Int. J. Dev. Biol. 55: 1007-1014
doi: 10.1387/ijdb.113394jf

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Ccbe1 expression marks the cardiac and lymphatic progenitor lineages during early stages of mouse development

JOÃO FACUCHO-OLIVEIRA[1,2], MARGARET BENTO[1,2,3] and JOSÉ-ANTÓNIO BELO*,[1,2,3]

[1]Regenerative Medicine Program, Departamento de Ciências Biomedicas e Medicina, University of Algarve, [2]IBB-Institute for Biotechnology and Bioengineering, Center for Molecular e Structural Biomedicine, University of Algarve, Campus de Gambelas, Faro, Portugal and [3]Instituto Gulbenkian de Ciência, Oeiras, Portugal

ABSTRACT The mammalian heart is a complex organ composed of diverse components and various cell types. Heart organogenesis requires the contribution of distinct pools of heart progenitors positioned in separate embryonic regions and subject to particular developmental signals. Moreover, these embryonic heart lineages have different transcriptional profiles expressing specific genes which activate pathways involved in heart lineage specification. Understanding the molecular control of heart organogenesis has major implications for treating congenital and adult heart diseases since specific heart lineages have been associated with particular human cardiovascular malformations. Collagen and calcium-binding EGF-like domain 1 (Ccbe1) was identified in our laboratory using an Affymetrix GeneChip system approach to identify the transcriptome of chick heart/hemangioblast precursor cells. Here, we present a detailed and systematic analysis of the expression of *Ccbe1* during early mouse development using whole-mount *in situ* hybridization (WISH), immunohistochemistry and histological techniques. *Ccbe1* mRNA was initially detected in the early cardiac progenitors of the two bilateral cardiogenic fields (E7.0) and in the cardiogenic mesoderm (E7.5 to E8.0). *Ccbe1* mRNA was then persistently detected in the pericardium and transiently expressed in the myocardial tissue of the primitive heart tube (E8.25), being later expressed in the proepicardium. By E9.5, the Ccbe1 and Prox1 proteins were found to be expressed in common regions, including the septum transversum and in the proximity of the anterior cardinal vein. Here, it is shown that *Ccbe1* is expressed in the FHF, SHF and proepicardium during heart organogenesis (E7.0 to E8.75). Later in development, *Ccbe1* expression is localized in the septum transversum and in the vicinity of the anterior cardinal vein, embryonic structures related to hepatic and lymphatic development, respectively.

KEY WORDS: *Ccbe1*, *cardiogenic mesoderm*, *proepicardium*, *cardiogenesis*, *lymphangiogenesis*

Cardiogenesis is dependent on three major pools of embryonic heart progenitors that are spatially and temporally segregated in the developing embryo and give rise to distinct cardiac structures (Harvey, 2002; Laugwitz *et al*., 2008). The cardiogenic mesoderm is the first major source of heart cell precursors to be identified during heart development (embryonic day (E) 7.0) and consists of two different population of heart progenitors, the first heart field (FHF) and the second heart field (SHF). The FHF is derived from the anterior splanchnic mesoderm and gives rise to the primitive heart tube that ultimately generates the bulk of the atrial chambers and the left ventriculum (Laugwitz *et al*., 2008; Vincent and Buckingham, 2010). The SHF is derived from the pharyngeal mesoderm and contributes to the outflow tract, the right ventricular region and the main parts of the atrial tissue (Laugwitz *et al*., 2008, Vincent and Buckingham, 2010). Another cardiogenic region is the proepicardium that locates posterior and dorsally to the heart tube and migrates onto the outer cardiac surface to form the epicardium mantle, contributing with myocytes during development of the ventricular septum and atrial and ventricular walls (E9.5; Manner *et al*., 2001; Cai *et al*., 2008). The proepicardium differentiates from a lateral rim of the cardiogenic mesoderm in close contact with the precursors of the septum transversum that are required for hepatic

Abbreviations used in this paper: Ccbe1, collagen and calcium-binding EGF-like domain 1; FHF, first heart field; LECs, lymphatic endothelial cells; SHF, second heart field; WISH, whole mount *in situ* hybridization.

***Address correspondence to:** José António Belo. Centre for Molecular and Structural Biomedicine, Biomedical and Medicine Sciences Department, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal. Fax +351-289-818-419. e-mail: jbelo@ualg.pt

Accepted: 29 November 2011. *Final, author-corrected PDF published online:* 22 December 2011.

ISSN: Online 1696-3547, Print 0214-6282

and pancreatic specification (Mommersteeg *et al.,* 2010, Burke and Oliver, 2002). Almost simultaneously with the epicardium formation, the cardiac neural crest cells migrate from the dorsal neural tube into the heart to give rise to the vascular smooth muscle of the aortic arch, ductus arteriosus and great vessels (Epstein and Buck, 2000). Understanding the tight spatial and temporal control of differentiation and unravel novel marker genes for these heart cell lineages is critical for a better knowledge of the molecular control of cardiogenesis which in turn has major implications for treating both congenital and adult heart diseases.

We have previously reported a differential screening using Affimetrix GeneChip® Chicken Genome arrays to identify novel genes required for the correct development and differentiation of the vertebrate heart and hemangioblast precursor cell lineages (Bento *et al*., 2011). A construct containing EGFP expression under the control of a 2.5 kb fragment upstream the ATG of chick *Cerberus* (c*Cer*; Tavares *et al*., 2007) was used to electroporate chick embryos and isolate early cardiac progenitors. The genetic profiling provided relevant data of the chick heart/hemangioblast precursor lineage identity and led to the detection of 301 uncharacterized genes (119 unknown genes and 182 annotated genes) that were upregulated in the heart/hemangioblast precursors in comparison to embryonic control cells (Bento *et al*., 2011). Among the annotated genes potentially involved in heart development, chick Collagen and calcium-binding EGF-like domain 1 *(c**Ccbe1)* was found to be upregulated by 7.8 fold.

Recently, mouse (m)*Ccbe1* has been shown to be essential for budding and/or migration of lymphatic endothelial cells (LECs) from the anterior cardinal veins to form the lymph sacs (E10.5 to E13.5) and give rise to the lymphatic vasculature (Bos *et al*., 2011). Indeed, homozygous m*Ccbe1* knockout mice showed a reduction of the number of Prox-1^{+} and Lyve-1^{+} LECs at the level of the cardinal vein and lymph sacs which lead to defective lymphatic vasculature, severe edema and prenatal death (Bos *et al*., 2011). In zebrafish, z*ccbe1* transcripts were also found to be located along the migratory routes of lymphangioblast that bud from the cardinal veins to seed the horizontal myoseptum region and subsequently generate the primordial lymphatic vessels. Moreover, homozygous mutation in the calcium-binding EGF-like domain (exon 4) of z*ccbe1* gene was shown to result in absence of thoracic duct and longitudinal lymphatic vessels which in turn resulted in severe edema and high lethality rate of mutant fish (Hogan *et al.,* 2009). In humans, *CCBE1* gene has also been associated with Hennekam syndrome, a disorder characterized by abnormal lymphatic system development causing generalized lymphedema, intestinal lymphangiectasias and mild to moderate levels of growth and mental retardation. Homozygous cysteine to serine mutation in a human *CCBE1* (h*CCBE1*) gene region (exon 3) highly conserved across vertebrates has been shown to result in a generalized lymphatic dysplasia leading to significant morbidity and high mortality prevalence (Connell *et al*., 2009; Alders *et al*., 2009). While the importance of mCcbe1 for the development of the lymphatic system appears to be indisputable, its role in cardiac development has not been investigated despite the increasing evidence of a potential function in cardiogenesis. Indeed, analyses of m*Ccbe1* heterozygous knockout embryos have shown X-Gal staining in the mesothelium of the heart at E12.5 (Bos *et al*., 2011). Furthermore, some of the Hennekam syndrome patients carrying a mutated h*CCBE1* gene were shown to possess congenital heart defects including hypertrophic cardiomyopathy and ventricular septal defects (Connell *et al*., 2009; Alders *et al*., 2009).

