUNEL assay was performed using the in situ Cell Death Detection kit (POD Roche Cat Nr: 1 684 817).

To visualize proliferation, 100μL of 10mg/mL 5’-Bromo-2’-Deoxyuridine (BrdU; Sigma) in physiological salt solution was injected into the egg yolk directly under the embryo. After 1 hour of incubation, the embryos were fixed in Methanol/Acetone/Water (40:40:20 vol/vol), dehydrated, embedded in paraplast and serially sectioned at 7μm. Images were recorded sequentially using a laser scanning microscope (BIORAD, MRC1024).

Dil-labeling
For in vivo Dil-labeling, embryos were labeled via an opening in the egg shell. As previously described,7 embryos were exposed to Dil (Molecular Probes) using a picospritzer (General Valve Corp.) and a micromanipulator with a pulled glass capillary tube. Bright-field and fluorescent photos were made immediately after labeling and during further incubation. In total, 17 embryos were injected at the right vitelline vein of which 11 reached the 3 day stage. In 9 embryos the left vitelline vein was labeled from which 6 reached the 3 day stage.

Western blot analysis
Rat cardiomyocyte-like H10-cells8 were cultured at 80% density. After serum-starvation for 24 hours, the cells were rinsed with EBSS (37°C) and incubated with BMP2 (R&D, 50ng/ml), FGF2 (Peprotech, 50ng/ml), and/or U0126 (Mek1/2 inhibitor, Sigma, 10μM). The same growth factors and concentrations were used to stimulate pro-epicardia. For this analysis, pro-epicardia were isolated from chicken embryos. Per group 12 pro-epicardia were used.
which were starved for half an hour in EBSS at room temperature and stimulated for an
other 30 minutes with growth factors and/or inhibitor in CM199 (M199 culture medium
(Life Technologies) supplemented with penicillin/streptomycin (Life Technologies), 5μg/
ml insulin, 5μg/ml transferrin 5ng/ml selenium (ITS, Collaborative Research Inc.), 2mM
glutamine (Life Technologies), and 1% chicken serum) in Eppendorf tubes. After stimulation,
the incubation was stopped by adding preheated (80°C) lysisbuffer (10 mM Tris, 1 % SDS
solution). The lysed cells were aspirated, collected, boiled and centrifuged at 300 rpm for
15 minutes. The supernatant was passed 10-times through a 21G needle and the protein
concentration was determined using the BCA (Thermo Scientific) assay. Laemmli sample
buffer was added (5 minutes at 100°C) and equal amounts of protein were separated on a
10% SDS-PAGE gel. After separation, the proteins were transferred to Hybond-P membrane
(Amersham) using semi dry blotting, methanol transfer buffer and the Bio-Rad Trans-Blot
SD. After blocking (5% chicken serum in TBST), antibody staining with anti-Erk1/2, anti-P-
Erk1/2, anti-Smad1/5/8, anti-P-Smad1/5/8 or anti-Tubulin was performed. Antibody binding
was visualized using ECLplus western blotting detection (Amersham). Blots were visualized
in a LAS3000 western blot imager.

In vitro explant assay
Collagen gels were prepared as described previously. After isolation of HH16 embryos,
pro-epicardia were cut at their base, positioned on top of a collagen gel which was pre-
incubated with CM199 culture medium. After overnight attachment CM199 medium was
added. Explants were treated with 50ng/ml BMP2, 50ng/ml FGF2 and/or 5μM U0126. Only
pro-epicardial explants that showed monolayer formation and no rhythmically contracting
cells after the overnight incubation were included for further analysis. After incubation (1 to
5 days), the explants were fixed and immuno-stained using a rabbit polyclonal antiserum
directed to cardiac Troponin I (cTnl, HyTest, 4T21/2, Breda, The Netherlands, 1:1000) and
Alexa568-conjugated goat-anti-rabbit (1:250) as secondary antibody to visualize the
cardiomyocytes. Sytox-Green was used to visualize all nuclei.

The total area occupied by cardiomyocytes and the total number of nuclei were
determined using user-written macros in NIH-image (area, version 1.62) and Image Pro-
Plus (cell count, version 6.2), respectively. Subsequently, factor correction was applied to
the data to remove multiplicative variation between-sessions. The data are presented as
mean (±SEM) per experimental group. Non-parametric one-way ANOVA analysis (Kruskal-
Wallis test) was used to determine the statistical differences between groups for myocardial
area and cell number. To determine the number of cells in a pro-epicardium at isolation,
3D-reconstructions from proepicardia of stage HH16 embryos were prepared and the
number of cells was counted.
qRT-PCR

Samples were collected of freshly isolated proepicardia, sinus venosus, atrium and ventricle of 3 days old embryos (stage 16), epicardium of 5 days old embryos (stage 24), cultured proepicardia (1, 3 and 5 days of culture) and cultured pro-epicardia stimulated with FGF2, BMP2+FGF2 and U0126+BMP2. RNA extraction was performed using a RNA isolation kit (NucleoSpin Bioke).

