The proepicardium keeps a potential for glomerular marker expression which supports its evolutionary origin from the pronephros

Running title: glomerular potential of the proepicardium

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Abstract

The proepicardium is the embryonic primordium of the epicardium. This transient structure is essential for cardiac development giving rise to the epicardium and supplying the heart with vascular and cardiac connective tissue progenitors. However, their nature and evolutionary origin are poorly-known. We have suggested elsewhere (Pombal et al. *Evol. Dev.* 10:210–216, 2008; Cano et al. *J. Dev. Biol.* 1:3–19, 2013) that the proepicardium is an evolutionary derivative of the primordium of an ancient external pronephric glomerulus, devoid of its original excretory function. In this study, we describe for the first time expression of two podocyte markers in the chick proepicardium (glepp1 and synaptopodin) and we have shown how these podocyte markers as well as the intermediate mesoderm marker Pax2 are strongly upregulated when the proepicardium is cultured with nephrogenic inducers. Retinoic acid treatment also induced in the proepicardium expression of Hoxb4, a gene which confers to intermediate mesoderm competence to respond to nephrogenic signals. Thus, a latent nephrogenic potential persists in the proepicardium and also that its original glomerular fate can be partially rescued. The transcription factor Wt1, essential for kidney and epicardial development, plays opposite roles in both tissues, inducing epithelial-mesenchymal transition in the proepicardium and promoting epithelialization in the kidneys (Essafi et al., *Dev. Cell* 21:559–574, 2011). Consistently with this antithetical function of Wt1, we have observed an upregulation of podocalyxin in the epicardium of mouse embryos with conditional deletion of the *Wt1* gene, while this protein is transcriptionally activated by Wt1 in podocytes.

Key Words: proepicardium, epicardium, pronephros, podocytes, Pax2, Hoxb4, Wt1.

INTRODUCTION

The proepicardium is the embryonic primordium of the epicardium, the outer layer of the heart. The proepicardium consists of a cluster of coelomic and mesenchymal cells located in the posterior limit of the heart. Proepicardial cells migrate on the cardiac surface, spread and give rise to the epicardium, which in turn supplies vasculogenic and fibroblastic cells to the heart (Männer et al. 2001; Carmona et al. 2010). Proepicardium and epicardium are essential tissues for cardiac development, and a number of mutations affecting epicardial development have lethal consequences due to a failure to undergo myocardial maturation and/or maldevelopment of the coronary vascular system.

The mechanism for coelomic investment of the heart, based in the transfer of the proepicardial primordium, is unique among animals. A hypothesis about the evolutionary origin of the proepicardium was forwarded by us (Pombal et al. 2008) on the base of the epicardial development in lampreys (*Petromyzon*), representatives of the Agnathans, the most primitive living lineage of vertebrates. This hypothesis proposed that the proepicardium is the remain of an ancient pronephric external glomerulus, which lost its excretory function but was kept in the evolution of the gnathostomes (jawed vertebrates) to supply the heart with coelomic cells and vascular progenitors. In fact, the lamprey proepicardium, differently to the transient structure present in all the other vertebrates, gives rise to the right pronephric external glomerulus, which remains functional during the entire larval life.

Our hypothesis accounts for the large number of genes which are involved in the development of both, the proepicardium and the nephric glomerulus. For example, Wt1, Tcf21, Tbx18, nephrin and podoplanin are all expressed by proepicardial, epicardial and nephric tissue, and in most cases their loss of function causes cardiac and kidney abnormalities (reviewed in Cano et al. 2013).

Despite these parallelisms in gene expression, the hypothesis of a glomerular origin of the proepicardium has not received yet experimental support. The aim of this paper is to provide experimental evidence for the latent glomerular potential of the proepicardium and to show how this potential can be partially rescued by exposing isolated chick proepicardia to factors which are known to be involved in the induction of nephrogenesis. We have used three inducers, coculture with murine embryonic kidney tissue, an activator of the canonical Wnt pathway and retinoic acid. It has been well established that activation of the canonical and non canonical Wnt signalling pathways are required for nephrogenesis (Iglesias et al., 2007; Schmidt-Ott and Barasch, 2008, Burn et al., 2011). On the other hand, retinoic acid (RA) is a main factor for specification of the intermediate mesoderm (Oeda et al., 2013) and its gradient through the anteroposterior axis establishes the anterior limit of the kidney morphogenetic field (Preger-Ben Noon et al., 2009). RA is required for pronephros differentiation in *Xenopus* (Cartry et al., 2006) and zebrafish, where RA is essential for podocyte differentiation and induces expression of Wt1 (Micelli et al., 2014). Additionally, we also describe expression in the chick proepicardium of novel specific glomerular markers (*Glepp*-1/*Ptpro*, synaptopodin) and we speculate about the mechanisms that might be repressing glomerular differentiation in this tissue.

MATERIAL AND METHODS

Animal manipulation. The animals used in our research program were handled in compliance with institutional and European Union guidelines for animal care and welfare. Chick eggs were kept in a rocking incubator at 37°C. Embryos were staged according to the Hamburger and Hamilton stages of chick development.

