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Induction of proepicardial marker gene expression by the liver bud

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Cells of the coronary vessels arise from a unique extracardiac mesothelial cell population, the proepicardium, which develops posterior to the sinoatrial region of the looping-stage heart. Although contribution of the proepicardial cells to cardiac development has been studied extensively, it remains unresolved how the proepicardium is induced and specified in the mesoderm during embryogenesis. It is known, however, that the proepicardium develops from the mesothelium that overlays the liver bud. Here, we show that the expression of proepicardial marker genes – *Wt1*, capsulin (epicardin, *pod1*, *Tcf21*) and *Tbx18*, can be induced in naïve mesothelial cells by the liver bud, both in vitro and in vivo. Lateral embryonic explants, when co-cultured with the liver bud, were induced to express these proepicardial marker genes. The same induction of the marker genes was detected in vivo when a quail liver bud was implanted in the posterior-lateral regions of a chick embryo. This ectopic induction of marker gene expression was not evident when other endodermal tissues, such as the lung bud or stomach, were implanted. This inductive response to the liver bud was not detectable in host embryos before stage 12 (16-somite stage). These results suggest that, after a specific developmental stage, a large area of the mesothelium becomes competent to express proepicardial marker genes in response to localized liver-derived signal(s). The developmentally regulated competency of mesothelium and a localized inductive signal might play a role in restricting the induction of the proepicardial marker gene expression to a specific region of the mesothelium. The data might also provide a foundation for future engineering of a coronary vascular progenitor population.

KEY WORDS: Proepicardium, Epicardium, Coronary vessel, Liver bud, Transcription factor, Wilms tumor 1, Capsulin (Epicardin, Pod1, Tcf21), Tbx18, Cfc1, Pax2, Paracrine signal, Heart development, Chick

INTRODUCTION

Cells of the coronary vessels and epicardium all arise from an extracardiac source, the proepicardium (PE) (Dettman et al., 1998; Mikawa and Fischman, 1992; Mikawa and Gourdie, 1996; Pérez-Pomares et al., 1998; Reese et al., 2002; Vrancken Peeters et al., 1999). In the avian embryo, the PE develops as multiple epithelial villi protruding from the pericardial serosa immediately posterior to the sinoatrium at embryonic day 2 (E2) [stage 14 (Hamburger and Hamilton, 1951)]. At this developmental stage, the embryonic tubular heart is composed exclusively of cardiomyocytes and endocardial endothelial cells (Manasek, 1968; Nahirney et al., 2003), and there are no detectable coronary vessels. Prior to coronary vessel formation, the myocardial wall of the embryonic heart is nourished by the diffusion of oxygen and nutrients through the endocardium, which forms extensive trabecular channels. Villous projections of the PE extend towards the looping-stage heart between E2 and E3 (stage 14-18), then attach to and spread over the myocardial surface, forming the epicardium (Hiruma and Hirakow, 1989; Ho and Shimada, 1978; Manasek, 1969; Männer, 1992; Nahirney et al., 2003; Shimada et al., 1981). As the epicardial sheet forms, some PE/epicardial cells undergo an epithelial-mesenchymal transformation (Dettman et al., 1998; Männer, 1992), giving rise to the vascular precursors of the coronary vessels and to cardiac connective tissue cells (reviewed in Mikawa, 1999; Männer et al., 2001). Capillary plexi are formed first, followed by arterial vessels (Bogers et al., 1989; Waldo et al., 1990). The connection of the coronary orifices to the aorta and closure of the coronary vascular bed is completed after E14 (Rychter and Ostadal, 1971).

Physical blockage of PE cell entry to the embryonic heart results in the loss of epicardial and coronary vessel formation (Gittenberger-de Grott et al., 2000; Männer, 1993; Pennisi et al., 2003). Although these studies demonstrate that the PE is the major bona fide source of the coronary vasculature and that there is a specific pathway through which PE cells reach the heart, nothing is known about how a particular mesothelial cell population is induced to differentiate into the PE. Genetic experiments suggest that PE development requires both cell autonomous and non-autonomous activities. Epithelial-to-mesenchymal transition from the epicardium requires Fog2 (friend of Gata2) expression in the myocardium (Tevosian et al., 2000). Targeted inactivation of the Wilms tumor gene *Wt1* results in incomplete epicardial coverage of the heart with fewer subepicardial cells present (Moore et al., 1999). Migration of PE-derived cells is also impaired by overexpression and antisense-mediated knockdown of the T-box transcription factor gene *Tbx5* in chick (Hatcher et al., 2004). Disruption of the zinc-finger transcription factor gene *Gata4* results in defective PE formation (Watt et al., 2004). Recent studies suggest the involvement of secreted signaling molecules in the specification and maintenance of the PE cell fate. In mouse, the activity of the *Gata4* lateral plate enhancer is attenuated by the bone morphogenetic protein (BMP) antagonist noggin, suggesting a role for BMP signaling in PE development (Rojas et al., 2005). In vitro culture experiments suggest that BMP and fibroblast growth factor (FGF) signaling activities can influence differentiation of the PE (Kruithof et al., 2006; Schlueter et al., 2006).

Although the above and other studies have provided significant insights into the molecular basis of PE development, little is known about mechanisms that initiate PE formation within the embryonic

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mesothelium. However, it is known that the PE develops from a mesodermal cell population that overlays the liver bud endoderm (Männer, 1992; Männer et al., 2001; Nahirney et al., 2003; Virágh et al., 1993). Here, we tested the potential role of the liver bud in PE induction. Data are presented to demonstrate that the liver bud can induce expression of the PE marker genes *Wt1*, capsulin (also known as *Tcf21*, epicardin and *pod1*) and *Tbx18*, both in vitro and in vivo. The competency of mesothelium to respond to an inductive cue from the liver bud is developmentally regulated. These data suggest that a developmentally regulated competency of mesodermal cells to a localized inductive signal might play a role in induction of the proepicardial marker genes within the mesothelium.

MATERIALS AND METHODS

Embryos

Chick (*Gallus gallus domesticus*) and quail (*Coturnix coturnix japonica*) embryos were incubated at 38°C in a humidified incubator and were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Histology

Embryos were fixed with 10% formalin in phosphate-buffer (pH 7.2), dehydrated through graded series of ethanol, embedded in paraffin and sectioned at 6 μm. The sections were stained with hematoxylin and eosin.

In situ hybridization

Using the following primers, we carried out reverse transcriptase (RT)-PCR to obtain DNA templates for the synthesis of digoxigenin (*Wt-1* and capsulin) or fluorescein (*Hex*, also known as *Prh* and *Probox*)-labeled riboprobes. For *Wt1*, 5′-CTGGCACTCGTCGGACATCT-3′ and CGGACCCCCTACAACAGTGA; for capsulin, 5′-TCACACACGCTCACTCACTGACTC-3′ and 5′-TCTGGACCTGCTTTCCTTCCTG-3′; for *Hex*, 5′-GACAAAGAAGCAACGGACTC-3′ and 5′-GGCAAAATCCTCCAAAATGCG-3′. After PCR products were ligated into the pCRII-TOPO plasmid vector (Invitrogen, CA), the sequence and orientation of the inserts were confirmed by sequencing. The plasmid used to synthesize the digoxigenin-labeled *Pax2* probe (Obara-Ishihara et al., 1999) was a gift from Dr Herzlinger (Cornell University Medical College, New York, NY). We used a 1 kb partial cDNA clone of *Tbx18* and 1 kb full-length cDNA of *Cfc1* (Schlueter et al., 2006) kindly provided by Dr Brand (University of Würzburg, Germany). Normal or implanted embryos were fixed with 4% paraformaldehyde and processed for single or double whole-mount in situ hybridization (Hurtado and Mikawa, 2006). For double in situ hybridization, modifications were made as follows. A riboprobe for *Wt1* was first detected using anti-digoxigenin antibody and NBT/BCIP substrate. After the removal of the antibody by incubating twice with glycine-HCl (pH 2.2), the *Hex* probe was detected using anti-fluorescein antibody and BCIP substrate. After color development was completed, embryos were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), embedded in wax and sectioned at 10 μm using a microtome.