Here, we report that during early mouse development, m*Ccbe1* mRNA is initially expressed in the early cardiac progenitors that emerge from the primitive streak to form the two bi-lateral cardiogenic fields at E7.0 and in the cardiogenic mesoderm of the FHF and SHF between E7.5 to E8.0. m*Ccbe1* mRNA is then expressed in the pericardium and in the primitive heart tube at E8.25 and in the proepicardium, septum transversum and somites between E8.75 and E10.0. In addition, immunohistochemistry demonstrated that mCcbe1 and Prox1 proteins were detected at the level of the septum transversum and in the vicinity of the anterior cardinal vein, a primordial site for the onset of lymphangiogenesis. Adding to the reported role in the development of the lymphatic vasculature, the consistent expression of m*Ccbe1* in tissues containing the most relevant cardiac progenitor lineages put this gene forward as an important marker of heart precursor cell lineages.

Results

c*Ccbe1* was identified during a gene expression profiling of chick heart/hemangioblast precursors using Affymetrix® GeneChip Arrays (Bento *et al*., 2011). Gene expression analysis using whole mount *in situ* hybridization (WISH) confirmed the c*Ccbe1* is expressed in the early cardiac progenitors of the heart forming region and continue to be expressed in major cardiogenic lineages during chick heart development (unpublished data). In addition, *cCcbe1* is also expressed in the anterior and posterior cardinal veins and in the somites (unpublished data). Moreover, *Ccbe1* gene is conserved across vertebrates with chick *Ccbe1* aminoacid (a.a.) sequence being 79% and 69% identical to the mouse and zebrafish Ccbe1 protein, respectively. In mouse, m*Ccbe1* encodes a 408 a.a. predicted secreted protein that contains a signal peptide, a collagen domain and a calcium binding EGF-like domain. To characterize the expression of m*Ccbe1* during mouse development, a blast search was conducted to identify several plasmid clones suitable for synthesis of WISH anti-sense probes. A plasmid DNA clone containing an 854 bp sequence fragment of the 3'UTR of m*Ccbe1* gene was ordered. Here we present a comprehensive analysis of the expression pattern of m*Ccbe1* during mouse development (E7.0 to E10.5) with particular focus on the process of morphogenesis and organogenesis of the heart. Overall expression of m*Ccbe1* was determined by WISH and more detailed analysis of tissue specific expression was examined using histological techniques. Single WISH using probes for the proepicardium marker *Tbx18* and double WISH using probes for m*Ccbe1* and the SHF marker *Isl1* were carried out to determine the exact cardiogenic lineages expressing m*Ccbe1*. In addition, immunohistochemistry using antibodies for mCcbe1 and Prox1 was performed to confirm expression of mCcbe1 at the level of the septum transversum and in the domains surrounding the anterior cardinal vein.

mCcbe1 expression in the cardiogenic mesoderm

m*Ccbe1* mRNA is firstly detected at E7.0 forming two fields at an anterior-lateral position under the primitive head fold region and on either side of the notochordal plate (Fig. 1A and 1B, arrows). Between E7.25 to E7.5, m*Ccbe1* messages are extended from the initial bi-lateral fields to a more anterior and medial position forming a crescent-like shape under the head folds (Fig. 1C to

1F). Histological sections demonstrate that m*Ccbe1* expression is located at the cardiogenic mesoderm subjacent to the head fold (Fig. 1D' and 1D"). At E7.5, *mCcbe1* continues to be expressed in the cardiogenic mesoderm that will give rise to the future cardiogenic plate (arrow, Fig. 1F') and in the mesothelial cells that give rise to the intra-embryonic coelomic cavity (arrowhead, Fig. 1F" and 1F'"). At the primitive heart tube stage (E8.0), m*Ccbe1* mRNA staining continues to generate a well defined crescent-like shape under the head folds (Fig. 1G to 1J). Expression is located at the cardiogenic plate (arrow, Fig. 1H') and myocardial tissue forming the primitive heart tube and in the mesothelium lining the coelomic cavity (prospective pericardial cavity; arrowhead, Fig. 1H") and the left- and right-horn of the coelomic canals (future left and right pericardio-peritoneal canals; Fig.1H'"). Moreover, double WISH performed to detect m*Ccbe1* mRNA (light blue) and *Isl1* mRNA (purple) at E8.0 demonstrated that m*Ccbe1* is mainly expressed in FHF progenitors (Fig. 1I and 1J). Nevertheless, less intense m*Ccbe1* staining was also detected in *Isl1* positive regions suggesting that the cardiac progenitors of the SHF also express *Ccbe1* (Fig. 1I and 1J). Histological sections at the level of the cardiogenic plate shows that *Isl1* transcripts are specifically located in the SHF precursors of pharyngeal mesoderm whereas *mCcbe1* transcripts are mostly expressed by the FHF progenitors located at a more ventral position (Fig. 1J'). Sections at a more posterior region, also demonstrate that the most medial limit of m*Ccbe1* expression in the cardiogenic mesoderm lining the intra-embryonic coelomic cavity (purple arrow, Fig. H'" and J'") is contained within the Isl1 expression domain (blue arrow, Fig. J'"). At E8.25, m*Ccbe1* mRNA was detected in the ventral mesothelium of the pericardium (Fig. 1L', 1L", 1N' and 1N") and mesoderm lining the intra-embryonic coelomic cavity (Fig.1L'" and 1N'"). Noteworthy, less intense m*Ccbe1* staining was also detected in the heart tube tissue adjacent to the ventral pericardium (arrows, Fig. 1L' and 1N'). At this stage, m*Ccbe1* mRNA continues to be weakly colocalised with *Isl1* (Fig. 1L' and 1N'). Indeed, staining at the level of the heart tube further relates m*Ccbe1* with the FHF progenitors.

mCcbe1 expression in the proepicardium

At E8.5, m*Ccbe1* expression outlines a V-like shape staining that delineates the pericardial cavity and fuse at a posterior position to the heart (Fig. 2A and 2B). At this stage of development, m*Ccbe1* staining localizes in the mesothelium of the pericardial cavity (Fig. 2B'). m*Ccbe1* expression in the heart is residual and most likely restricted to cardiac cells derived from m*Ccbe1*-expressing cardiac