Proepicardia isolation. Chick embryos were incubated at 37°C until stages HH16-17. At this stage, the embryos were excised, extensively washed in EBSS and their proepicardia dissected using small forceps and tungsten needles. The isolated proepicardia were fixed for immunohistochemistry, kept in RNAzol (Sigma) for RNA extraction or seeded in culture.

Culture. For each experiment 20-25 proepicardia were cultured in a hanging drop system. The culture medium was DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin. 5-10 proepicardia were collected in a drop of medium placed in the inner surface of the lid of a sterile 35 mm Petri dish containing 1 mL of sterile water. The proepicardia were cultured for 48 hours at 37°C and 5% CO_2 in a humidified incubator. For the treatment of the proepicardia, the medium was supplemented with 1 μM of retinoic acid (RA) or with 2,8 μM of BIO ((2′Z,3′E)-6-Bromoindirubin-3′-oxime), a GSK3 inhibitor. In the coculture assay, the proepicardia were cultured also in hanging drop culture with an embryonic kidney isolated from a 14.5 dpc C57BL/6J mouse embryo.

RNA extraction and RT-PCR. RNA was isolated from excised proepicardia using RNAzol (Sigma) according to the manufacturer's guidelines. Equal amounts of RNA were used to synthesize cDNA with the First Strand Amplification Kit (Roche) and PCR was performed using the following primer pairs:

Gapdh: (F) GTCTGGAGAGAAACCAGCCAAG / (R) ACCAGGAAACAAGCTTGACG;
Wt1: (F) CCGATCAGCAAGTGAGACAA / (R) GTCTTTGGTGCCGTTTCAGT;
Pax2: (F) AAAGTAGCGACCCCCAAAGT / (R) CTGTGCTGGGCACGATAGTA;
Synaptopodin: (F) CCTGCCCGTAACTTCCGTG / (R) GAGCGGCGGTAGGGAAAAG;
Glepp1: (F) ACCCTTTGGGACCTACTGCT / (R) ATGGTGACCCGGAAATTGTA;
Hoxb4: (F) ATGAAGCGATGTACCAGCCG/ (R) TTGGGGTTTACCGTGCTTACA;

The following PCR program was used for amplification: 95°C for 5 minutes; 95°C for 45 seconds; 60°C for 45 seconds; 72°C for 45 seconds; 72°C for 5 minutes; 30-36 cycles. RNA from an HH17 whole embryo was used as positive control, and water instead of cDNA as negative control.

Proepicardial blockage *in vivo*. Small openings were made with tungsten needles through the vitelline and chorionic membranes in HH16-17 chick embryo to expose the coelomic cavity. A small piece of the eggshell membrane was inserted between the inner curvature of the heart and the junction between OFT and aortic sac. The eggs were then reincubated for 72 hours. When the proepicardial adhesion to the heart chick embryos is partially blocked, we can still observe proepicardial villi in late embryos (HH29).

Real-time PCR of FACS-sorted GFP$^+$ epicardial cells. The methodology used to generate epicardial-specific *Wt1* knock-out mice has been described previously (Martínez-Estrada *et al.* 2010). The ventricles of *Cre*$^+$ (*Wt1*$^{loxP/gfp}$) and *Cre*$^-$ (*Wt1*$^{loxP/gfp}$) mouse embryos at stage E13.5 were dissected. The ventricles of *Wt1*$^{+/gfp}$ embryos at different stages of development (E11.5, E13.5, E14.5 and E16.5) were also dissected. Ventricles were trypsinized at 37°C until single cell suspensions were obtained. GFP$^+$ epicardial cells were isolated by FACS (Velecela et al. 2013). RNA was isolated from GFP$^+$ epicardial cells (Arcturus® PicoPure® RNA isolation kit), checked for quality (Agilent 2100 Bioanalyzer), amplified (Arcturus® RiboAmp®) and reverse-transcribed (SuperScript® III, Invitrogen). Analysis of gene expression was carried out by SYBR green real-time PCR. The levels of *Podocalyxin* (*Podxl*) were normalized to that of the housekeeping gene 18S (TATABiocenter). The primers used for *Podxl* are: (F) TCCTTGTTGCTGCCCTCT / (R) CTCTGTGAGCCGTTGCTG.

Immunohistochemistry. Chick embryos for laminin immunostaining were dissected, fixed in 4% PFA/PBS overnight at 4°C, embedded in paraffin and sectioned (10 μm). Deparaffinized slides were washed with Tris-PBS and nonspecific binding sites were saturated for 30 min with 16% sheep serum, 1% bovine serum albumin, and 0.5% Triton X-100 in Tris-PBS (SBT). The slides were incubated overnight at 4°C in the primary antibodies diluted in SBT: laminin (Sigma L9393, 1:100). Incubation of the primary antibody was followed by incubation in a secondary, Cy5-conjugated anti-rabbit. Nuclei were counterstained with DAPI (Sigma).