Explant culture

Chick lateral embryonic fragments (cLEF) were isolated from the 4- to 7-somite level of stage 10-11 embryos, excluding the hepatogenic endoderm demarked by *Hex* expression according to Zhang et al. (Zhang et al., 2004). The quail liver buds (qLiB) and PE (qPE) were isolated from stage 17 embryos using a fine glass capillary and finely sharpened forceps. Lung buds (qLuB) were isolated from stage 20-21 quail embryos and freed from *Wt1*-positive mesothelium (Carmona et al., 2001). These embryonic tissue fragments were either cultured alone or co-cultured with various combinations in a hanging drop (20 μl) of Medium 199 (Invitrogen, CA) for 24 or 44 hours in a CO_2 incubator.

RNA isolation, RT-PCR and qRT-PCR

Total RNA was isolated from cultured explants with Trizol (Invitrogen, CA) according to the manufacturer's instructions. RNA (20-70 ng) was reverse transcribed in 10 μl total volume using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, CA) with an oligo $(dT)_{12-18}$ primer. A negative control was done in parallel without SuperScript II reverse transcriptase. PCR amplification was performed in a 25 μl total volume using Taq DNA polymerase (New England Biolabs, MA). Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used to normalize the template input (1/60-1/320 of the total reverse-transcription reaction). The number of cycles was optimized for each primer pair to remain within the linear range of amplification. Electrophoresis of PCR samples (5 μl) was conducted on a 1.5% agarose gel. Kodak EDAS 290 (Kodak, NY) was used to capture digital images of the gel. Relative levels of band intensity were quantified by using Multi-Analyst version 1.0.2 software (Bio-Rad Laboratories, CA). Primers used for PCR were as follows. For chick/quail *Wt1* (*c/qWt1*), 5′-TCTAGGGGACCAGCAGTACTC-3′ and 5′-GATATGGTTTTTCACCAGTGTGC-3′; for chick/quail capsulin (c/qcapsulin), 5′-GGGTCCTCTCTGGATCTATATCAC-3′ and 5′-GCCATTCTCGCCATTGGAT-3′; for chick/quail *Pax2* (*c/qPax2*), 5′-AAGTAGCGACCCCCAAAGTAGTG-3′ and 5′-CCCTCGGATACATCTTCATCACG-3′; for chick/quail *Gapdh* (*c/qGapdh*), 5′-CAGCCTTCACTACCCTCTTG-3′ and 5′-ACGCCATCACTATCTTCCAG-3′; for chick *Gapdh* (*cGadph*), 5′-GGCATTGCACTGAATGACCAT-3′ and 5′-TCTCCCACCTCCCCCAGGTG-3′; for quail *Gapdh* (*qGadph*), 5′-GGCATTGCACTGAATGACAAC-3′ and 5′-TCTCCCACCTCCCCCAGGCT-3′; for chick *Wt1* (*cWt1*), 5′-ACAATTTGTACCAAATGACGTCACAA-3′ and 5′-GATGGGACAGCTTGAAGTATCG-3′; for chick *Tbx18* (*cTbx18*), 5′-GGAATTTGAAGACAAATAACACA-3′ and 5′- AAGGGAATTTCTCATACTGCG-3′; for chick *Cfc1* (*cCfc1*), 5′-GTCTGCGCTGATTTGCCTTCTCAC-3′ and 5′-ACAATGAAAATTCAATACTTAAGGCATAG-3′. The same primer sets were used for quantitative real-time PCR (qRT-PCR) using the ABI 7900HT sequence detection system (Applied Biosystems, CA) and BIO-RAD iTaq SYBR Green Supermix with ROX (Bio-Rad Laboratories, CA). Relative levels of gene expression were quantified based on the threshold cycle obtained from three independent reactions and were normalized to *cGapdh*.

Implantation and whole-embryo culture

Chick embryos were set-up in whole-embryo culture as previously described [EC method (Chapman et al., 2001)]. Briefly, the embryos were attached to pieces of filter paper and placed on agar/albumen gel (50% thin albumen, 0.0615 M NaCl, 0.3% Bacto-Agar, Becton Dickinson, MD) after removal of the yolk. Donor tissues, such as the liver bud, lung bud, stomach and sinoatrial myocardium were isolated from quail embryos at stages 17 and 21-22. Stomach was identified as a swelling of the gut tube immediately posterior to the lung bud. After isolation, it was cut into four pieces to obtain similar-sized implants as the liver bud and lung bud. Sinoatrial myocardium was isolated from stage 17 embryos. In our hands, isolation of these endodermal tissues at or before stage 16 was unsuccessful. Implants were placed in various sites of posterior lateral regions of the chick host at various developmental stages. Operated embryos were incubated at 38°C for 20 hours in a humidified chamber. Implantation was made mainly on the left side of the host embryo to avoid the prominent *Wt1* expression on the right side of the embryo (Schlueter et al., 2006); the contralateral side acted as a negative control.

Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde in PBS for 2-4 hours at 4°C, cryoprotected by 30% sucrose in PBS and embedded in OCT compound for frozen sectioning. Frozen sections 12 μm thick were cut, rehydrated in PBS and incubated with blocking solution (1% bovine serum albumin and 0.5% TritonX-100 in PBS). Sections were incubated at 4°C overnight with primary antibodies diluted in blocking solution. Primary antibodies used in this study were the following: monoclonal QCPN antibody (supernatant, Developmental Studies Hybridoma Bank, IA) diluted 1:8, rabbit anti-Wt1 antibody (C19, Santa-Cruz, CA) diluted 1:50, and rabbit anti-Pax2 antibody (Zymed, CA) diluted 1:50. After three washes with PBS, the sections were incubated with secondary antibodies (goat anti-mouse IgG conjugated with Alexa-Fluor-488 and/or goat anti-rabbit IgG conjugated with Alexa-Fluor-594; Molecular Probes, OR) and 4′,6-diamidino-2-phenylindole (DAPI; for counter staining) for 2 hours at room temperature. Sections were washed three times in PBS and mounted with Aqua Poly/Mount (Polysciences, PA). Images were captured by using a Spot RT Slider 2.3.1 (Diagnostic

Instruments, MI) and Spot software 3.5.9 (Diagnostic Instruments, MI). Adobe Photoshop 7.0.1 (Adobe Systems, CA) was used to pseudocolor and superimpose the captured images.

RESULTS

The close proximity of the liver bud and developing PE

At the time when the PE develops, the inflow tract region contains distinct mesodermal cell populations, including the mesothelial and mesenchymal components of the PE and a stratified cuboidal cell layer adjacent to the endothelial lining of the inflow tract (Nahirney et al., 2003). Recent gene expression analysis demonstrated that the common PE marker genes *Tbx18*, *Cfc1* and *Wt1* are highly expressed in the PE mesothelium but not in the mesenchymal core or myocardium (Schlueter et al., 2006). Although morphological and histological studies have documented the proximity of the PE and liver bud (Männer, 1992; Männer et al., 2001; Nahirney et al., 2003; Virágh et al., 1993) (see Fig. S1 in the supplementary material), it remains uncertain whether the liver bud endoderm is in direct contact with specific cell populations marked by PE marker genes. Thus, we first examined the spatial relationship between the developing PE and liver bud molecularly, using double in situ hybridization (Fig. 1). The well-established marker genes *Wt1*, capsulin, *Tbx18* and *Cfc1* were used as PE markers (Carmona et al., 2001; Hidai et al., 1998; Moore et al., 1999; Schlueter et al., 2006; Schulte et al., 2007; von Scheven et al., 2006). *Hex* [also called *Prh* (Zhang et al., 2004) and *Probox*] was used as a marker for the liver bud. All PE marker genes examined in the present study exhibited strong signals at known sites of PE development immediately posterior to the tubular heart (Fig. 1), where the most-intense signals were detected on the surface of the right inflow tract (data not shown). *Hex* expression was detected in the underlying liver bud. Semi-sagittal sections of whole-mount-stained embryos showed that all PE marker genes are expressed in mesodermal tissue in direct contact with the tip of the *Hex*-positive liver bud endoderm (Fig. 1D,H,L,P). This close proximity between the PE and liver bud suggested inductive interactions in their development. In the present study, we investigated the in vitro and in vivo PE-inducing activity of the liver bud to test a model in which the liver primordium acts as an inducer of the PE.