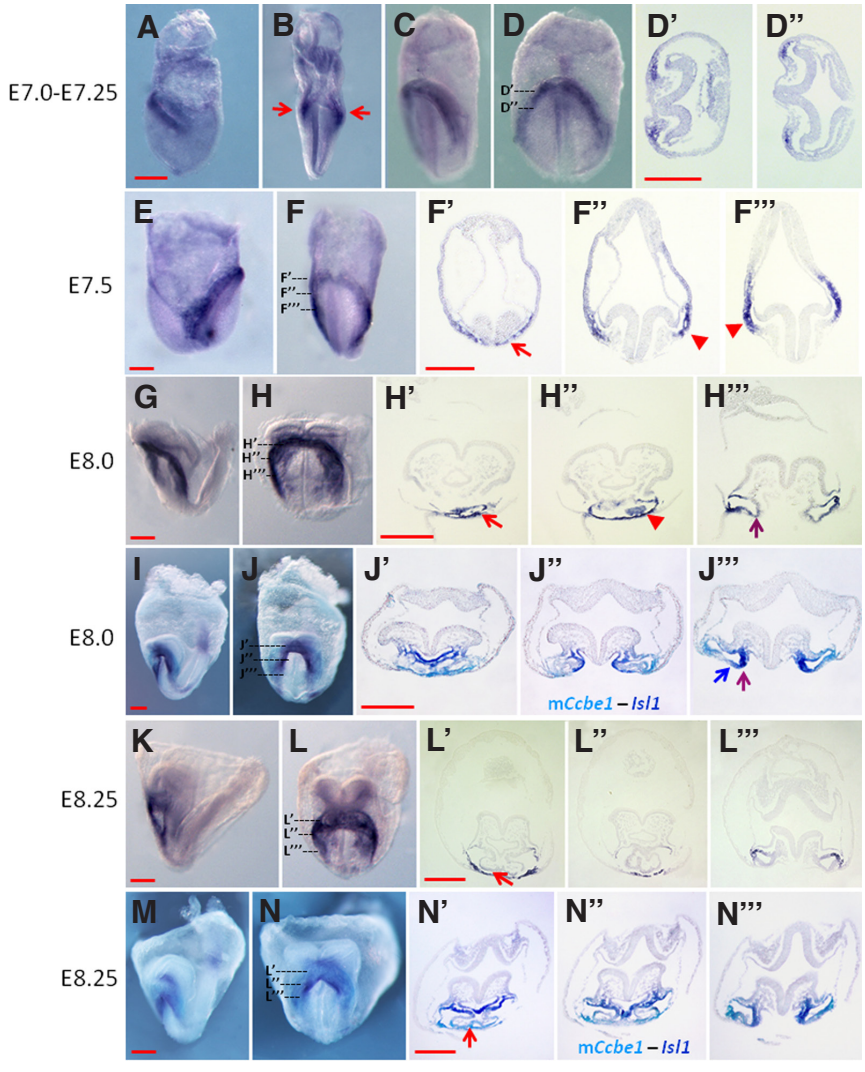

Fig. 1. Whole mount *in situ* hybridization analysis of m*Ccbe1* expression during E7.0 to E8.25. *Histological transverse sections were performed at 8 μm. Lateral* **(A)** *and anterior* **(B)** *views at E7.0 showed expression of* mCcbe1 *at two bilateral fields in either side of the primitive streak. Lateral* **(C)** *and anterior* **(D)** *views at E7.25 showed crescent-like shape staining under the head folds. Transverse sections at the head fold level showed that* mCcbe1 *is expressed in the cardiogenic mesoderm (D', D"). At stage E7.5,* mCcbe1 *mRNA staining continues to delineate a crescent shape in anterior view* **(E,F)**. *A series of transverse sections extending from the head fold level towards to a more caudal positions showed that* mCcbe1 *is expressed in the cardiogenic plate (arrow in F') and in the mesothelial precursors of the intra-embryonic coelomic cavity (arrow in F", F'"). Lateral* **(G)** *and anterior* **(H)** *views of whole mount* in situ *hybridization at the early heart tube stage (E8.0). Transverse histological section showed* mCcbe1 *mRNA located in the cardiogenic plate (arrow, H'), myocardial tissue of the primitive heart tube and mesothelium of the pericardial cavity (arrow, H") and in the left- and right-horn of the coelomic canals corresponding to the prospective left and right pericardio-peritoneal canals (arrow, H'"). Double WISH performed to detect* mCcbe1 *mRNA (light blue) and* Isl1 *mRNA (purple) at E8.0 demonstrated that* mCcbe1 *is mainly expressed in FHF progenitors; see grey arrow in* **(I,J)**, *although restricted overlap of* mCcbe1 *and* Isl1 *staining confirms that* mCcbe1 *is also expressed in the SHF (blue arrow, I and J'"). At E8.25,* mCcbe1 *continues to delineate the pericardium cavity; lateral view,* **(K,M)**; *anterior views* **(L,N)**. mCcbe1 *mRNA was detected in the ventral mesothelium of the pericardium (L",N'), heart tube tissue adjacent to the ventral pericardium (arrows, L' ,N') and mesoderm lining the intra-embryonic coelomic cavity (L'" , N'"). Double WISH reveals that* mCcbe1 *mRNA (light blue) continues to be partially colocalized with* Isl1 *(purple) at the level of the pharyngeal mesoderm (L' , N'). Scale bars, 200 μm.*

progenitors that have not yet completely downregulated m*Ccbe1* expression (Fig. 2B'). Moreover, cross sections of E8.5 embryos at the level of the sinus venosus demonstrated the presence of two clusters of m*Ccbe1*-expressing cells that are continuous with the pericardial cavity and most likely compose the right and left anlagen of the developing proepicardium (arrows, Fig. 2B"). Indeed, in mouse, the proepicardium is derived from two anlagen of pericardial mesothelium which appear on either side of the venous pole of the pericardial cavity at E8.5. These cells then migrate to the embryonic midline where they merge to give rise to the mature proepicardium at E9.0 (Manner *et al.*, 2001, Mommersteeg *et al.,* 2010). Indeed, transverse sections of embryos between E8.5 and E8.75 at the level of the sinus venosus showed that m*Ccbe1* has a wide expression extending laterality from the cells adjacent to the right horn to the cells adjacent to the left horn of the sinus venosus (Fig. 2D" and 2E'). Moreover, histological sections of double WISH performed to detect m*Ccbe1* mRNA (light blue) and *Isl1* mRNA (purple) revealed that m*Ccbe1* staining is partially overlapped with *Isl1* staining at the level of the primitive proepicardium (arrows, Fig.1D"). In addition, m*Ccbe1* mRNA staining is also detected in the dorsal-lateral side of the mesothelium of the right and left pericardio-peritoneal canal where *Isl1* is also expressed (arrows, Fig. 2B"' and 2D"'). However, no co-localization of *Isl1* and m*Ccbe1* were detected at the level of the pharyngeal mesoderm (Fig. 1D'). To further confirm that m*Ccbe1* expression at the level of the venous pole of the heart is related to the proepicardium organ, m*Ccbe1* and *Tbx18* mRNA expression was compared in littermate embryos. Indeed, analysis of whole mount embryos and sagital sections clearly demonstrate that m*Ccbe1* and *Tbx18* expression coincide at the level of the proepicardium (Fig. 2F to 2F"' and 2G to 2H"').