For podocalyxin immunostaining, 7-µm-thick paraffin wax sections were mounted onto glass slides (SuperfrostTM Plus) and antigens were retrieved by boiling samples in a pressure cooker for 1 min in TEG buffer (10 mM Tris and 0.5 mM EGTA, pH 9.0). Slides were then blocked in 1% BSA, 0.2% gelatin and 0.05% saponin. Samples were incubated overnight at 4°C with antibodies against Podxl (1:100; AF1556, R&D). Incubation of the primary antibody was followed by incubation using a secondary anti-goat antibody (MolecularProbes®). Nuclei were counterstained with DAPI (Sigma).

RESULTS

As described previously, Wilms' tumor gene 1 (*Wt1*) is expressed by the chick proepicardium *in vivo*. This expression is maintained after culturing of the proepicardium for 48 h. *Pax2*, a marker of the intermediate mesoderm, is not expressed in the proepicardium *in vivo*. This confirms previous data (Ishii et al. 2007). However, *Pax2* expression is detected in the proepicardium by RT-PCR after 48 h in culture (Figure 1). Localization of Pax2 protein in cultured proepicardium was confirmed by immunohistochemistry (Figure 2). Given this upregulation of *Pax2*, we decided to check if the expression of two podocyte markers (the phosphatase *Glepp1* and the cytoskeletal protein synaptopodin) were also upregulated in cultured chick proepicardia. Surprisingly, we found that both, *Glepp1* and synaptopodin were expressed at the same levels by both, fresh and cultured proepicardia (Figure 1).

We next performed experiments treating the cultured proepicardia with nephrogenic factors. We used RA, as explained above, and BIO (6-bromoindirubin-3'-oxime), an activator of the β-catenin canonical pathway that can mimic nephrogenic signals (Davies and Garrod, 1995) in the same way that Wnt proteins do (Herzlinger et al. 1994; Kispert et al. 1998). We also cocultured the proepicardia with mouse fetal kidney, after checking that the primers used were inactive in mouse tissue. We found that in all cases the expression of nephrogenic or podocyte markers was clearly upregulated in treated proepicardia (Figure 1). This upregulation was particularly strong when the proepicardia were treated with RA.

It has been described that competence of intermediate mesoderm to respond to nephrogenic signals is established by expression of Hoxb4, a gene whose anterior limit of expression is located about the sixth somite in the chick embryo, much posterior to the proepicardial area (Preger Ben Noon et al., 2009; Barak et al., 2012). Thus, we check expression of Hoxb4 in the proepicardium and we found that RA treatment induces this expression after 48 h in culture (Figure 1). Fresh or cultured proepicardium does not express Hoxb4.

Since untreated, isolated proepicardia already showed an upregulation of *Pax2*, we speculated that the contact of the proepicardium with the heart could be repressing the nephrogenic differentiation of this structure. Thus, we performed experiments to partially block the proepicardial adhesion to the myocardium in chick embryos, thus allowing for the persistence of proepicardial villi by the stage HH29, when the proepicardium has normally disappeared. As we already demonstrated previously (Cano et al. 2013) we observed that the expression of laminin was strongly upregulated in these late proepicardial villi, suggesting a mesenchymal to epithelial transition (Figure 2). Laminin was not expressed in the normal epicardium by these stages, but it was strongly expressed by the developing glomerulus where a mesenchymal to epithelial transition is occurring.

The Wt1 transcription factor is expressed in the epicardium and in the kidney mesenchyme, but it plays opposite roles in both embryonic tissues, as described in the discussion. Wt1 promotes epithelial-mesenchymal transition in the epicardium and mesenchymal to epithelial transition in the kidney (Essafi et al., 2011). To test if this antithetical function is also correlated with the repression and induction of nephrogenesis, we checked the expression of podocalyxin, a podocyte marker directly activated by Wt1 (Palmer et al., 2001) in mice embryos with conditional deletion of Wt1 in the epicardium (Martínez-Estrada et al., 2010). Podocalyxin is expressed in epicardial cells isolated from early embryos (E11.5), but the expression is normally downregulated in later stages (Figure 3). However, this downregulation seems to be Wt1-dependent, since we found an upregulation of podocalyxin in the Wt1-deficient epicardium by RT-PCR and also by immunohistochemistry (Figure 3).

Discussion

We have previously proposed the hypothesis of an evolutionary origin of the proepicardium from an ancestral external pronephric glomerulus which lost its excretory function but whose primordium transiently appears in the embryonic development of gnathostomates to supply the heart with vascular and connective tissue progenitors (Cano et al., 2013). The reasons for this proposal, as described in the introduction, are 1) the origin of the epicardium from the primordium of the right pronephric external glomerulus in agnathans (lamprey larvae) and 2) the large number of genes shared by proepicardial and kidney development. In this study we provide experimental support to our hypothesis since we have described for the first time expression in the proepicardium of some genes characteristic of podocytes and, more importantly, because we have demonstrated that treatment of the isolated propicardium with factors that induce nephrogenesis greatly increases the expression of nephric markers. These studies were performed with chick proepicardia, since the mouse proepicardium is far more difficult to use for experimental studies.