Although all four PE marker genes were detectable in our in situ hybridization condition, none of these markers is exclusive to the PE. Expression of both *Wt1* and capsulin were also detected in the intermediate mesoderm (Fig. 1A,C,E,G and Fig. 2A,B). *Tbx18* was expressed not only in the PE but also in the somites, intermediate mesoderm and limb bud (Fig. 1I,K and Fig. 2C), consistent with previous reports (Haenig and Kispert, 2004). *Cfc1* expression was widely detectable in the lateral plate mesoderm and the myocardium of looping heart tube in addition to the PE (Fig. 1M,N and Fig. 2D), as reported (Schlueter et al., 2006). Therefore, there appears to be no single PE marker that can distinguish the induction or specification of the PE fate from those of other mesodermal tissues, such as the intermediate mesoderm. However, the expression of a transcription factor, *Pax2*, was detected in the intermediate mesoderm, as previously described (Obara-Ishihara et al., 1999), but was absent in the PE cells (Fig. 2E). Consistent results were obtained from our immunohistochemical analysis (Fig. 2F,G). The intermediate mesoderm is immunoreactive to both anti-Wt1 and anti-Pax2 antibodies. By contrast, the PE showed a detectable level of immunoreactivity for Wt1 but not for Pax2. Thus, the differential expression of *Pax2* allowed us to distinguish the induction of characteristics of the PE from those of the intermediate mesoderm in the present study.

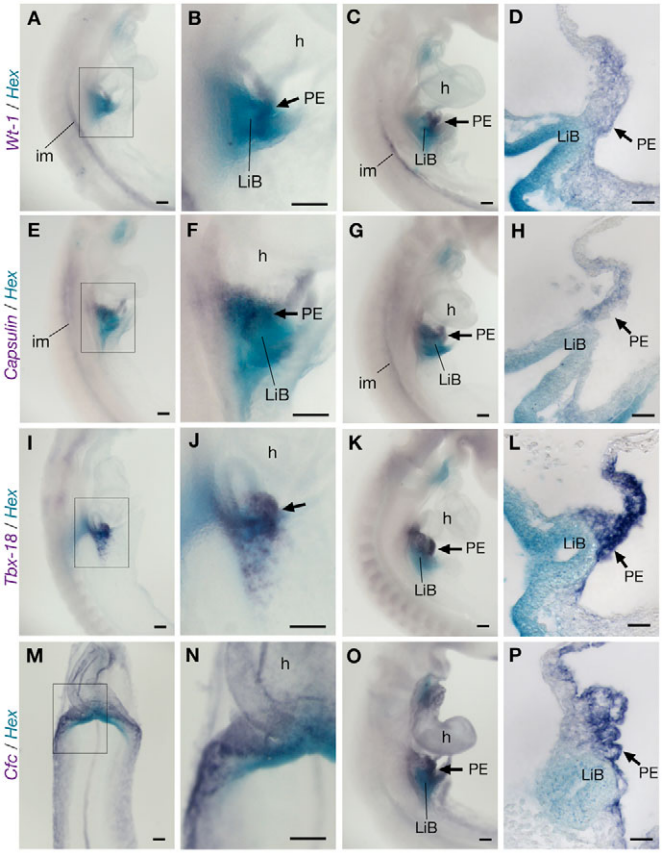

Fig. 1. Close proximity between the PE and liver bud as shown molecularly by double in situ hybridization. (**A-P**) Chick embryos at stage 11 (M,N), stage 12 (I,J), stage 14 (A,B,E,F) and stage 16 (C,D,G,H,K,L,O,P) stained for expression of a PE marker gene (purple) [*Wt1* (A-D), capsulin (E-H), *Tbx18* (I-L) or *Cfc1* (M-P)] in conjunction with for expression of a liver marker gene, *Hex* (turquoise). Right-side view (A-C,E-G,I-K,O) and ventral side view (M,N) of the whole-mount-stained embryos. B,F,J and N are high-magnification images of boxed regions in A,E,I and M, respectively. (D,H,L,P) Semi-sagittal sections of whole-mount-stained embryos at stage 16, showing that all PE marker genes are expressed in mesodermal tissue in direct contact with the *Hex*-positive liver bud. PE, proepicardium; LiB, liver bud; h, heart; im, intermediate mesoderm. Scale bars: 100 μm (A-C,E-G,I-K,M-O) and 20 μm (D,H,L,P).

In vitro induction of PE marker expression

To examine whether the liver bud has the capacity to induce PE marker gene expression in mesodermal cells, we developed a simple explant co-culture assay system (Fig. 3A). In brief, a chick lateral embryonic fragment was isolated as the responder tissue at stage 10-11 (prior to overt induction of bona fide PE cells) and was co-cultured with the quail liver bud as the inducer tissue. Using reverse transcriptase (RT)-PCR, relative amounts of PCR products of the positive PE marker genes *Wt1* and capsulin, and of a negative marker gene, *Pax2*, were examined (Fig. 3B,C). Under our RT-PCR conditions, weak signals for *Wt1*, capsulin, and *Pax2* were detected in a control explant, which contained a responder tissue alone. By contrast, significantly stronger signals of *Wt1* and capsulin were obtained when a responder lateral tissue was co-cultured with an inducer liver bud (Fig. 3B), exhibiting a two- to three-fold increase

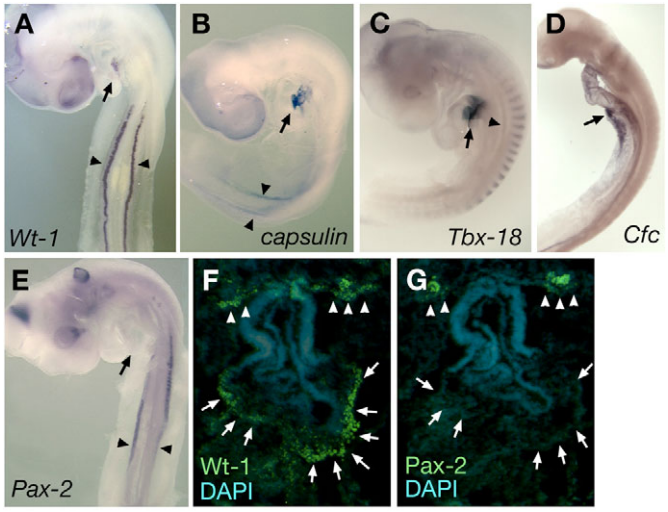

Fig. 2. Expression of positive and negative PE markers. (**A-E**) Whole-mount in situ hybridization of chick embryos at stage 18 (A), stage 16 (B), stage 17 (C,E) and stage 15 (D) with probes for *Wt1* (A), capsulin (B), *Tbx18* (C), *Cfc1* (D) and *Pax2* (E). Arrows, proepicardium (PE); arrowheads, intermediate mesoderm. (**F**) A transverse frozen section through the PE of a stage 17 embryo. The section was immunostained with the anti-Wt1 antibody (green) and was counterstained with DAPI (blue). (**G**) A sister section of that shown in F stained with the anti-Pax2 antibody (green) and DAPI (blue). Notice that Wt1 but not Pax2 is detectable in the PE, whereas similar levels of staining are seen in the intermediate mesoderm.

of signal intensity (Fig. 3C). Importantly, no significant increase of an intermediate mesoderm marker, *Pax2*, was induced by co-cultured liver bud (Fig. 3B,C).