The samples were amplified in 384-well plates in a Roche LightCycler480. The qPCR reaction was done in 10 μl with a primer concentration of 1 μM and SYBR Green qPCR Master Mix (Roche). The sequences of the forward and reverse primers as well as the amplicon lengths are listed in Supplemental Table 3. These samples were measured in at least three separated runs using the following protocol; 5 min 96°C, 45x (10 sec 95°C, 20 sec 58°C, 20 sec 72°C). The resulting amplification curves were analyzed using the qPCR data analysis program LinRegPCR which imports raw fluorescence data, performs a baseline subtraction, determines the PCR efficiency per amplicon and calculates an estimate of the starting concentration expressed in fluorescence units. Differences in yield of the different RT reactions were removed using the factor correction program for removal of multiplicative between-session variation using RT reaction as the session and the amplicon and tissue sample as combined condition. Thereafter, all gene expression levels were normalized by dividing the value of the gene of interest by the geometric mean value of three housekeeping genes (APC6, GAPDH en NDUFB3) measured in the same sample. The gene expression level of each gene is given as mean (and SEM) of at least three replicate measurements per tissue. For each gene of interest, a one-way analysis of variance was used to test the difference between samples.
References

10. Ruijter JM, Thygesen HH, Schoneveld OJLM, Das AT, Berkhout B, Lamers WH. Factor correction as a tool to eliminate between-session variation in replicate experiments: application to molecular biology and retrovirology. Retrovirology. 2006;3:2
Supplemental Figures and Tables

Supplemental Figure 1. Myocardium formation and number of cells in proepicardial explant during the culture period

In control and BMP2-treated proepicardial explant cultures, myocardium formation starts after 24h of culture. In FGF2- and BMP2+FGF2-treated cultures hardly any myocardium formation is observed even after 5 days of culture. The number of cells in control, BMP2- or FGF2-treated cultures is similar at all time point analyzed while in cultures treated with BMP2+FGF2 at all time point more cells are present, see also Suppl Table 1.
Supplemental Figure 2. P-Erk and P-Smad expression in control and U0126+BMP2-treated embryos

In control embryos P-Erk (a) is present predominantly in the cells of the villi of the proepicardium. Upon U0126+BMP2-treatment, P-Erk expression was decreased to background levels (b). P-Smad is found in nuclei both in control (c) and U0126 +BMP2-treated (d) embryos. The staining intensity is, however, stronger in the proepicardium and inflow myocardium of U0126+BMP2-treated embryos. In the U0126 +BMP2-treated embryos P-Smad staining is predominantly found in the nucleus, whereas in controls P-Smad is also found in the cytoplasm of the cells of the villi of the pro-epicardium.
Supplemental Figure 3. BMP2 or U0126 alone does not inhibit epicardium formation
Serial sections of embryos treated for 24 (a,b) or 48 hours (c,d) with either BMP2 (a,c) or U0126 (b,d). Panels a-d show an overview and a''-d'' an enlargement of a sagittal section at the level of the pro-epicardium in which the myocardium is visualized. Panels a''-d'' show the same region on an adjacent section that was stained for Tbx18. After 24h of BMP2- or U0126-treatment the (pro-)epicardium has developed (a,b). After 48h of treatment with BMP2 (c) or U0126 (d), an epicardium has developed which envelops a substantial part of the heart (red arrow).
Supplemental Figure 4. Control and treated embryos after isolation
Overview images of embryos treated for 24 or 48 hours with growth factor solvent (control), U0126+BMP2, and FGF2.
### Supplemental Table 1. Myocardial area and number of cells during pro-epicardial culture

Summary of the quantification of the total number of cells and the myocardial area in the cultured pro-epicardial explants treated with different growth factors. Statistical differences were indentified using a Kruskal Wallis nonparametric 2-way ANOVA analysis followed by a multiple comparison of groups. For both tests, a significance level of 0.05 was used. A filled triangle indicates significantly higher, whereas an open triangle indicates significantly lower compared to the time-matched control. In all groups we started with 174 ± 16 cells, the number of cells present in a freshly isolated pro-epicardium.

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### Supplemental Table 2. Myocardial area and number of cells after 5 days of culture with or without Mek1/2-inhibition (U0126)

Summary of the quantification of the myocardial area and the total number of cells in 5 days cultured pro-epicardial explants treated with different growth factors in the presence or absence of the Mek1/2 inhibitor U0126. Statistical differences were indentified using a Kruskal Wallis nonparametric 2-way ANOVA analysis followed by a multiple comparison of groups. For both tests, a significance level of 0.05 was used. A filled dot indicates significantly higher, whereas an open dot indicates significantly lower compared to the respective culture without U0126. A filled triangle indicates significantly higher, whereas an open triangle indicates significantly lower compared to the control.

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**Supplemental Table 3. qRT-PCR primers**

Chicken gene-specific primer sequences used in the quantitative RT-PCR analysis. Indicated are the transcript name, Genebank accession number, primer sequences in the 5’ to 3’ direction, and amplicon length. We designed Primers that span an exon-exon junction, are located close to the 3’ end of the mRNA, and have an annealing temperature of approx. 60°C.
### Stage 21 Chicken Embryo

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#### preset views

#### credits

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