Later in development, at E9.5, m*Ccbe1* expression is maintained in the proepicardium (Fig. 3A to 3C). Histological analysis of transverse and sagital sections confirm that the expression of m*Ccbe1* is residual or non-existent in the heart components (Fig. 3B') but rather strongly expressed in the heart progenitors of the proepicardium (arrowheads, Fig 3B", 3B"', 3C" and 3C"'). In addition, transverse sections of E9.5 embryos stained for m*Ccbe*1 or *Tbx18* mRNA at the level of the hepatic diverticulum (region of foregut-midgut junction) showed that m*Ccbe1* expression encompasses a region wider than the *Tbx18* expression and extends towards the septum transversum (Fig. 3B"' and 3E"'). To confirm that m*Ccbe1* expression at the level of the hepatic diverticulum was not only restricted to the proepicardium but also present in the septum transversum, immunohistochemistry for mCcbe1 and the septum transversum marker Prox1 was carried out in histological sections (Fig. 4). Indeed, mCcbe1 protein was detected in the extracellular domain of some Prox1$^+$ cells. However, mCcbe1 was not detected in the extracellular domain of all Prox1$^+$ cells but rather more concentrated around the more central Prox1$^+$ cells of the septum transversum (Fig. 4B). In addition, histological sections at the interface between the septum transversum and the embryonic gut shows high levels of mCcbe1 protein localized around the Prox1$^+$ cells and lower levels of mCcbe1 protein within the lumen of the gut (Fig. 4C). Expression of m*Ccbe1* in both the proepicardium and the septum transversum is consistent with the fact that these two transient organs are in close contact with each other and derive from adjacent progenitor

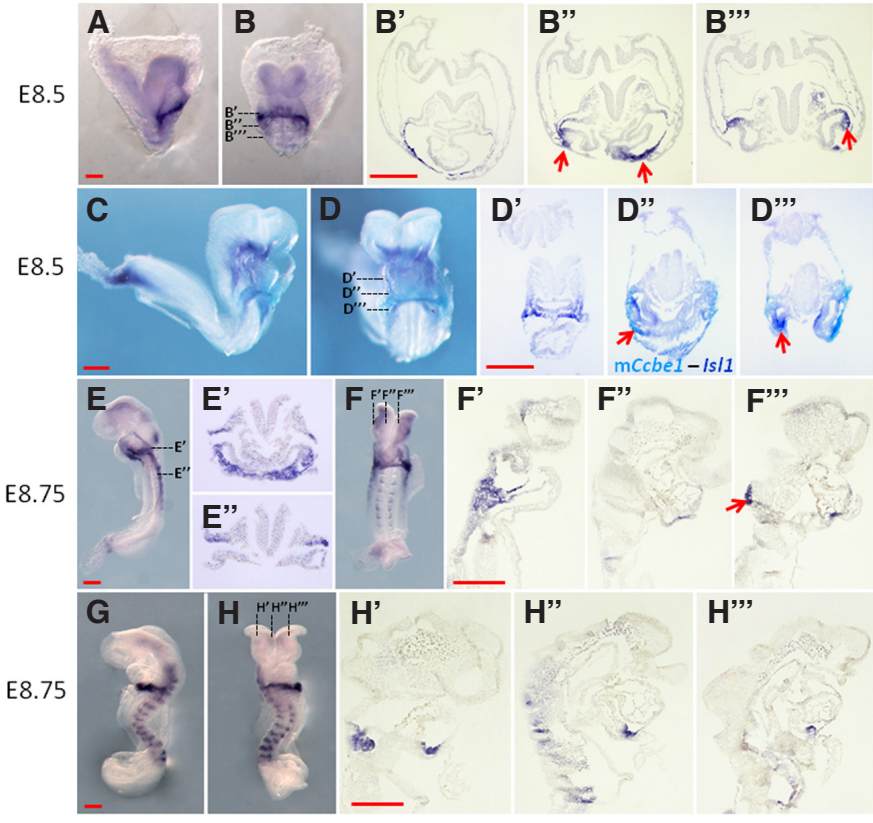

Fig. 2. Whole mount *in situ* hybridization analysis of *mCcbe1, Isl1* and *Tbx18* expression at E8.5 and E8.75. *Histological transversal and sagital sections were performed at 8 μm. Lateral* **(A)** *and anterior* **(B)** *views show that m*Ccbe1 *mRNA staining is detected at a ventral and dorsal position to the heart forming a V-shape that fuses at the level of the sinus venosus. Transverse sections showed that* mCcbe1 *expression localizes at the mesothelium of the pericardial cavity* **(B')**, *in the right and left anlagen of the developing proepicardium (arrows; B") and in the dorsal-lateral side of the mesothelium of the right and left pericardio-peritoneal canal (B"'). Lateral* **(C)** *and anterior* **(D)** *view of double WISH demonstrated that m*Ccbe1 *(light blue) and* Isl1 *(purple) staining were not co-localized at the level of the pharyngeal mesoderm* **(D')** *but partially overlapped at the level of the proepicardium (arrow; D") and in the dorsal-lateral side of the mesothelium of the right and left pericardio-peritoneal canal (arrow; D"'). Lateral view at E8.75* **(E)** *shows that m*Ccbe1 *staining continues to delineate the pericardial cavity. Transverse histological sections at the level of the sinus venosus showed that m*Ccbe1 *is expressed at the proepicardium (E'). Transverse section at a more posterior position (E") showed that m*Ccbe1 *is expressed in lateral plate mesoderm (somatopleure). Sagital sections represented in the anterior view* **(F)** *further demonstrate that m*Ccbe1 *is expressed in the proepicardium (F'-F"') at 8.75. Comparative analysis of litter mates processed by WISH showed that m*Ccbe1 **(E,F)** *and* Tbx18 **(G,H)** *are expressed in the proepicardium. Sagital sections at a lateral position showed m*Ccbe1 *(F') and* Tbx18 *(H') expressed at the lateral-dorsal region of the proepicardium. Sagital sections at a more medial positions showed m*Ccbe1 *(F" and F"') and* Tbx18 *(H", H"') expression is located in a region posterior to the venous pole of the heart corresponding to a more visceral region of the proepicardium. Scale bars, 200 μm.*

