Developmental Biology 390 (2014) 68–79

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Mesodermal Nkx2.5 is necessary and sufficient for early second heart field development

Lu Zhang [a,1], Aya Nomura-Kitabayashi [a,1], Nishat Sultana [a,1], Weibin Cai [a], Xiaoqiang Cai [a], Anne M. Moon [b], Chen-Leng Cai [a,*]

[a] Department of Developmental and Regenerative Biology, The Mindich Child Health and Development Institute, and The Black Family Stem Cell Institute, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, New York, NY 10029, USA
[b] Weis Center for Research, 100 North Academy Avenue, Danville, PA 17822, USA

ARTICLE INFO

Article history:
Received 20 June 2013
Received in revised form
13 February 2014
Accepted 24 February 2014
Available online 5 March 2014

Keywords:
Nkx2.5
Pharyngeal endoderm
Pharyngeal mesoderm
Second heart field
Heart development

ABSTRACT

The vertebrate heart develops from mesoderm and requires inductive signals secreted from early endoderm. During embryogenesis, Nkx2.5 acts as a key transcription factor and plays essential roles for heart formation from *Drosophila* to human. In mice, Nkx2.5 is expressed in the early first heart field, second heart field pharyngeal mesoderm, as well as pharyngeal endodermal cells underlying the second heart field. Currently, the specific requirements for Nkx2.5 in the endoderm *versus* mesoderm with regard to early heart formation are incompletely understood. Here, we performed tissue-specific deletion in mice to dissect the roles of Nkx2.5 in the pharyngeal endoderm and mesoderm. We found that heart development appeared normal after endodermal deletion of *Nkx*2.5 whereas mesodermal deletion engendered cardiac defects almost identical to those observed on *Nkx*2.5 null embryos (*Nkx*2.5$^{-/-}$). Furthermore, re-expression of *Nkx*2.5 in the mesoderm rescued *Nkx*2.5$^{-/-}$ heart defects. Our findings reveal that Nkx2.5 in the mesoderm is essential while endodermal expression is dispensable for early heart formation in mammals.

Introduction

The induction and development of the early embryonic heart is a dynamic process in vertebrates and require inductive signals secreted from the endoderm. During gastrulation, the cardiac precursors reside in the anterior region of the primitive streak. They migrate anterolaterally and form the lateral plate mesoderm that divides into somatic and splanchnic mesoderm (Buckingham et al., 2005; Abu-Issa and Kirby, 2007; Nakajima et al., 2009). As embryo folding occurs, the splanchnic mesoderm cells fuse at the midline to form a cardiac crescent. Subsequently, the first heart field (FHF) precursors in the cardiac crescent further fuse to form a linear heart tube that develops to the left ventricle (Buckingham et al., 2005; Abu-Issa and Kirby, 2007; Nakajima et al., 2009). The second heart field (SHF) is a subpopulation of cells located in the pharyngeal mesoderm. It contains precursors that give rise to the outflow tract, right ventricle, and atria (Abu-Issa and Kirby, 2007; Kelly et al., 2001; Cai et al., 2003; Mjaatvedt et al., 2001; Waldo et al., 2001).

The lateral plate and cardiac crescent mesoderm lie sub-adjacently to the endoderm. Studies from amphibians, birds and mice suggested that early endoderm elaborates heart-inducing factors (*e.g.*, Bmps, Fgfs, and Wnt inhibitors) that induce nascent mesoderm toward cardiac fates (Nascone and Mercola, 1995; Schultheiss et al., 1995; Alsan and Schultheiss, 2002; Schneider and Mercola, 2001; Marvin et al., 2001; Tzahor and Lassar 2001; Brand, 2003; Lough and Sugi, 2000; Madabhushi and Lacy, 2011). During SHF development, endoderm-derived signals continue to play essential roles to govern SHF formation and deployment in a non-cell autonomous fashion. In mice, sonic hedgehog (Shh) is expressed in the pharyngeal endoderm (E7.5–10.5). Ablation of *Shh* in the endoderm or its receptor smoothened (*Smo*) in the SHF leads to aortic arch and outflow tract malformations (Lin et al., 2006; Goddeeris et al., 2007). Shh is required for SHF cell proliferation and survival, and regulates migration of hedgehog-responsive cells from SHF into atrial septum and pulmonary trunk (Dyer and Kirby, 2009; Hoffmann et al., 2009). It is largely unknown, however, whether and how the other pharyngeal endodermal signals contribute to heart formation during SHF development. This is an important and intriguing question given that several genes critical for SHF development are expressed in both pharyngeal mesoderm and endoderm (*e.g.*, *Fgf*8 and *Tbx*1), and their endodermal expression is essential for proper SHF formation

* Corresponding author. Tel.: +1 212 824 8917.
E-mail address: chenleng.cai@mssm.edu (C.-L. Cai).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.ydbio.2014.02.023
0012-1606

(Macatee et al., 2003; Park et al., 2006; Arnold et al., 2006; Ilagan et al., 2006; Zhang et al., 2006).