The simple isolation of the proepicardium in culture for 48 h leads to the expression of *Pax2*. This gene is expressed in the intermediate mesoderm and its function is required for nephrogenesis but it is not expressed in the *in situ* chick proepicardium (Ishii et al. 2007, and our results). *Pax2* appears sufficient to specify renal tissue within the early mesoderm (Dressler 2011), and ectopic expression can expand the region fated to become renal epithelia (Bouchard et al. 2002). *Pax2* is also involved in the mesenchymal to epithelial transition, and this can be related with the upregulation of laminin that we have described in proepicardial villi when they are prevented to attach to the myocardium. In contrast, the epicardium attached to the heart by the same stage does not express laminin. These results suggest that the contact with the heart can be repressing in some way epithelial differentiation in the proepicardium and epicardium.

Activation of the β-catenin canonical signaling pathway in the cultured proepicardium and especially treatment with retinoic acid sharply increased the expression of Pax2 and podocyte markers such as the phosphatase Glepp-1 and the cytoskeletal protein synaptopodin. Glepp1 is a protein tyrosine phosphatase involved in the regulation of foot process structure and function (Thomas et al. 1994), while synaptopodin is an actin-binding protein directly involved in the formation of the podocyte foot processes (Mundel et al. 1997).

The effect of RA treatment seems paradoxical since the proepicardium itself expresses the RA-synthesizing enzyme Raldh2, thus being a site of RA production. We think that the effect of exogenous RA on the proepicardium can be related with the anteroposterior gradient of RA which contributes to pattern the intermediate mesoderm, establishing the anterior boundary of the nephric tissue, which in chick embryos is located at the level of the sixth somite (Preger Ben Noon et al., 2009). This gradient of RA determines the anterior expression boundary of Hoxb4, a gene which is key to establish the competence to respond to nephrogenic inducers such as activin (Preger Ben Noon et al., 2009; Barak et al., 2012). As described by these authors, RA treatment induces ectopic expression of Hoxb4 in intermediate mesoderm anterior to the sixth somite (ie., non nephrogenic). In the same way, we showed that treatment of the proepicardium with RA induced expression of Hoxb4, while this expression is not found in fresh or isolated proepicardium as expected. Thus, the lack of normal nephrogenic differentiation of the proepicardium must be related with the comparatively low local concentration of RA which is not enough to induce Hoxb4 expression making the proepicardium unresponsive to nephrogenic induction. Exogenous RA treatment "posteriorizes" the proepicardium, leading to expression of Hoxb4, acquisition of competence to respond to local nephrogenic inducers (which are present all along the trunk, Barak et al., 2005) and upregulation of Pax2 and podocyte markers. Interestingly, the anterior boundary of the Hox gene cluster corresponding to the group 4 is much anterior in embryos of the agnathan *Lethenteron* (Takio et al., 2007) as compared with the chick embryos. Hox4 genes are expressed in the cardiac area, thus explaining why the agnathan proepicardium, but not the proepicardium of gnathostomes, persists as a functional pronephric glomerulus.

As previously suggested (Cano et al. 2013) the transcription factor encoded by the Wilms' tumor suppressor gene *Wt1* could be repressing the nephrogenic potential of the proepicardium at the same time that *Wt1* is promoting nephrogenesis in the intermediate mesoderm. This paradoxical function can be explained by the dual role of *Wt1*, which promotes mesenchymal to epithelial transition in the kidney and the reverse process (epithelial to mesenchymal transition) in the epicardium, through a mechanism known as chromatin flip-flop (Essafi et al. 2011). In order to check if this dual role also affects to the expression of podocyte differentiation markers, we analyzed the expression of podocalyxin, a Wt1 target in podocytes (Palmer et al., 2001). Podocalyxin is a major component of the glycocalyx of the podocytes (Kerjaschki et al. 1984). Accordingly with the antithetic role played by Wt1, podocalyxin, which is expressed in the early epicardium but downregulated soon, maintains and increases its expression in Wt1-deficient epicardial cells, being detectable by immunohistochemistry. We think that the promotion by Wt1 of EMT and MET in the epicardium and developing kidney, respectively, does not only affect to cell phenotype, but it is also associated to repression and induction of podocyte markers such as podocalyxin.