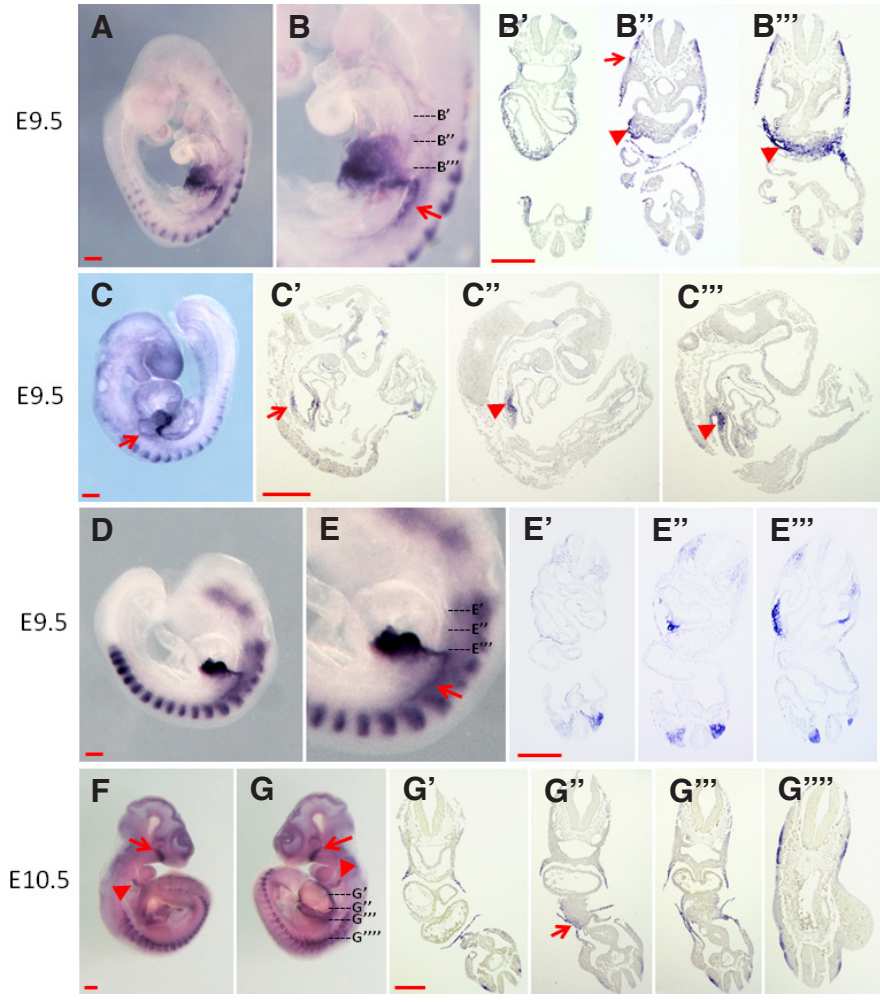

Fig. 3. Whole mount *in situ* hybridization analysis of m*Ccbe1* and T-box 18 expression during E9.5 to E10.5. *Histological transversal and sagital sections were performed at 8 μm. Lateral view of E9.5 embryo showed that m*Ccbe1 *is expressed in the proepicardium, in the anterior cardinal veins (arrow) and in the somites* **(A,B)**. *Transverse sections show that m*Ccbe1 *expression in the heart is residual or non-existent (B') but rather highly expressed in the proepicardium (arrowhead; B'', B'''), in the anterior cardinal vein (arrow; B'', B''') and dermomyotome of the cervical somites (B'', B'''). Sagital sections further demonstrate that m*Ccbe1 *staining is located in the vicinity of the anterior cardinal vein (arrow) which during mouse development gives rise to important veins of the cardiovascular and lymphatic systems* **(C)**. *Comparative analysis of whole mount and histological sections of littermate embryos processed to detect m*Ccbe1 *(A to B''') and* Tbx18 *mRNA* **(D, E)** *showed that m*Ccbe1 *expression is mostly coincident with* Tbx18 *expression (B''', E'''). A lateral view of a mouse embryo stained for m*Ccbe1 *mRNA at E10.5* **(F,G)**. *Transverse sections showed that* mCcbe1 *is expressed at the maxilary component of the first branchial arch containing trigeminal (V) neural crest tissue (G'), in the mesothelium of the pericardium and in a restricted region of the septum transversum (arrow; G'') and in the dermomyotome of the somites (G''''). Scale bars, 200 μm.*

regions at the lateral zone of the cardiogenic mesoderm (lateral rim of the splanchnic mesoderm; Mommersteeg *et al.*, 2010, Burke and Oliver, 2002). Moreover, expression of m*Ccbe1* at the septum transversum is consistent with the previous expression at the right and left pericardio-peritoneal canals at E8.5 (Fig.2B''') as the septum transversum derives from the fusion of the left and right pericardio-peritoneal to individualize the pericardial and peritoneal cavities. At E10.5, expression of m*Ccbe1* is then maintained at the mesothelium of the pericardial cavity and in the septum transversum (Fig. 3F to 3G'''). However, expression of m*Ccbe1* in the septum transversum at this stage of development is restricted to a small number of cells (arrow, Fig. 3G''). Downregulation of m*Ccbe1* at the level of proepicardium and septum transversum at this stage of development is coincident with the major translocation of cardiac progenitors from proepicardium to the heart and subsequent fading of the transient proepicardium organ (Zhou *et al.*, 2008).

***Ccbe1* expression in non-cardiogenic regions**

Apart from the consistent expression of m*Ccbe1* in cardiac progenitor tissues, m*Ccbe1* mRNA was also detected in the subset of cells located in a dorsal position to the heart, and the proepicardium of the mouse embryo at E9.5 (arrow, Fig. 3B and 3C). Transverse and sagital sections demonstrate that m*Ccbe1* mRNA staining is located in the vicinity of the anterior cardinal vein (arrow, Fig. 3B''and 3C'). Indeed, m*Ccbe1* expression in embryonic tissues close to the anterior cardinal veins has recently been demonstrated using X-gal staining of heterozygous *mCcbe1* knockout embryos (Bos *et al.*, 2011). In addition, double immunohistochemistry analysis detected mCcbe1 protein and Prox1$^+$ cells in vicinity of the anterior cardinal vein (yellow arrows, Fig. 4A). These Prox1$^+$ cells represent LECs that bud from the anterior cardinal vein to give rise to the lymph sacs and the primordial lymphatic system (Bos *et al.*, 2011). Here, it is reported that mCcbe1 protein is expressed in the tissues surrounding the anterior cardinal vein where Prox1$^+$ LECs are also present. m*Ccbe1* expression was also detected in the head mesenchyme between E8.25 and E8.75 (Fig. 1L, 2B and 2F') and dermomyotome of somites between E9.5 and E10.5 (Fig. 3B''' and 3G''''). Interestingly, expression of m*Ccbe1* at level of the somites appears to be much more intense in the cervical somites than in the anterior occipital or posterior thoracic somites (Fig. 3A and 3F) suggesting that m*Ccbe1* mRNA is possibly upregulated during a specific stage of somite maturation or otherwise more particularly involved in cervical somite differentiation. On the other hand, Flk1, a marker of endothelial and hematopoietic progenitors, is also expressed in the somites and in the cardiogenic mesoderm precursors and has been shown to contribute to the cardiac muscle cell lineages (Ema *et al.*, 2006; Motoike *et al.*, 2003). In addition, m*Ccbe1* is also expressed at the maxilary component of the first branchial arch containing trigeminal (V) neural crest tissue (arrow, Fig. 3F and 3G) and at the second branchial arch cleft at E10.5 (arrowhead, Fig. 3F and 3G).

Discussion

Expression analysis showed that m*Ccbe1* mRNA was initially

detected at an anterior-lateral position on either side of the notochordal plate at E7.0 (arrow, Fig. 1B). m*Ccbe1* mRNA staining at this stage of development coincides with the commitment of mesodermal cells that emerge from the anterior region of the primitive streak to form two bilateral fields with cardiogenic potential. Although, cardiac progenitors have been mapped at the anterior region of the streak, these cells retain a certain level of plasticity and become much clearly committed to cardiac fate upon migration into an anterior-lateral direction to form two groups of cells on either side of the midline (Tam *et al.*, 1997). During E7.25 to E8.0, expression of m*Ccbe1* mRNA forms a crescent like-shape at the cranial and cranial-lateral regions of the embryo resembling that observed in staining performed using markers of the FHF and SHF such as *Hand1* and *Isl1*, respectively (Cai *et al.*, 2003, Biben and Harvey, 1997). Indeed, histological section showed that m*Ccbe1* is expressed in the cardiogenic mesoderm (Fig. 1D' and 1F") and subsequently in the cardiogenic plate (arrow, Fig. 1H') as well as in the mesothelial cells of the intra-embryonic coelomic cavity that later gives rise to the pericardial cavity and pericardio-peritoneal canals where m*Ccbe1* is also expressed (Fig. 1H"', 1J"' and 1L"'). Moreover, double WISH showed that m*Ccbe1* is mainly expressed in the FHF although low levels of m*Ccbe1* mRNA were also detected in the *Isl1* positive SHF (Fig. 1J' to 1J"' and 1N' to 1N"').