Nkx2.5 is a homeobox transcription factor and plays fundamental roles for early heart formation and function from *Drosophila* to human (Harvey et al., 2002). Mice lacking *Nkx*2.5 (*Nkx*2.5$^{-/-}$) die at E9.5–10.5 with severely underdeveloped heart (Lyons et al., 1995; Tanaka et al., 1999). Haploinsufficiency for *NKX*2.5 in humans also causes congenital heart defects with a variety of malformations including atrial septal defect (ASD), double-outlet right ventricle (DORV) and tetralogy of Fallot (Schott et al., 1998; Prall et al., 2007). During early embryogenesis, *Nkx*2.5 is expressed in the FHF, SHF pharyngeal mesoderm and its adjacent endodermal cells. In this study, we performed genetic analysis and attempted to determine specific requirements of *Nkx*2.5 in the endoderm and mesoderm with regard to early SHF development.

Material and methods

Animal models

Foxa2^{MerCreMer} (denoted as *Foxa2*$^{MerCreMer/+}$) (Park et al., 2008), *Mesp1*^{Cre} (denoted as *Mesp1*$^{Cre/+}$) (Saga et al., 1999), *Nkx*2.5$^{flox/flox}$ (denoted as *Nkx*2.5$^{f/f}$) (Pashmforoush et al., 2004) and *Rosa26*^{LacZ} reporter (denoted as *R26R*$^{LacZ/LacZ}$) (Soriano, 1999) mouse lines were described previously. To generate a conditional Nkx2.5 expression mouse model, a floxed *H2B-GFP-4XpolyA* cassette followed by 3*XFLAG* was targeted into *Nkx*2.5 start codon in mice. 3*XFLAG* sequence is fused in frame with *Nkx*2.5 1st exon (Fig. 7). Tamoxifen (Sigma, T5648) was administrated to the pregnant mice through oral gavage (Park et al., 2008). Mouse husbandry was carried out according to an approved IACUC protocol at the Icahn School of Medicine at Mount Sinai.

Whole mount RNA in situ hybridization, histology and immunohistochemistry

Whole mount RNA *in situ* hybridization of mouse embryos was carried out according to Wilkinson's protocol (Wilkinson, 1992). For histology, whole mount mouse embryos were fixed in 4% paraformaldehyde, dehydrated through graded methanol and embedded in paraffin. Paraffin sections were cut at 8-μm thickness with a Leica RM 2255 microtome. Immunostaining of anti-Nkx2.5 antibody (Abcam ab35842, 1:50) was carried out on 8-μm paraffin sections with citrate-based antigen retrieval procedure (10 mM sodium citrate, pH 6.0).

X-gal staining

Mouse embryos were fixed in 4% paraformaldehyde for 30 min. After permeabilization (0.02% Na deoxycholate, 0.01% NP-40 in PBS), embryos were stained in X-gal solution (5 mM K-ferricyanide, 5 mM K-ferrocyanide, 2 mM $MgCl_2$, and 1 mg/ml X-gal in PBS) for 12 h.

Scanning Electron Microscopy (SEM) analysis

A standard SEM sample preparation procedure was applied to process mouse embryos (Cai et al., 2003). In brief, embryos were primarily fixed with 3% glutaraldehyde with a 0.2 M Na cacodylate buffer followed by fixation in 1% osmium tetroxide for 2 h, dehydratred in graded steps of ethanol and then critical point dried, sputter coated with gold–palladium and observed with a Hitachi S13300 scanning electron microscope at Mount Sinai.