Our findings clearly supports that the proepicardium is an evolutionary derivative of an external pronephric glomerulus. Thus, its anatomical association with the septum transversum and liver should be purely contingent and due to the evolutionary posteriorization of the intermediate mesoderm and the kidneys. The liver bud has been shown to induce expression of proepicardial markers in lateral mesoderm (Ishii et al. 2007), but this could be a consequence of the persistence of the proepicardial inductive signal in an area where the intermediate mesoderm existed in primitive vertebrates and which was later occupied by the septum transversum and the liver. Related with this, a recent report described the contribution of somatic mesoderm to the proepicardium (Schlueter and Brand 2013). We think that this interesting observation is not in contradiction with our proposal of a pronephric origin of the proepicardium. The somatic mesodermal area providing cells to the proepicardium (laterodorsal to the heart) would correspond to the very same area where the intermediate mesoderm is located in primitive vertebrates such as agnathans. We think that posteriorization of intermediate mesoderm, probably due to a decrease in the local RA concentration, lead to transformation of the most anterior intermediate mesoderm into the somatic mesoderm that would contribute to the proepicardium. The mechanism of induction of the right external glomerulus was kept, allowing for the formation of a non-excretory proepicardium. It is important to remark that we are proposing that the ancient origin of the proepicardium is a primitive anterior domain of intermediate mesoderm which disappeared along the evolution of gnathostomes, and not the splanchnic lateral mesoderm as previously thought.

The contribution of the glomerular/proepicardial cells to the developing heart solved the problem of providing vasculogenic cells to an organ which in vertebrates rapidly acquires high metabolic demand. Is it important to consider that other organs like the lungs, liver, gut or spleen always have a splanchnic mesodermal contribution in order to develop vessels and connective tissue, but the heart is directly developed from the splanchnic mesodermal sheet itself. The association of an ancestral excretory glomerulus with a heart is reminiscent of the association of a pulsatile structure with a glomerulus in hemichordates, in the so-called heart-kidney complex (Balser and Ruppert, 1990). This complex, located in the proboscis of enteropneusts, the most anterior part of the body, is composed of a contractile vessel associated to a glomerulus consisting of podocytes. However, the lack of expression of Nkx2-5 in the heart kidney complex (Lowe et al., 2006), argues against this possibility. In fact, the heart-kidney complex, together with the axial organ of Echinoderms, have been regarded as a feature unrelated with other pumping organs of metazoans such as the vertebrate heart (Xavier Neto, in Rosenthal and Harvey). Anyway, the association of hearts with excretory structures, probably taking advantage of the increase of blood pressure provided by the heart, is a common trait in many animals, for example insects (pericardial nephrocytes, Weavers et al., 2009; Na and Cagan, 2013) or bivalve molluscs (Meyhofer et al., 1985). Unfortunately, the lack of an excretory system in urochordates and a true heart in cephalochordates precludes a comparative approach to the possibility of an ancestral relationship between the heart and the excretory system in chordates.

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References

Balser, E.J. and Ruppert, E.E. 1990. Structure, ultrastructure, and function of the preoral heart-kidney in *Saccoglossus kowalevskii* (Hemichordata, Enteropneusta) including new data on the stomochord. Acta Zool., *71*, 235-249.

Barak, H., Preger-Ben Noon, E., and Reshef, R. 2012. Comparative spatiotemporal analysis of Hox gene expression in early stages of intermediate mesoderm formation. Dev. Dyn. *241*, 1637-1649.

Barak, H., Rosenfelder, L., Schultheiss, T.M., and Reshef, R. 2005. Cell fate specification along the anterior-posterior axis of the intermediate mesoderm. Dev. Dyn. *232*, 901-914.

Bouchard, M., Souabni, A., Mandler, M., and Neubüser, A. 2002. Nephric lineage specification by Pax2 and Pax8. Genes Dev. *16*, 2958–2970.

Burn, S.F., Webb, A., Berry, R.L., Davies, J.A., Ferrer-Vaquer, A., Hadjantonakis, A.K., Hastie, N.D., and Hohenstein, P. 2011. Calcium/NFAT signalling promotes early nephrogenesis. Dev. Biol. *352*, 288-298.

Cano, E., Carmona, R., and Muñoz-Chápuli, R. 2013. Evolutionary Origin of the Proepicardium. J. Dev. Biol. *1*, 3–19.

Carmona, R., Guadix, J. A., Cano, E., Ruiz-Villalba, A., Portillo-Sánchez, V., Pérez-Pomares, J.M., and Muñoz-Chápuli, R. 2010. The embryonic epicardium: an essential element of cardiac development. J. Cell. Mol. Med. *14*, 2066–2072.

Cartry, J., Nichane, M., Ribes, V., Colas, A., Riou, J.-F., Pieler, T., Dollé, P., Bellefroid, E.J., and Umbhauer, M. 2006. Retinoic acid signalling is required for specification of pronephric cell fate. Dev. Biol. *299*, 35–51.

Davies, J.A., and Garrod, D.R. 1995. Induction of early stages of kidney tubule differentiation by lithium ions. Dev. Biol. *167*, 50–60.

Dressler, G.R. 2011. Patterning and early cell lineage decisions in the developing kidney: the role of Pax genes. Pediatr. Nephrol. *26*, 1387–1394.

Essafi, A., Webb, A., Berry, R.L., Slight, J., Burn, S.F., Spraggon, L., Velecela, V., Martinez-Estrada, O.M., Wiltshire, J.H., Roberts, S.G.E., et al. 2011. A wt1-controlled chromatin switching mechanism underpins tissue-specific wnt4 activation and repression. Dev. Cell *21*, 559–574.