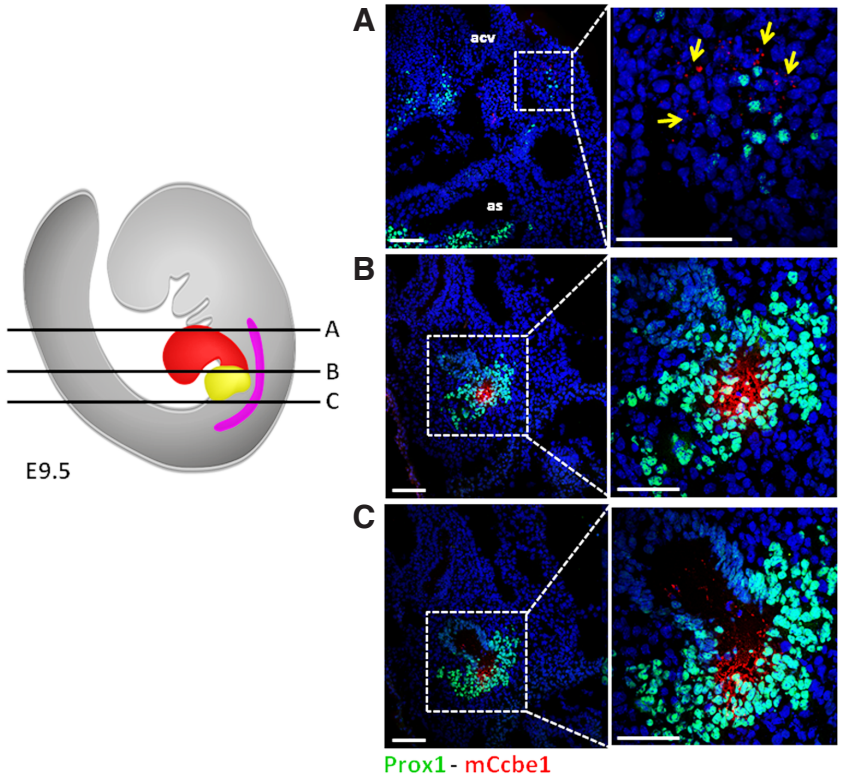

Fig. 4 Immunohistochemistry analysis of mCcbe1 and Prox1 expression at E9.5. *Histological transversal sections were performed at 10 μm. Transverse sections at the most posterior level of the aortic sac (as)* **(A)**, *showed mCcbe1 protein (red; yellow arrowheads) and Prox1 (green) localized in the vicinity of the anterior cardinal vein (acv). Histological section at the level of the septum tranversum* **(B)** *shows mCcbe1 protein expression within a subset of Prox1⁺ cells. Higher magnification detail shows mCcbe1 protein localized in the extracellular domain of the septum transversum. Histological section at the interface between the septum transversum and the embryonic gut* **(C)** *shows mCcbe1 protein localized around the Prox1+ cells and lower levels of mCcbe1 protein within the lumen of the embryonic gut. Scale bars, 100 μm.*

Although, m*Ccbe1*-expressing cells could be detected in the myocardial tissue forming the primordia of the primitive heart tube at E8.0 (arrows, Fig 1H" and 1N'), expression in the cardiac tissue in more advanced stages of heart morphogenesis was residual or nonexistent (Fig. 2B'). Continuous expression of m*Ccbe1* in the cardiogenic mesoderm and residual levels in the primordial myocardial tissues followed by absence of expression at later stages of heart development suggests that expression of m*Ccbe1* is limited to multipotent and highly proliferative progenitors and downregulated upon cellular commitment into more specific cardiac phenotypes. Indeed, restricted expression in the primordia of the heart tube is possibly limited to a subset of cardiac cells that derived from m*Ccbe1*-expressing cardiac progenitors that have not yet completely downregulated m*Ccbe1* expression.

Later in development, m*Ccbe1*-expressing cells were located at the developing and mature proepicardium and septum transversum (arrows, Fig. 2B", 3E', 4B and 4C). As previously described, these two populations arise from adjacent progenitor regions but contribute to the development of different organs. The proepicardium cells arise from *Nkx2.5*⁺/*Isl1*⁺ progenitors at the lateral zone of the cardiogenic mesoderm (lateral rim of the splanchnic mesoderm; Zhou *et al.*, 2008; Mommersteeg *et al.,* 2010) and originates the epicardium, the majority of the cardiac interstitium, and the coronary vasculature (Poelmann *et al.*, 2002) whereas the septum transversum can be identified through the expression of Prox1 (Fig. 4B and 4C) and is involved in hepatic and pancreatic development (Burke and Oliver, 2002).

During E9.5 to E10.5, m*Ccbe1*-expressing cells were also detected in the proximity of the anterior cardinal vein (arrow, Fig. 3B and 3C). Expression of mCcbe1 at the level of the cardinal veins have previously been reported in mouse at E10.5 and E12.5 using X-gal staining of m*Ccbe1* knockout embryos (Bos *et al.*, 2011) and in zebrafish at 36 h to 48 h post fertilization (hpf) using WISH (Hogan *et al.*, 2009). In addition, m*Ccbe1* knockout embryos and *zccbe1* mutant zebrafish have been shown to have a highly defective lymphatic system which accounts for a generalized edema and ultimately to premature death (Hogan *et al.*, 2009; Bos *et al.*, 2011). Adding to this, here we demonstrate that mCcbe1 protein was detected in the extracellular domain of tissues containing Prox1⁺ LECs at the level of the anterior cardinal vein (yellow arrows, Fig. 4A). Moreover, the detection of mCcbe1 protein near Prox1⁺ LECs further supports the previously raised hypothesis that Ccbe1 protein might act as an extracellular guidance molecule regulating the budding and migration of lymphangioblasts from the anterior cardinal vein (Hogan *et al.*, 2009). In humans, homozygous and compound heterozygous mutations of h*CCBE1* gene have also been shown to result in generalized lymphatic dysplasia associated with the very debilitating and highly deadly Hennekam syndrome (Connell *et al.*, 2009; Alders *et al.*, 2009). Despite the above mentioned and more preeminent defects in

lymphangiogenesis, cysteine to serine mutation of h*CCBE1* gene (C75S) has also been shown to be associated with in hypertrofic cardiomyopathy and congenital heart defects (Connell *et al*., 2009; Alders *et al*., 2009). This raises the possibility of a role of *Ccbe1* in the organogenesis of the heart from cardiac progenitors of the FHF, SHF and proepicardium where m*Ccbe1* is now reported to be expressed. Demonstration of a role in heart organogenesis must wait for further studies on genetic interactions of m*Ccbe1* with other genes involved in cardiac development.

In conclusion, we presented a comprehensive analysis of m*Ccbe1* expression during early mouse development. m*Ccbe1* was shown to be expressed in three major populations of embryonic cardiac progenitors namely the FHF, SHF and proepicardium. Moreover, m*Ccbe1* was expressed in these cardiogenic regions but rather not expressed in their derivative and more mature heart components suggesting that *Ccbe1* is downregulated as the progenitor cells differentiate towards more definitive cardiac phenotypes. As such, m*Ccbe1* appear to be an exclusive and common marker of cardiac precursors of the FHF, SHF and proepicardium. Despite the role of *Ccbe1* on the onset of lymphangiogenesis being irrefutable, the potential involvement of *Ccbe1* in heart morphogenesis still needs to be investigated.