The above results suggest that the co-presence of a liver bud with a lateral embryonic fragment promotes the expression of PE marker genes but not of an intermediate mesoderm marker gene. However, it was still unclear whether the upregulated expression of *Wt1* and capsulin in a co-culture was indeed induced only in the responder chick tissue and not in the inducer quail tissue. It was also unclear whether the inductive influence is specific to the liver bud or whether other endoderm-derived tissues would exert similar inductive effects. To test these possibilities, the PE marker gene expression in a responder chick tissue was examined using chick-specific primer sets for *Wt1*, *Tbx18* and *Cfc1* (Fig. 3D,E). In addition, the inductive activity of non-liver endodermal tissues was examined using a lung bud. Our chick-specific primer set successfully amplified *Wt1*, *Tbx18* and *Cfc1* mRNA in chick tissues but not in quail tissues (Fig. 3D), demonstrating that these mRNAs can be detected in a sequence-specific manner. Quail-specific GAPDH primers were used to ensure that the number of cells was similar between different inducers. Significant increases of mRNA levels of chick *Wt1* (*cWt1*; five- to six-fold increase in signal intensity) and chick *Tbx18* (*cTbx18*; four- to eight-fold increase) were detected in a co-culture with the liver bud. Although less prominent than *cWt1* and *cTbx18*, chick *Cfc1* (*cCfc1*) also showed an increased signal level (approximately twofold) in co-culture with the liver bud. The lung bud also enhanced the expression of PE marker genes, but its inductive activity was lower than the liver bud (two- to three-fold increase of *cWt1* and *cTbx18*). Quantative real-time (qRT)-PCR analysis confirmed the results of the above-mentioned end-point PCR, demonstrating a six- to seven-fold increase in *cWt1* mRNA level when in co-culture with the liver bud and a three- to four-fold increase when in co-culture with the lung bud (Fig. 3F). These results suggest that, under our co-culture system, the liver bud exerts an inductive influence that upregulates PE marker gene expression in the lateral embryonic fragment.

In vivo liver bud implantation leads to ectopic expression of PE marker genes

The above results suggest that the liver bud has a capacity to induce PE marker expressions in a co-cultured lateral fragment of early embryos. However, we still do not know whether the liver bud can exert such inductive effects in vivo. It also remains unclear whether all or a part of responding tissues are induced to express PE marker genes. We addressed these points in the in vivo implantation assay, which provides spatial resolution of the competent tissues. The in vivo inductive potency of the liver bud on PE marker expression was tested by implanting single donor quail liver buds into host chick embryo and analyzing ectopic expression of PE marker genes in host tissues at the site of implantation (Fig. 4). A quail liver bud was implanted into 13- to 20-somite levels of the host chick embryo at stages 12-14 (Fig. 4A,B). Implanted embryos were allowed to develop on an agar-albumen gel (Chapman et al., 2001) for 20 hours. To control for the effects of implantation, a lung bud was implanted into different embryos. Ectopic expression of PE marker genes at the implanted site was examined by whole-mount in situ hybridization. Ectopic capsulin gene expression was detected at the implanted site in 55.0% of liver bud-implanted embryos (6/11; Fig. 4C,D), whereas ectopic expression of *Tbx18* was found in 37.5% (3/8; Fig. 4E,F). In no case was *Pax2* expression induced at the site of liver bud implantation (Fig. 4G,H). In situ hybridization for *Hex* transcripts in implanted embryos showed positive signals at the implanted site in all cases (n=5) (Fig. 4I,J), suggesting that implanted liver bud retains its hepatic identity. In addition, none of the lung-implanted embryos (capsulin, n=4; *Tbx18*, n=6) showed ectopic expression of these markers (Fig. 4K-N). The data suggested that ectopic PE marker expression is induced by implanted liver bud in a tissue-specific manner, rather than as a result of either a surgical operation or the presence of exogenous tissue.

These data, however, did not rule out the possibility that ectopic expression of PE marker genes might be due to the gene expression in donor cells, such as contaminating bona fide PE progenitor cells, rather than to induced expression in surrounding host cells. In our hands, it was difficult, if not impossible, to precisely identify a donor quail tissue in a host chick embryo using in situ hybridization. To clarify whether induction occurs in the host-derived tissue or in donor cells, we used immunohistochemistry, which provided higher resolution for the analysis of both the distribution of donor cells and marker gene expression in implanted embryos. The donor cells were detected using the QCPN antibody, which is specific for quail-derived implant tissue (Selleck and Bronner-Fraser, 1995), and induced expression of a PE marker was identified using a monoclonal antibody specific for Wt1 (Figs 5, 6).

Out of 18 liver bud-implanted embryos, five developed with malformations, and 13 developed without any detectable malformation (Fig. 5A,B). Only the latter population was processed for subsequent immunohistochemical inspection. Strong fluorescent signals of endogenous Wt1 were detected in the intermediate mesoderm and dorsal mesothelium (Fig. 5C,D; asterisks), as seen in the above whole-mount in situ hybridization data (Fig. 2). The data are also consistent with the previous studies (Carmona et al., 2001). In addition, ectopic nuclear staining for Wt1 was detected adjacent to the QCPN-positive donor tissue (9/13; Fig. 5C), although

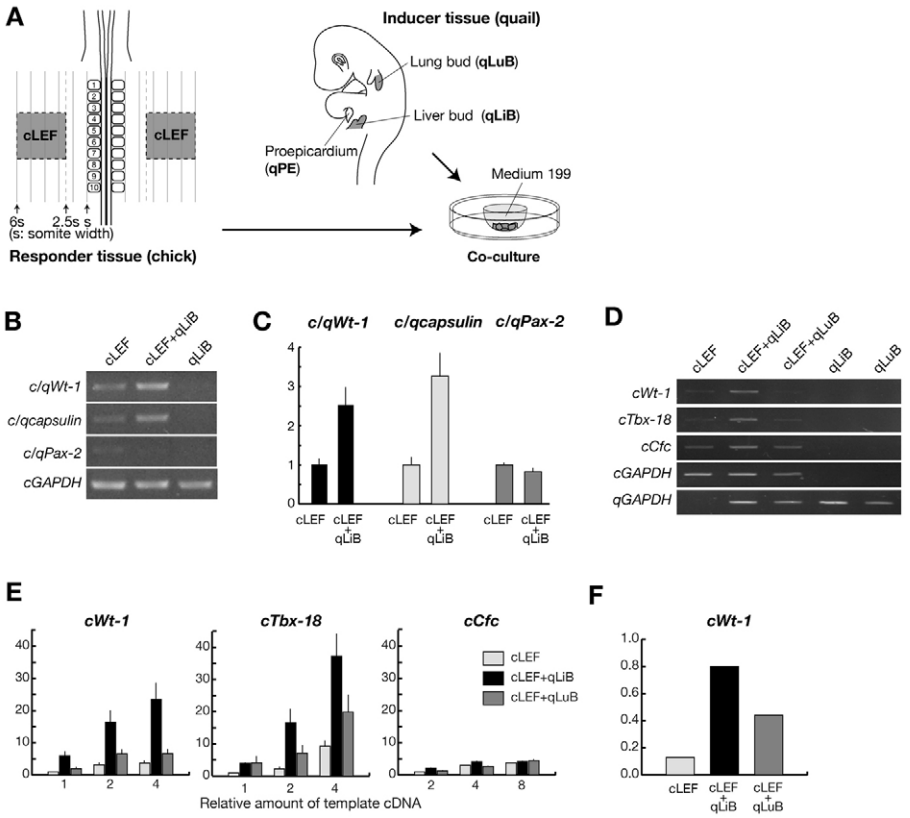

Fig. 3. In vitro explant culture assay for PE marker induction. (**A**) Schematic of the assay. Chick lateral embryonic fragments (cLEF) were isolated from the fourth to seventh (4-7) somite levels of stage 10-11 embryos. Width of the somite(s) was used as a morphological reference for mediolateral levels of the fragment. The fragment was cultured alone, or co-cultured with the quail liver bud (qLiB) or lung bud (qLuB) in a hanging drop of M199. (**B**) Reverse transcriptase (RT)-PCR analysis of mRNA isolated from cultured explants. Weak signals for *Wt1*, capsulin and *Pax2* are detectable in cLEF cultured alone. The level of signals for *Wt1* and capsulin, but not *Pax2*, increased significantly when cLEF was co-cultured with the liver bud. In our RT-PCR condition, mRNAs of all these markers were undetectable in the liver bud cultured alone. (**C**) Quantification of PCR products of *Wt1*, capsulin and *Pax2*, showing enhancement of proepicardium (PE) marker expression in co-culture with the liver bud. Standard deviation bars are shown. (**D**) RT-PCR analysis using chick-specific primers for *Wt1* (*cWt1*), *Tbx18* (*cTbx18*) and *Cfc1* (*cCfc1*). The liver bud has a strong capacity to upregulate PE marker gene expression in co-cultured cLEF. (**E**) Quantification of PCR products. We performed PCR changing the amount of the template cDNA to ensure linear amplification conditions. (**F**) Quantitative real-time (qRT)-PCR analysis of *cWt1*. Bars show the average of three independent PCR reactions.