Results and discussion

Nkx2.5 is expressed in the early pharyngeal endoderm during mouse embryogenesis

We performed whole mount RNA *in situ* hybridization to detect *Nkx*2.5 transcripts on E7.5–9.0 mouse embryos. As previously described (Searcy et al., 1998; Komuro and Izumo, 1993; Lints et al., 1993; Kasahara et al., 1998), *Nkx*2.5 is expressed in the early cardiac crescent at E7.5–7.75 (unnotched arrowheads in Fig. 1A1, B1). As development proceeds, the cardiac crescent cells fuse at the midline to form a linear heart tube, and then loops with addition of cells from the SHF (Fig. 1C,D,F). *Nkx*2.5 expression encompasses all the cardiac regions, including primitive left ventricle (asterisks in Fig. 1C1,D2,F2/4), SHF-derived outflow tract and right ventricle, as well as atria and sinus venosus (Fig. 1E–G). *Nkx*2.5 is also detected in the pharyngeal region adjacent to the heart (arrows in Fig. 1C1,D2,F1/4). Transverse sections of E7.75–9.0 embryos revealed that *Nkx*2.5^{+} cells are located in the pharyngeal mesoderm (notched arrows in Fig. 1C2,E,G) and its adjacent endoderm (unnotched arrows in Fig. 1C2,E,G) and ectoderm cells (brackets in Fig. 1B3,C2,E1/2). We examined a series of E7.5–8.0 embryos and found that *Nkx*2.5 is first detected in the pharyngeal endoderm at ~E7.75 (unnotched arrows in Fig. 1B2/3,A3). In addition, its pharyngeal expression is detected in the anterior but not in the posterior regions on E8.0–9.0 embryos (arrows in Fig. 1E,G).

Morphogenetic defects of *Nkx*2.5$^{-/-}$ hearts are apparent as early as E8.5 (Lyons et al., 1995; Tanaka et al., 1999). Given that *Nkx*2.5 is dynamically expressed in the pharyngeal endoderm and mesoderm (cardiac mesoderm and pharyngeal mesoderm), we investigated whether it has functions in both tissue layers during early cardiac development.

Elimination of Nkx2.5 in the pharyngeal endoderm does not affect heart formation

To determine potential roles for *Nkx*2.5 in the pharyngeal endodermal during heart development, we crossed *Nkx*2.5$^{f/f}$;*R26R*$^{LacZ/LacZ}$ mice to *Foxa2*$^{MerCreMer/+}$;*Nkx*2.5$^{f/f}$ mice. In this genetic cross, 50% embryos are anticipated to be mutants (*Foxa2*$^{MerCreMer/+}$;*Nkx*2.5$^{f/f}$;*R26R*$^{LacZ/+}$), and the remaining 50% are controls (*Nkx*2.5$^{f/f}$;*R26R*$^{LacZ/+}$). The *Foxa2*^{MerCreMer} allele has been shown to confer rapid, robust Cre activity in the pharyngeal endoderm, notochord, and floorplate after tamoxifen administration (Park et al., 2008). The *R26R*^{LacZ} reporter allele was used to document Cre efficiency.

To ensure complete Cre excision, we administrated tamoxifen daily to the pregnant dams for three consecutive days from E6.5 (0.12 mg/g body weight, oral gavage) (Park et al., 2008), and harvested the embryos at E9.5–13.5 (Fig. 2A). Robust and uniform X-gal staining was detected in the pharyngeal endoderm and other *Foxa*2-expressing cells (*e.g.*, notochord and floorplate) in mouse embryos at E9.5 (Figs. 2B,H and S1). We further performed Nkx2.5 immunostaining on mutant embryos at E9.0 and E8.0, and found that its expression was specifically removed in the pharyngeal endoderm, but not the heart or pharyngeal mesoderm (Fig. 3). These results indicate that *Foxa2*^{MerCreMer} indeed mediates effective Cre recombination in the pharyngeal endoderm of *Foxa2*$^{MerCreMer/+}$;*Nkx*2.5$^{f/f}$;*R26R*$^{LacZ/+}$ mice as early as E8.0.

We injected a total of 26 pregnant female mice and collected 73 E9.5 (17 litters), 10 E10.5 (2 litters), 8 E11.5 (2 litters), 13 E12.5 (2 litters) and 13 E13.5 (3 litters) mutant embryos. Interestingly, the mutant mice exhibited normal heart formation at all embryonic stages assayed (Fig. 2B,D,F), and they can survive to birth (even to adulthood). Cardiac shape of the mutant embryos was indistinguishable from their