Herzlinger, D., Qiao, J., Cohen, D., Ramakrishna, N., and Brown, A.M. 1994. Induction of kidney epithelial morphogenesis by cells expressing Wnt-1. Dev. Biol. *166*, 815–818.

Iglesias D.M., Hueber, P.A., Chu, L., Campbell, R., Patenaude, A.M., Dziarmaga, A.J., Quinlan, J., Mohamed, O., Dufort, D., and Goodyer, P.R. 2007. Canonical WNT signaling during kidney development. Am. J. Physiol. Renal Physiol. *293*, F494-500.

Ishii, Y., Langberg, J.D., Hurtado, R., Lee, S., and Mikawa, T. 2007. Induction of proepicardial marker gene expression by the liver bud. Development *134*, 3627–3637.

Kerjaschki, D., Sharkey, D.J., and Farquhar, M.G. 1984. Identification and characterization of podocalyxin--the major sialoprotein of the renal glomerular epithelial cell. J. Cell Biol. *98*, 1591–1596.

Kispert, A., Vainio, S., and McMahon, A.P. 1998. Wnt-4 is a mesenchymal signal for epithelial transformation of metanephric mesenchyme in the developing kidney. Development *125*, 4225–4234.

Lowe, C.J., Terasaki, M., Wu, M., Freeman, R.M. Jr, Runft, L., Kwan, K., Haigo, S., Aronowicz, J., Lander, E., Gruber, C., Smith, M., Kirschner, M., and Gerhart, J. 2006. Dorsoventral patterning in hemichordates: insights into early chordate evolution. PLoS Biol. *4*, e291.

Männer, J., Pérez-Pomares, J.M., Macías, D., and Muñoz-Chápuli, R. 2001. The origin, formation and developmental significance of the epicardium: a review. Cells Tissues Organs *169*, 89–103.

Martínez-Estrada O.M., Lettice L., Essafi A., Guadix, J.A., Slight, J., Velecela, V., Hall, E., Reichmann, J., Devenney, P. S., Hohenstein, P., Hosen, N., Hill, R. E., Muñoz-Chapuli, R., and Hastie, N. D. 2010. Wt1 is required for cardiovascular progenitor cell formation through transcriptional control of Snail and E-cadherin. Nat. Genet. *108*, 89–93.

Miceli, R., Kroeger, P., and Wingert, R. 2014. Molecular mechanisms of podocyte development revealed by zebrafish kidney research. Cell. Dev. Biol. *3*, pii: 1000138.

Monte, G. del, Casanova, J.C., Guadix, J.A., MacGrogan, D., Burch, J.B.E., Pérez-Pomares, J.M., and de la Pompa, J.L. 2011. Differential Notch signaling in the epicardium is required for cardiac inflow development and coronary vessel morphogenesis. Circ. Res. *108*, 824–836.

Mundel, P., Heid, H.W., Mundel, T.M., Krüger, M., Reiser, J., and Kriz, W. 1997. Synaptopodin: an actin-associated protein in telencephalic dendrites and renal podocytes. J. Cell Biol. *139*, 193–204.

Na, J., Cagan, R. 2013. The Drosophila nephrocyte: back on stage. J. Am. Soc. Nephrol. *24*, 161-163.

Oeda, S., Hayashi, Y., Chan, T., Takasato, M., Aihara, Y., Okabayashi, K., Ohnuma, K., and Asashima M. 2013. Induction of intermediate mesoderm by retinoic acid receptor signaling from differentiating mouse embryonic stem cells. Int. J. Dev. Biol. *57*, 383-389.

Palmer, R.E., Kotsianti, A., Cadman, B., Boyd, T., Gerald, W., and Haber, D.A. 2001. WT1 regulates the expression of the major glomerular podocyte membrane protein Podocalyxin. Curr. Biol. *11*, 1805-1809.

Pombal, M.A., Carmona, R., Megías, M., Ruiz, A., Pérez-Pomares, J.M., and Muñoz-Chápuli, R. 2008. Epicardial development in lamprey supports an evolutionary origin of the vertebrate epicardium from an ancestral pronephric external glomerulus. Evol. Dev. *10*, 210–216.

Preger-Ben Noon, E., Barak, H., Guttmann-Raviv, N., and Reshef, R. 2009. Interplay between activin and Hox genes determines the formation of the kidney morphogenetic field. Development *136*, 1995–2004.

Schlueter, J., and Brand, T. 2013. Subpopulation of proepicardial cells is derived from the somatic mesoderm in the chick embryo. Circ. Res. *113*, 1128–1137.

Schmidt-Ott, K.M., and Barasch, J. 2008. WNT/beta-catenin signaling in nephron progenitors and their epithelial progeny. Kidney Int. *74*, 1004-1008.

Takio, Y., Kuraku, S., Murakami, Y., Pasqualetti, M., Rijli, F.M., Narita, Y., Kuratani, S., and Kusakabe, R. 2007. Hox gene expression patterns in *Lethenteron japonicum* embryos--insights into the evolution of the vertebrate Hox code. Dev. Biol. *308*, 606-620.