fluorescent signals of the ectopic Wt1 were significantly weaker than those of endogenous Wt1 seen in intermediate mesoderm and dorsal mesothelium. Higher-magnification inspection revealed that ectopic Wt1 staining was predominantly detected in the host-derived cells in the mesothelial lining of the body cavity and was rarely seen in other host tissues, such as the underlying mesenchymal tissue, or in the implant itself (Fig. 5D). Sister sections double-immunostained with anti-Pax2 and QCPN antibodies showed no evidence for ectopic Pax2 expression in the host tissue at the implanted site (Fig. 5E,F).

We implanted inducer tissue at a site far distant to the intermediate mesoderm to distinguish endogenous and ectopically induced expression clearly. However, morphogenetic movement associated with the lateral body fold formation brought the implanted site medially to within close proximity of the intermediate mesoderm. Therefore, a chemotactic response of some mesothelial cells to migrate to the liver could not be ruled out. To test this possibility, we implanted donor tissue at more-posterior levels, in which the lateral body folding takes place later in development. As shown in Fig. 5G-L, ectopic Wt1 expression was detected near the implant, far from the intermediate mesoderm. This is consistent with the idea that ectopic expression of PE marker genes is due to the induction rather than migration of intermediate mesodermal cells to the implant, although the latter possibility cannot be ruled out.

Induction of PE marker genes was not evident when other endoderm-derived tissues, such as lung (n=6) and stomach (n=6), were used (Fig. 6A-L), although weak fluorescent signals above the background level were observed in some stomach-implanted embryos (Fig. 6G-L). These results demonstrate that the liver bud is capable of inducing surrounding mesodermal cells, particularly mesothelial cells, to express PE marker genes. The data also show that the mesodermal cell population preferentially responds to instructive cue(s) provided by the liver bud, more so than other endoderm-derived tissues.

In addition to the liver bud, the sinoatrial myocardium has been suggested to be a potential source of inductive signals (Majesky, 2004). Quail-derived sinoatrial myocardium was implanted into the posterior lateral region of stage 12-13 host chick embryos. After 20 hours of incubation, the embryos were fixed, cryosectioned and stained with anti-Wt1 or anti-Pax2 antibodies. No embryos (n=5) showed a detectable level of ectopic Wt1 or Pax2 expression adjacent to the myocardial implant (data not shown).

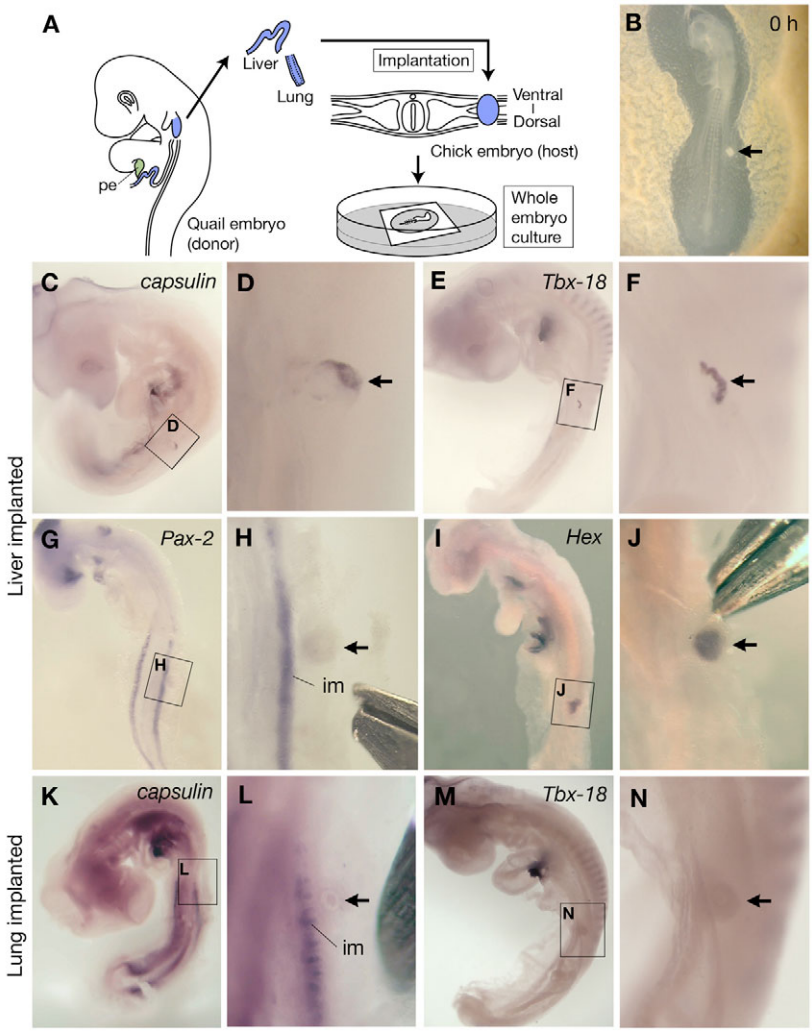

Fig. 4. Ectopic expression of proepicardium (PE) marker genes in embryos implanted with the liver bud. (**A**) Schematic of the implantation procedure. Liver bud or lung bud was isolated from a quail donor and implanted ectopically in a host chick embryo. The host embryo was then cultured on an agar-albumen gel ventral side up. (**B**) A liver bud (arrow)-implanted embryo before whole-embryo culture. (**C-J**) In situ hybridization of liver bud-implanted embryos after 20 hours of whole-embryo culture. The embryos were stained for capsulin (C,D), *Tbx18* (E,F), *Pax2* (G,H) or *Hex* (I,J) transcripts. D,F,H and J are higher-magnification images of the boxed areas in C,E,G and I, respectively. Notice that ectopic expression of capsulin and *Tbx18*, but not of *Pax2*, is detectable at the site of implantation (arrows). The implant does not appear to affect endogenous expression for these markers in the intermediate mesoderm (im). (**K-N**) Lung bud-implanted embryos stained for capsulin (K,L) and *Tbx18* (M,N). L and N are higher-magnification images of the boxed areas in K and M, respectively. No ectopic expression of these PE markers is detectable at implanted sites.

Dynamic changes of PE-inducing activity and competency during embryogenesis

Whereas the liver continuously develops and exists throughout much of embryogenesis, PE formation takes place during a defined embryonic-stage window, stages 14-17. This suggested to us that the production of inducing signal(s) by the liver bud and/or competency of the mesoderm to respond to these signals is developmentally and spatially regulated. To address this possibility, induction of PE marker gene expression was monitored at various regions of host chick embryos at different developmental stages, by implanting liver buds obtained from the donor quail embryo at different developmental stages (Fig. 6M-X). Induction of Wt1 expression was not seen when host chick embryos were younger than stage 12 (0/11, Fig. 6M-R), regardless of implantation sites in the posterior-lateral region (Fig. 7, left). By contrast, induction of Wt1 expression was evident with a higher frequency (5/11; Fig. 7, middle) when stage 17 liver bud (Fig. 5) was implanted in host embryos at stages 12-13^{-}. The inductive activity of liver fragments from stage 21-22 embryos was less prominent, as judged by signal intensity in ectopic Wt1-positive cells and by the number of these cells (Fig. 6S-X).