Materials and Methods

A gene expression profiling of chick heart/hemangioblats precursors followed by a protocol for direct amplification of small amounts of mRNA (Gonçalves *et al*., 2011) and GeneChip Arrays (Affymetrix ®) has been recently reported (Bento *et al*., 2011). Among the genes identified, c*Ccbe1* mRNA was found to be upregulated in the heart/hemangioblast gene pool being therefore potentially required for vertebrate heart development.

Embryo collection

C57BL/6 mice were maintained on a 7pm to 7am dark cycle and mated overnight. Mouse embryos were obtained by crossing C57BL/6 mice and embryonic development was staged according to gestational age, with noon of the day of vaginal plug detection being considered E0.5. Pregnant females were sacrificed by cervical dislocation and the *uteri* were surgically removed and placed in ice-cold PBS. Embryos were dissected out of the *decidua* with fine forceps as previously described (Hogan *et al*., 1994) and staged according to morphological landmarks (Downs and Davies, 1993).

Whole mount in situ hybridization and histological analysis

WISH and anti-sense probe preparation was carried out as described in Belo *et al.,* (1997). Briefly, dissected embryos at various stages of development (E7.0 to E10.5) were fixed overnight at 4°C in 4% paraformaldehyde, dehydrated through a graded methanol series and stored at -20°C until use. WISH was repeated at least 3 times for each developmental stage using an entire litter in each experiment (minimum of 6 embryos per litter (n=18)). Digoxigenin-labeled *mCcbe1* anti-sense RNA probe was synthesized by linearizing the RZPDp981D0934D clone (*mCcbe1*; ImaGenes, Berlin, Germany) with EcoRI restriction enzyme and transcribed using T3 RNA polymerase. The RZPDp981D0934D plasmid clone encodes an 854 bp fragment of the 3'UTR of *mCcbe1* gene. Digoxigenin-labeled *Tbx18* anti-sense RNA probe was synthesized by linearizing the pKS-*Tbx18* clone with HindIII restriction enzyme and transcribed using T7 RNA polymerase. The fluorescein-labeled *Isl1* anti-sense RNA probe was synthesized to perform two-color double WISH staining. *Isl1* plasmid clone was linearized using XhoI restriction enzyme and transcribed using T3 RNA polymerase. The anti-sense probes were run on a 2% agarose gel to verify yield and length. Double staining was performed by simultaneous hybridization of digoxigenin-labeled *mCcbe1* anti-sense RNA probe and the fluorescein-labeled *Isl1* anti-sense RNA probe. Embryos were initially incubated with the alkaline phosphatase (AP)-conjugated anti-DIG Fab antibody (Roche Applied Sciences, Penzberg, Germany) overnight at 4°C. After several washes, specimens were incubated with AP-substrate BM purple solution (NBT/BCIP ready-to-use solution; Roche) and allowed to develop through chromogenic reaction to detect *Ccbe1* mRNA expression. Following thorough staining, AP enzymes were inactivated through a 72u61904C incubation in MABT (100mM Maleic Acid; 150mM NaCl; 0.1% Tween 20, pH7.5) for 1 hour. Samples were then incubated overnight at 4°C with the AP-conjugated anti-fluorescein Fab antibodies (Roche). After another series of washes, a solution containing the AP-substrate BCIP (187.5 μg/ml; Roche) was used to perform staining of the fluorescein-labeled probe. Finally, embryos were refixed in 4% paraformaldehyde and photographed using a Lumar V12 stereoscope and the Axiocam MRC digital camera (Carl Zeiss). Embryos selected for histological analysis (minimum of 4 embryos per developmental stage (n=4)) were embedded in paraffin and sectioned at 8 μm using a Leica RM2135 microtome. The sections were examined and photographed using a Axio Observer Z2 Fluorescence microscope (Carl Zeiss) and the digital camera AxioCam ICc 3.

Immunohistochemistry

Mouse embryos (E9.5) were dissected as described above, embedded in OCT (Tissue Tek, Torrance, CA) and frozen overnight at -80u61904C. Cryosections were cut at 10 μm using a Leica C3050 cryostat microtome (Leica Microsystems, Wetzlar, Germany), mounted onto superfrost slides and stored at –80°C until use. After thawing, the slides were fixed in 1% paraformaldehyde in PBS for 2 minute and washed in 0.1% Tween in PBS (PBS-T). The sections were then incubated in blocking solution (0.2% BSA (Roche Diagnostics, Mannheim, Germany) PBS-T) for 1 hour. Primary antibody incubations were performed overnight at 4u61904C using 1.5 μg/mL of the Ccbe1 anti-mouse antibody (Sigma-Aldrich, Sintra, Portugal) diluted in blocking solution in combination with 2 μg/mL of the Prox1 anti-goat antibody (R&D systems, Minneapolis, MN). The sections were then washed three times in PBS-T for 5 minutes and incubated for 1 hour at RT with 2 μg/mL AlexaFluor-488 donkey anti-goat antibody (Molecular probes, Paisley, UK) and 2 μg/mL AlexaFluor- 594 donkey anti-mouse antibody (Molecular Probes) diluted in blocking solution. Control experiments were performed using no primary antibodies against mCcbe1 or Prox1 but using the secondary antibodies at the corresponding concentrations. The sections were further washed three times with PBS-T for 5 minutes and placed onto slides using mounting medium containing DAPI (Vectashield; Vector Labs, Peterborough, England). Confocal fluorescent microscopy was performed using the laser scanning microscope LSM710 Confocal microscope (Carl Zeiss) and images captured using the Zen 2009 software at 100x, 200x and 400x magnification. A minimum of 3 embryos were analyzed by immunohistochemistry in 2 separate experiments (n=6)). Negative controls were used to determine the appropriate exposure time and prevent the generation of false positives in the samples.

Acknowledgements

We thank Prof. Andreas Kispert and Prof. Margaret Buckingham for the mTbx18 *and* mIsl1 *plasmid clones and Dr José Inácio, Tiago Justo and Sara Marques for critical reading of this manuscript. J.M Facucho-Oliveira is a F.C.T. post-doctoral fellow. This work was supported by research grants from F.C.T., from IGC/FCG and from IBB/CBME, LA to J.A. Belo.*

References

ALDERS M., HOGAN B.M., GJINI E., SALEHI F., AL-GAZALI L., HENNEKAM E.A., HOLMBERG E.E., MANNENS M.M., MULDER M.F., OFFERHAUS G.J., PRESCOTT T.E., SCHROOR E.J., VERHEIJ J.B., WITTE M., ZWIJNENBURG P.J., VIKKULA M., SCHULTE-MERKER S., HENNEKAM R.C. (2009). Mutations in CCBE1 cause generalized lymph vessel dysplasia in humans. *Nat Gene*t 12: 1272-1275.

BELO J. A., BOUWMEESTER T., LEYNS L., KERTESZ N., GALLO M., FOLLETTIE M., DE ROBERTIS E. M. (1997). Cerberus-like is a secreted factor with neutral-

izing activity expressed in the anterior primitive endoderm of the mouse gastrula. *Mech Dev* 68: 45- 57.

BENTO M., CORREIA E., TAVARES A.T., BECKER J.D., BELO J.A. (2011). Identification of differentially expressed genes in the heart precursor cells of the chick embryo. *Gene Expr Patterns* 11: 437-447.

BIBEN C. AND HARVEY R. P. (1997). Homeodomain factor Nkx2–5 controls left/right asymmetric expression of bHLH gene eHand during murine heart development. *Genes Dev* 11: 1357-1369.