Thomas, P.E., Bryan, L., Goyal, M., Wiggins, J.E., Holzman, L.B., and Wiggins, C. 1994. GLEPP1 , a renal glomerular epithelial cell (podocyte) membrane protein-tyrosine phosphatase. J. Biol. Chem. *269*, 19953–19962.

Velecela, V., Lettice, L.A., Chau, Y.Y., Slight, J., Berry, R.L., Thornburn, A., Gunst, Q.D., van den Hoff, M., Reina, M., Martínez, F.O., Hastie, N.D., and Martínez-Estrada O.M. 2013. WT1 regulates the expression of inhibitory chemokines during heart development. Hum. Mol. Genet. *22*, 5083-5095.

Weavers, H., Prieto-Sánchez, S., Grawe, F., Garcia-López, A., Artero, R., Wilsch-Bräuninger, M., Ruiz-Gómez, M., Skaer, H., and Denholm, B. 2009. The insect nephrocyte is a podocyte-like cell with a filtration slit diaphragm. Nature. 457, 322-326.

Xavier-Neto, J., Davidson, B., Simoes-Costa, M.S., Castro, R.A., Castillo, H.A., Sampaio, A.C. and Azambuja, A.P. 2010. Evolutionary origins of hearts. In N. Rosenthal and R.P. Harvey (eds.). Heart Development and Regeneration. Academic Press, Boston. pp. 3–45.

Figures and Figure Legends

Figure 1. Top: Expression of nephrogenic markers in fresh chick proepicardia (F), cultured proepicardia for 48 h (C), cultured proepicardia treated with retinoic acid (RA), or a Gsk3 inhibitor (BIO) and proepicardia cocultured with fetal mouse kidney (CO). C+ is a positive control (whole chick embryo). A strong increase of nephrogenic and podocyte markers is observed when the proepicardia are treated with RA and BIO. **Bottom**: Retinoic acid treatment induces Hoxb4 expression in cultured proepicardia. Fresh or non treated cultured proepicardial do not express Hoxb4.

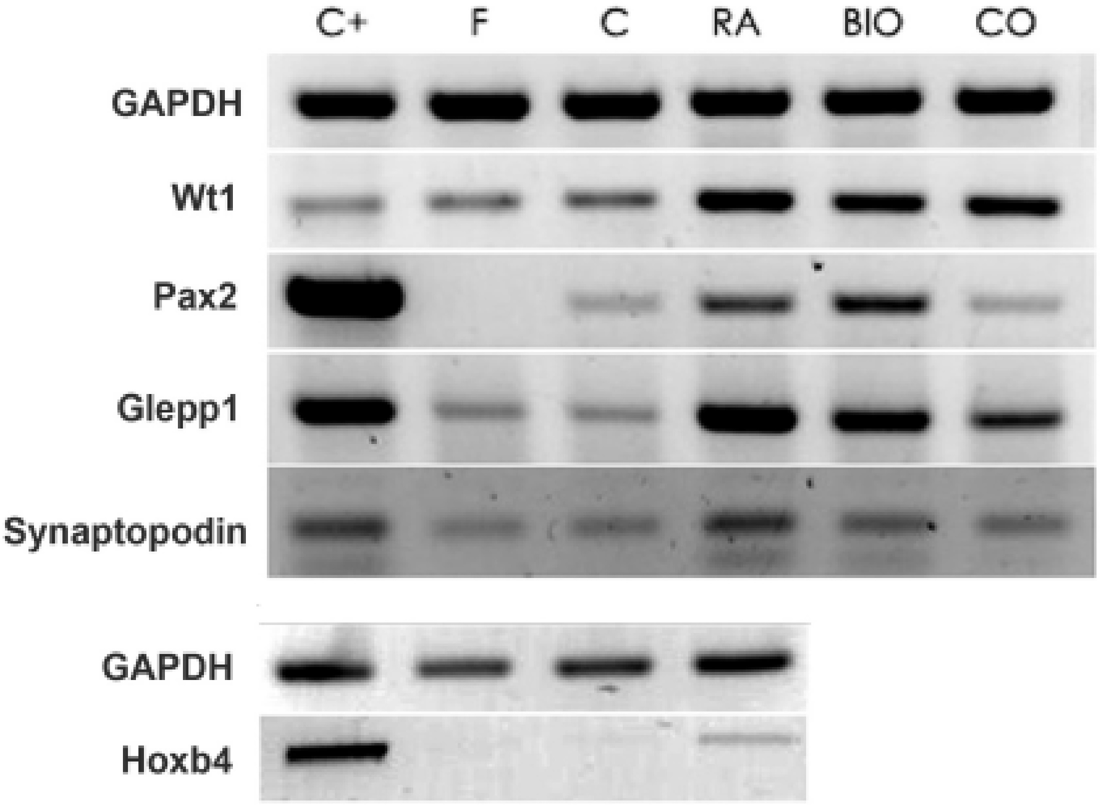

Figure 2. A-B. Immunolocalization of laminin in proepicardial villi (PE) of HH29 chick after partial blockage of proepicardial adhesion to the heart. Laminin expression is upregulated in these late proepicardial villi (arrowhead) and even in cells inside the villi (arrow). However no expression of laminin is detected in the epicardium (EP). **C.** The kidney glomeruli (GL) shows a strong laminin expression by these stages. A previous version of this figure was published in Cano et al. (2013), and it is reproduced with permission. D. Pax2 immunolocalization in a chick proepicardium cultured for 48 h. No expression of Pax2 was detected in fresh proepicardia (not shown).