It should be noted that the frequency of induction was low (1/5) when the liver was implanted posterior to the vitelline arteries (Fig. 7, middle). By contrast, when host chick embryos were at or older than stage 13, Wt1 expression was frequently induced not only anterior to the vitelline arteries (6/9) but also posterior to these arteries (5/6) (Fig. 7, right). The above results demonstrate that both localized liver-derived inductive signal(s) and the competency of mesodermal cells are developmentally regulated. Our data show that, after a specific developmental stage, a large area of the mesothelium becomes competent to express *Wt1* in response to liver-derived inductive signal(s).

DISCUSSION

The PE is the major source of coronary vasculature. Cell lineage and fate mapping studies using *lacZ* retrovirus and adenovirus have demonstrated the proepicardial origin of the endothelial and smooth muscle cells of coronary vessels (Dettman et al., 1998; Mikawa and Fischman, 1992; Mikawa and Gourdie, 1996; Pérez-Pomares et al., 1998; Vrancken Peeters et al., 1999). Genetic studies in mice have identified several molecules required for PE development and PE cell entry to the heart. However, the exact mechanisms that induce the PE fate within the mesoderm are poorly understood. Our data presented in the present study is consistent with a model in which a liver bud-derived instructive cue(s) induces a mesothelial cell population to express PE marker genes in a temporally and spatially specific manner.

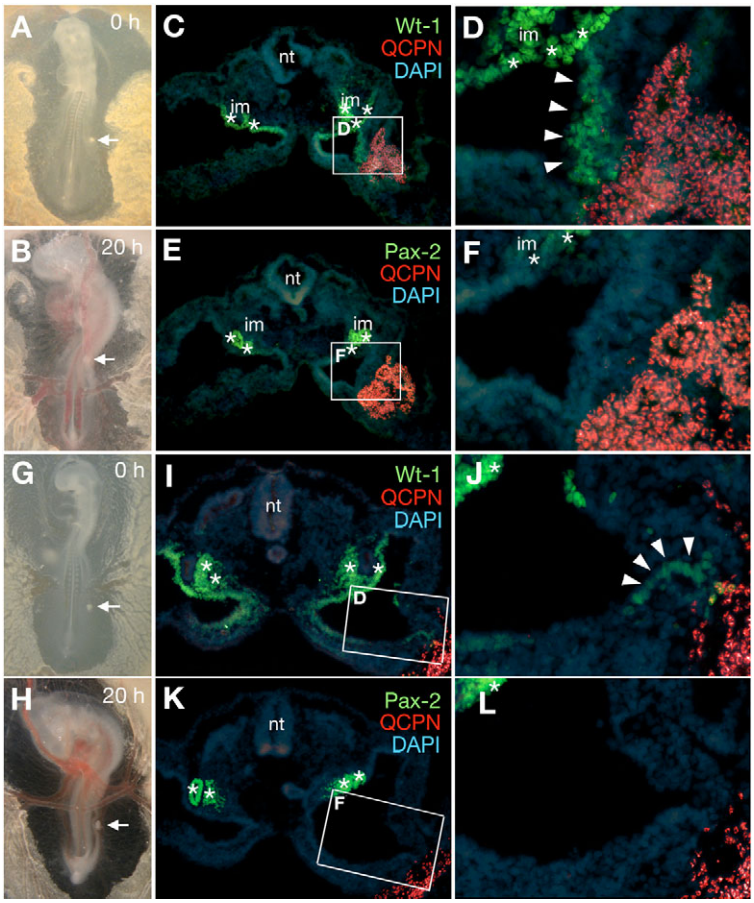

Fig. 5. Ectopic induction of Wt1 expression by the liver bud as shown by immunohistochemistry. (**A**) A chick embryo implanted with a quail liver bud (arrow) at stage 13; (**B**) after 20 hours of whole-embryo culture. (**C-F**) Transverse sections of the embryo shown in B at the level of the implant. The sections were stained with anti-Wt1 (C,D) or anti-Pax2 (E,F) antibody (green). The sections were also stained with a quail-specific QCPN antibody (red) and DAPI (blue; for counterstaining). D and F are high-magnification images of the boxed regions in C and E, respectively. Bona fide expression of Wt1 and Pax2 was detected in the intermediate mesoderm and dorsal mesothelium (asterisks). Wt1 (arrowheads in D), but not Pax2 (F), was detected ectopically in the ventrolateral lining of the body cavity. The signal was observed in the nuclei of the host-derived cells adjacent to the QCPN-positive donor tissue. (**G-L**) Embryos implanted with the liver at a more-posterior level, showing that ectopic Wt1 expression becomes detectable far form the intermediate mesoderm prior to the completion of the lateral body folding. im, intermediate mesoderm; nt, neural tube.

Inductive interaction(s) between the liver bud and mesothelium

The close association between PE formation and liver development has been shown previously by morphological studies (Männer, 1992; Männer et al., 2001; Nahirney et al., 2003; Virágh et al., 1993). Consistent with these reports, our histological and in situ hybridization analyses have revealed the close proximity between developing PE and liver bud. Transcripts of PE marker genes in the PE-forming region become detectable by stage 11-12, just before the PE development becomes morphologically detectable. *Wt1*-positive cells were seen in the mesothelium on the right side of the embryo in close proximity to the *Hex*-positive liver bud, which is also more pronounced on the right side. Close association between the liver bud and PE was also seen at later stages, when PE villi were seen in the vicinity of underlying liver bud or liver primordium. Thus, the spatial proximity of the liver bud and PE-forming site is consistent with inductive interactions between these two embryonic tissues.

To our knowledge, the present study provides the first experimental evidence for an inductive role of the liver bud in initiating PE marker gene expression within the competent mesodermal cells. Our in vitro co-culture experiments have shown that the liver bud can upregulate expression of the PE marker genes *Wt1*, capsulin and *Tbx18*, but not of an intermediate mesoderm marker, *Pax2*, in a lateral embryonic explant. Our in vivo implantation experiments have demonstrated that the liver bud is capable of inducing ectopic expression of *Wt1*, capsulin and *Tbx18*, but not *Pax2*, in the adjacent mesothelial cells. Although *Wt1*, capsulin, *Tbx18*, *Cfc1* and other molecules, such as retinalaldehyde dehydrogenase 2 (Raldh2, Aldh1a2) and cytokeratin, are often used as PE markers, they are all expressed in additional non-epicardial tissues, which limits their individual usefulness as PE markers. For example, cytokeratin is distributed widely in various epithelial tissues (Page, 1989), and Raldh2, *Tbx18* and *Cfc1* are all expressed in other heart tissues, such as the myocardium and endocardium (Haenig and Kispert, 2004; Kraus et al., 2001; Plageman, Jr and Yutzey, 2005; Schlueter et al., 2006; Xavier-Neto et al., 2000).

Although expression of *Wt1* and capsulin is not detectable in the myocardium or endocardium, they are both highly expressed in the intermediate mesoderm (Carmona et al., 2001; Hidai et al., 1998; Moore et al., 1999; von Scheven et al., 2006). Therefore, *Wt1*, capsulin and *Tbx18* alone cannot discriminate induction of the PE from that of non-PE cell types, including the intermediate mesoderm. However, we found that expression of an intermediate mesoderm marker, *Pax2*, is not detectable in the PE. Thus, the present study monitored the inductive role of the liver bud by analyzing the expression of both *Wt1*, capsulin and *Tbx18* as positive PE markers and *Pax2* as a negative PE marker. Identification of more molecular markers specific to PE cells will be needed to assign a definitive role to liver bud-derived cue(s) in PE induction.