BOS F.L., CAUNT M., PETERSON-MADURO J., PLANAS-PAZ L., KOWALSKI J., KARPANEN T., VAN IMPEL A., TONG R., ERNST J.A., KORVING J., VAN ES J.H., LAMMERT E., DUCKERS H.J., SCHULTE-MERKER S. (2011). CCBE1 is essential for mammalian lymphatic vascular development and enhances the lymphangiogenic effect of vascular endothelial growth factor-C in vivo. *Circ Res* 109: 486-491.

BURKE Z. AND OLIVER G. (2002). Prox1 is an early specific marker for the developing liver and pancreas in the mammalian foregut endoderm. *Mech Dev* 118: 147-155.

CAI C.L., LIANG X., SHI Y., CHU P.H., PFAFF S.L., CHEN J., EVANS S. (2003). Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. *Dev Cell* 5: 877-889.

CAI C.L., MARTIN J.C., SUN Y., CUI L., WANG L., OUYANG K., YANG L., BU L., LIANG X., ZHANG X., STALLCUP W.B., DENTON C.P., MCCULLOCH A., CHEN J., EVANS S.M. (2008). A myocardial lineage derives from Tbx18 epicardial cells. *Nature* 454:104-108.

CONNELL F., KALIDAS K., OSTERGAARD P., BRICE G., HOMFRAY T., ROBERTS L., BUNYAN D. J., MITTON S., MANSOUR S., MORTIMER P., JEFFEREY S. (2009). Linkage and sequence analysis indicate that CCBE1 is mutated in recessively inherited generalized lymphatic dysplasia. *Hum Gene*t 127: 231-241.

DOWNS K. M. AND DAVIES T. (1993). Staging of gastrulation mouse embryos by morphological landmarks in the dissecting microscope. *Development* 118: 1255-1266.

EMA M., TAKAHASHI S., ROSSANT J. (2006). Deletion of the selection cassette, but not cis-acting elements, in targeted Flk1-lacZ allele reveals Flk1 expression in multipotent mesodermal progenitors. *Blood* 107: 111-117.

EPSTEIN J. A. AND BUCK C. A. (2000). Transcriptional regulation of cardiac development: implications for congenital heart disease and DiGeorge syndrome. *Pediatr Res* 48: 717-724.

GONÇALVES L., FILIPE M., MARQUES S., SALGUEIRO A. M., BECKER J. D., BELO J. A. (2011). Identification and functional analysis of novel genes expressed in the Anterior Visceral Endoderm. *Int J Dev Biol* 55: 281-295.

HARVEY R. P. (2002). Patterning the vertebrate heart. *Nat Rev Genet* 3: 544-556.

HOGAN B., BEDDINGTON R., COSTANTINI F., LACY, E. (second Edition)(1994). Manipulating the mouse embryo: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.

HOGAN B. M., BOS F. L., BUSSMANN J., WITTE M., CHI N.C., DUCKERS H. J., SCHULTE-MERKER S. (2009). *ccbe1* is required for embryonic lymphangiogenesis and venous sprouting. *Nat Genet* 4: 396-398.

LAUGWITZ K.L., MORETTI A., CARON L., NAKANO A., CHIEN K.R. (2008). Islet1 cardiovascular progenitors: a single source for heart lineages? *Development* 135: 193-205.

MANNER J., PEREZ-POMARES J. M., MACIAS D., MUNOZ-CHAPULI R. (2001). The origin, formation and developmental significance of the epicardium: a review. *Cells Tissues Organs* 169: 2205-2218.

MOMMERSTEEG M.T., DOMÍNGUEZ J.N., WIESE C., NORDEN J., DE GIER-DE VRIES C., BURCH J.B., KISPERT A., BROWN N.A., MOORMAN A.F., CHRISTOFFELS V.M. (2010). The sinus venosus progenitors separate and diversify from the first and second heart fields early in development. *Cardiovasc Res* 87: 92-101.

MOTOIKE T., MARKHAM D.W., ROSSANT J., SATO T.N. (2003). Evidence for novel fate of Flk1 progenitors: contribution to muscle lineage. *Genesis* 35: 153-159.

POELMANN R. E., LIE-VENEMA H. AND GITTENBERGER-DE GROOT A. C. (2002). The role of the epicardium and neural crest. *Tex Heart Inst J* 29: 255-261.

TAM P.P., PARAMESWARAN M., KINDER S.J., WEINBERGER R. P. (1997). The allocation of epiblast cells to the embryonic heart and other mesodermal lineages: the role of ingression and tissue movement during gastrulation. *Development* 124: 1631–1642.

TAVARES A.T., ANDRADE S., SILVA A.C., BELO J.A. (2007). Cerberus is a feedback inhibitor of Nodal asymmetric signaling in the chick embryo. Development 134: 2051-2060.

VINCENT S.D. AND BUCKINGHAM M.E. (2010). How to make a heart: the origin and regulation of cardiac progenitor cells. *Curr Top Dev Bio*l 90: 1-41.

ZHOU B., VON GISE A., MA Q., RIVERA-FELICIANO J., PU W.T. (2008). Nkx2-5- and Isl1-expressing cardiac progenitors contribute to proepicardium. *Biochem Biophys Res Commu*n 375: 450-453.

Further Related Reading, published previously in the *Int. J. Dev. Biol.*

Molecular mechanisms of lymphangiogenesis in development and cancer
Imke Albrecht and Gerhard Christofori
Int. J. Dev. Biol. (2011) 55: 483-494

Cell-based cardiovascular repair and regeneration in acute myocardial infarction and chronic ischemic cardiomyopathy – current status and future developments
Christian Templin, Thomas F. Lüscher and Ulf Landmesser
Int. J. Dev. Biol. (2011) 55: 407-417

Sox17-dependent gene expression and early heart and gut development in Sox17-deficient mouse embryos
Sabine Pfister, Vanessa J. Jones, Melinda Power, Germaine L. Truisi, Poh-Lynn Khoo, Kirsten A. Steiner, Masami Kanai-Azuma, Yoshiakira Kanai, Patrick P. L. Tam and David A. F. Loebel
Int. J. Dev. Biol. (2011) 55: 45-58

Movement and commitment of primitive streak precardiac cells during cardiogenesis
Carmen Lopez-Sanchez, Natividad Garcia-Masa, Carlos M. Gañan and Virginio Garcia-Martinez
Int. J. Dev. Biol. (2009) 53: 1445-1455

Building the vertebrate heart - an evolutionary approach to cardiac development
José M. Pérez-Pomares, Juan M. González-Rosa and Ramón Muñoz-Chápuli
Int. J. Dev. Biol. (2009) 53: 1427-1443

Embryonic development of the proepicardium and coronary vessels
Anna Ratajska, Elzbieta Czarnowska and Bogdan Ciszek
Int. J. Dev. Biol. (2008) 52: 229-236

Efficient Cre-mediated deletion in cardiac progenitor cells conferred by a 3'UTR-ires-Cre allele of the homeobox gene Nkx2-5
Edouard G Stanley, Christine Biben, Andrew Elefanty, Louise Barnett, Frank Koentgen, Lorraine Robb and Richard P Harvey
Int. J. Dev. Biol. (2002) 46: 431-439

The contribution of the proepicardium to avian cardiovascular development
JM Perez-Pomares, A Phelps, R Munoz-Chapuli, A Wessels
Int. J. Dev. Biol. (2001) 45: S155-S156

5 yr ISI Impact Factor (2010) = 2.961