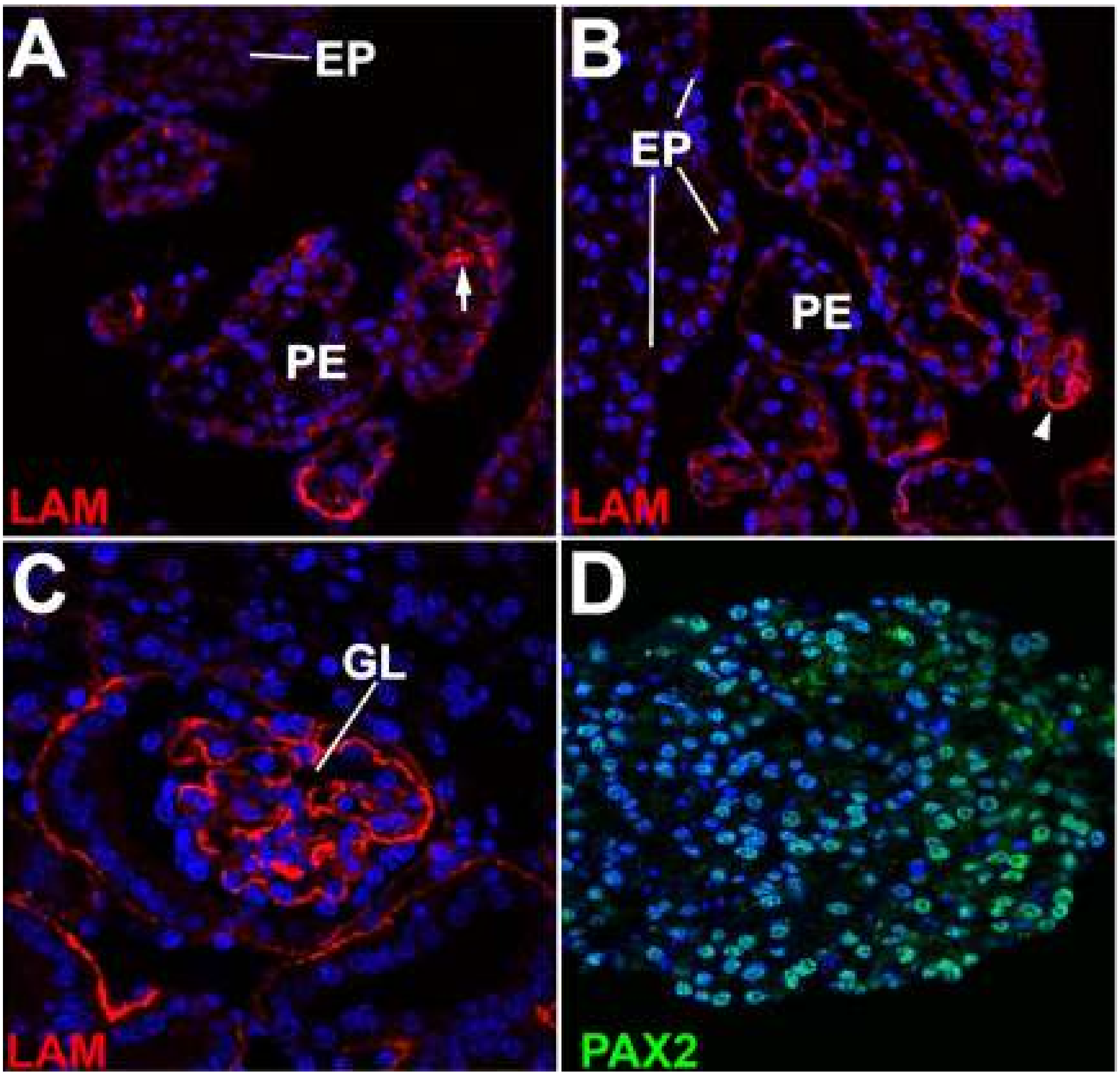

Figure 3. Top: The podocyte marker podocalyxin-1 is expressed by the earliest epicardial cells in mouse embryos, but the expression is downregulated in later stages. However, expression is significantly upregulated in embryonic epicardial cells where Wt1 is conditionally inactivated as shown in the right (epicardial cells isolated from Wt1 loxP/gfp Gata5Cre embryos). **Bottom:** This upregulation was confirmed by immunolocalization of podocalyxin in the ventricle of E15.5 Wt1 loxP/gfp Gata5Cre embryos with conditional inactivation of Wt1 in the epicardium.