Because the PE is closely apposed not only to the liver bud but also to the sinoatrial myocardium, both of these tissues have been suggested to play a role in inducing PE development (Majesky, 2004). Our data show that induction of ectopic *Wt1* expression can be triggered by the liver implant alone in mesoderm far away from the bona fide myocardium (e.g. posterior to the vitelline arteries), suggesting that a liver-derived signal(s) is sufficient for the induction of PE marker genes, without the need for a myocardium-derived signal(s). It should be noted, however, that morphological

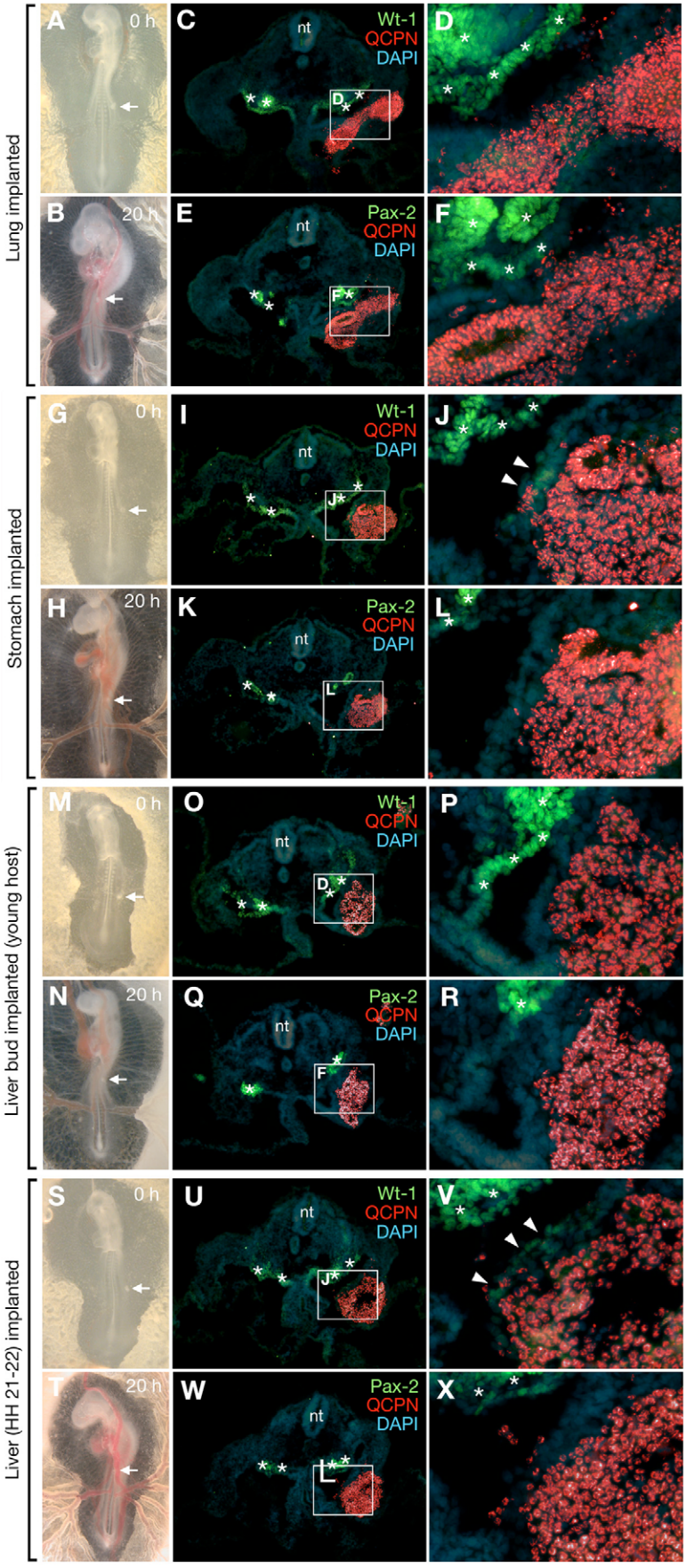

Fig. 6. Developmentally and spatially regulated inductive activity and competence. Embryos implanted with an inducer tissue (A,G,M,S) were cultured for 20 hours (B,H,N,T) and processed for immunohistochemistry. Transverse sections were cut and immunostained with anti-Wt1 (C,I,O,U) or Pax2 (E,K,Q,W) antibody (green), and with the QCPN antibody (red) and DAPI (blue). D,F,J,L,P,R,V,X are high-magnification images of the boxed regions in C,E,I,K,O,Q,U,W, respectively. Asterisks indicate bona fide expression of Wt1 or Pax2 in the intermediate mesoderm and dorsal mesothelium. nt, neural tube. (**A-L**) Inductive capacity of non-liver endodermal organs. (A-F) Embryo implanted with the lung bud. Ectopic induction of Wt1 expression is not detectable. (G-L) Embryo implanted with the stomach, showing a weak ectopic signal for Wt1 in a small number of cells in the host-derived tissue (arrowheads). No ectopic signal for Pax2 was detectable (E,F,K,L). (**M-X**) Developmetntally regulated inductive activity of the liver bud and of mesodermal competence to respond to this activity. (M-R) Young chick host (stage 11^{-}) implanted with quail liver bud. Induction of Wt1 expression is not evident. (S-X) A stage 13 embryo implanted with an older (stage 21-22) liver bud. A low level of ectopic Wt1 expression in mesodermal tissue adjacent to the implant (arrowheads in V) was observed. No ectopic signal for Pax2 was detectable (Q,R,W,X).

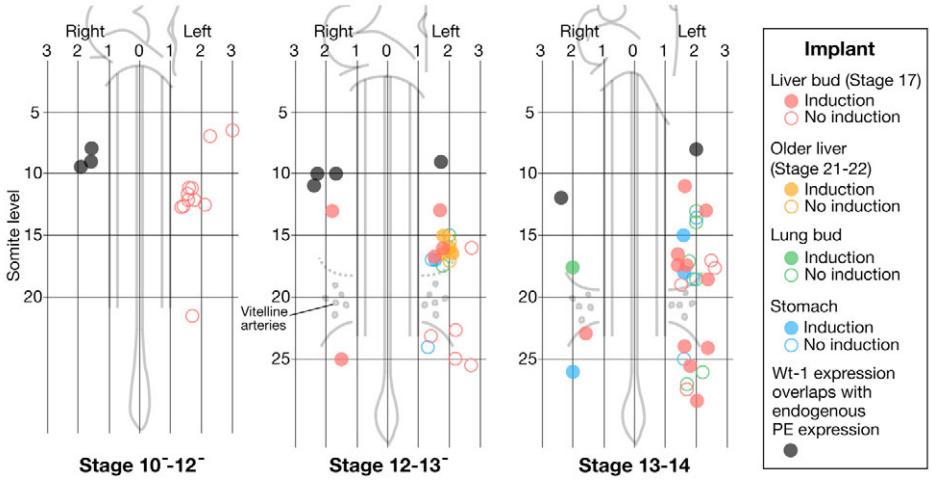

Fig. 7. Summary of immunohistochemical analysis of implanted embryos. Each filled circle represents one implantation that induced ectopic Wt1 expression. Open circles represent implantations in which induction was not detectable. Different colors represent different implanted tissue. Gray filled circles represent implantations in which induced expression and endogenous proepicardium (PE) expression could not be distinguished because of their close proximity. Somite level and the distance between the midline and lateral edge of the dorsal aorta were used to determine anteroposterior and mediolateral levels of the implant. Quail-derived inducer tissues were implanted into stage 10⁻-12⁻ (left), stage 12-13⁻ (middle) or stage 13-14 (right) chick host embryos at different sites. Induction of Wt1 expression in host tissue was examined by double immunohistochemistry using anti-Wt1 and QCPN antibodies.

characteristics of the PE, such as villous protrusion, were not evident at the site of implantation, suggesting that additional signals might be required for PE morphogenesis. It is intriguing to consider that a myocardium-derived signal(s) might control PE villus protrusion and its guided extension towards the myocardium. This idea is consistent with the appearance of an extracellular matrix bridge between the PE and the myocardium (Nahirney et al., 2003), which suggests interactions between these tissues in advance of their contact.

Developmental processes of PE formation vary among species (reviewed in Majesky, 2004; Männer et al., 2001; Muñoz-Chápuli et al., 2002). In mouse embryos, PE forms bilaterally and grows as such. In avian embryos, the left side regresses while the right side continues to grow. In accordance with this asymmetric morphology, the PE marker genes *Tbx18*, *Wt1* and *Cfc1* are expressed asymmetrically in chick, being more pronounced on the right side than the left side (Schlueter et al., 2006). This raises the issue of whether these asymmetries are due to localized inducing and/or inhibiting signals, or to a localized competence of the mesoderm. Our in vivo implantation study demonstrated that the mesoderm on the left side has the competence to respond to an ectopic liver bud-derived inductive influence, favoring the former possibility. However, before *Wt1* and capsulin expression becomes detectable, progenitors of the liver bud are located bilaterally (Matsushita, 1996; Rosenquist, 1971), similar to those in mouse embryos (Tremblay and Zaret, 2005), in which PE develops bilaterally. Therefore, it is possible that non-liver tissues negatively regulate PE development specifically on the left side of the pericardial mesoderm, although nothing is known about an activity that antagonizes PE development. Further investigations into tissue interactions at earlier stages will be necessary.

Inductive activity of the liver bud

Our data suggest that a high capacity to induce PE marker expression is predominantly held by the liver bud and is not shared by other endoderm-derived tissues, such as the lung bud or stomach. Although the basis for restricting PE development near the liver bud remains to be addressed, it is known that the mesothelial layer is dissociated from the endoderm by intervening mesenchymal tissue, which later gives rise to smooth muscle and supporting connective tissue. One exception is the liver bud, in which the endoderm undergoes outgrowth towards septum transversum mesenchyme (Rossi et al., 2001). This morphogenetic event results in closer contact between the liver bud endoderm and mesothelium (see Fig. S1 in the supplementary material). Thus, in addition to region-specific inductive cue(s), physical proximity might play a role in liver bud-dependent PE induction.

Although our data suggest that a signal(s) from the liver bud is sufficient to induce PE marker genes in a naïve mesodermal tissue, it remains unclear whether this organ rudiment is necessary for PE induction. It would be interesting to analyze a mutant animal model(s) in which the liver bud formation is specifically impaired. For example, mutant mouse embryos lacking both *Foxa1* and *Foxa2* genes do not form the liver bud (Lee et al., 2005). Unfortunately, the mutant embryos show a significant delay in the development of many organs at E9.5 (Lee et al., 2005), when the PE begins to develop in wild-type embryos (Majesky, 2004; Muñoz-Chápuli et al., 2002), and die shortly thereafter. Therefore, it still remains unsolved whether a liver bud-derived signal(s) is necessary for PE induction in the animal model.

Currently, molecules that mediate the PE-inducing activity of the liver bud are undefined. Recent studies have shown that BMP and FGF signaling can affect the differentiation of the PE. Both supplying and blocking BMP signaling result in a loss of PE marker gene expression and concomitant stimulation of cardiac myocyte differentiation (Schlueter et al., 2006). FGF2 can stimulate growth of the PE and inhibit its myocardial differentiation (Kruithof et al., 2006). Although these studies suggest that appropriate levels of BMP and FGF signals are required to maintain PE identity, the in vivo function of these proteins appears to be complicated by the complex expression patterns of different ligands in and around the PE-forming region (Kruithof et al., 2006; Schlueter et al., 2006;

Somi et al., 2004). Both BMP and FGF family member genes are highly expressed in the lung even before initiation of its branching morphogenesis (Bellusci et al., 1996; Bellusci et al., 1997; Narita et al., 2000; Sakiyama et al., 2000). Our data showing no or very low PE-inducing activity of the lung are inconsistent with the idea that BMPs and FGFs are key mediators of PE-inducing activity. Comprehensive analysis of molecular components specific to the liver bud might provide insights into inducing factor(s) responsible for PE development. To our knowledge, there is no published database regarding paracrine factors that are specifically expressed in the liver bud. Furthermore, our survey of the unpublished microarray data (H. Yoshitomi and K. S. Zaret, personal communication) that had kindly been provided to us did not reveal any paracrine factor expressed exclusively in a liver bud-specific manner.

Therefore, we examined several candidate paracrine factors that have been chosen based on the following four criteria: (i) implicated in patterning the heart and foregut endoderm (Bmp4, Bmp7, noggin, follistatin, Cripto (Cfc1), Wnt3a, Dkk1, and sFRP3); (ii) expressed in the endoderm (Shh); (iii) implicated in vessel development (Fgf2, Pdgf); and (iv) known liver-expressed paracrine factors (HGF, albumin as a control). We found that *Tbx18* was upregulated by many factors, including Bmp4, Dkk1, Fgf2, follistatin, Hgf, noggin, Pdgf, Shh, sFRP3 and Wnt3a. By contrast, *Wt1* was upregulated only by noggin, Shh and Wnt3a. None of these factors upregulated *Cfc1*. These data are presented as supplemental material (see Fig. S2 in the supplementary material). The results suggest that each PE marker gene exhibited a distinct spectrum of responsiveness to paracrine factors used, raising the possibility that PE development is induced by rather complex mechanisms involving multiple factors. Identification of true inducing factors will require future study.

Inductive response of the mesothelium to a liver bud-derived signal(s)

Whereas specification of the liver endoderm begins at about the 6-somite stage in chick and at the 7- to 8-somite stage in mouse (reviewed in Zaret, 2000), the overlaying mesothelium undergoes PE formation much later, in a defined developmental window – stages 14-17. Our implantation study suggests that the competency of naïve mesoderm, particularly mesothelial cells, to respond to the liver bud-derived inducing signal is developmentally regulated. Liver bud-dependent induction of *Wt1* in host embryos became detectable at stage 12, but not before. Thus, developmentally regulated competency of the mesothelium might play a role in restricting PE induction to a specific region and time during embryogenesis. It is currently unclear whether this competency is inherent in all mesothelial cells or whether it is induced within the mesothelium by other neighboring tissues. In mammalian embryos, it has been proposed that *Wt1*-positive serosal mesothelial cells migrate to cover the mesentery and, subsequently, the entire gut (Wilm et al., 2005). It will be important in the future to examine how the mode of mesothelium formation is related to the competency of mesothelium to respond to PE-inducing signals.

Concluding remarks

It is well-established that interactions between endoderm and mesoderm are crucial for heart and liver development. Induction of cardiogenic mesoderm depends upon paracrine signals from the underlying endoderm (Lough et al., 1996; Schultheiss et al., 1997) (reviewed in Lough and Sugi, 2000), and signals from the resulting cardiogenic mesoderm in turn initiate hepatic cell differentiation and liver bud formation in the ventral endoderm (Fukuda-Taira, 1981; Gualdi et al., 1996) (reviewed in Duncan, 2003). The results presented in this study suggest a subsequent inductive event in which the liver bud induces the overlying mesothelium to initiate PE development. Thus, reciprocal tissue interactions and a differential movement of the mesoderm and endoderm (Matsushita, 1996) might be crucial in coordinating the generation of the myocardium and epicardial/coronary vessel precursors from neighboring regions of the mesoderm at different stages of development.

Recent studies suggest that the PE is not only the major source of endothelial, smooth muscle and fibroblast cells of the coronary vasculature but that it also has the potential to produce many other cardiac cell types, such as blood, mesenchymal and myocardial cells, either in vivo or under certain experimental conditions (Kruithof et al., 2006; Schlueter et al., 2006; Tomanek et al., 2006) (reviewed in Wessels and Pérez-Pomares, 2004). Elucidating cellular and molecular mechanisms that control fate decisions of PE-derived cells will provide a foundation for rational therapeutics of coronary disorders in adults as well as the basis for understanding normal and aberrant coronary vessel development.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/20/3627/DC1

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