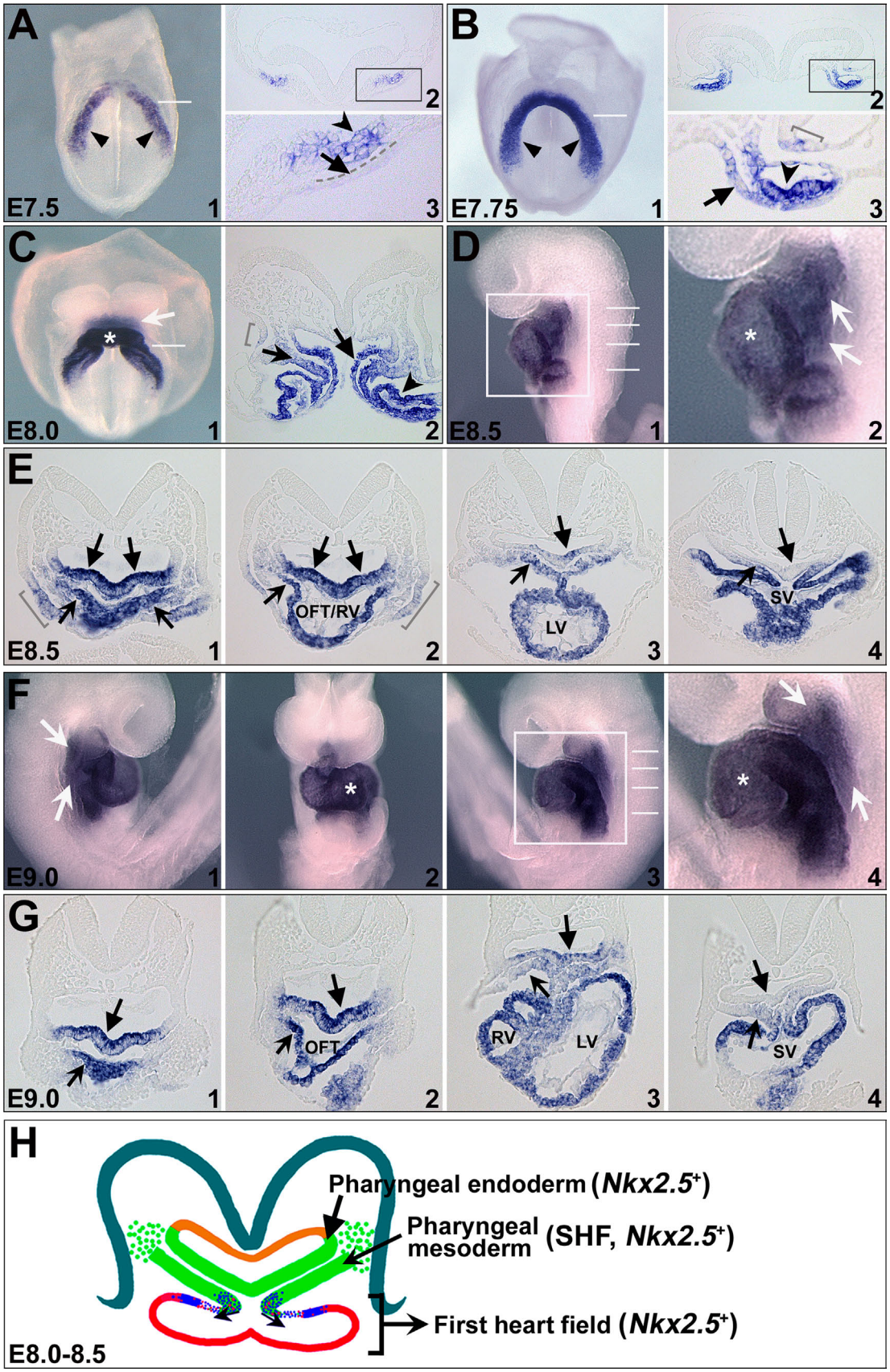

Fig. 1. Expression of *Nkx*2.5 in the FHF, SHF and pharyngeal endoderm during early mouse embryogenesis. (A–G) Whole-mount RNA *in situ* hybridization of *Nkx*2.5 expression in E7.5–E9.0 mouse embryos. A1/B1/C1 are frontal views and D1 is a left lateral view of mouse embryos at corresponding stages. F1/2/3 are right lateral, frontal and left lateral views of E9.0 embryo. A2/B2/C2/E1–5/G1–5 are transverse sections of embryos with approximate positions shown by white lines in A1/B1/C1/D1/F3. A3/B3/D2/F4 are high magnification images of A2/B2/D1/F3 in the square areas, respectively. Unnotched arrowheads in A1/B1 indicate cardiac crescent. Unnotched arrows in A3/B3 indicate splanchnic endoderm. Notched arrowheads in A3/B3 indicate cardiogenic mesoderm. Arrows in C/D/F indicate *Nkx*2.5 expression in the pharyngeal regions, including pharyngeal mesoderm (notched arrows in C2/E/G) and endoderm (unnotched arrows in C2/E/G) and ectoderm (brackets in B3,C2,E1/2). Asterisks in C/D/F indicate FHF cells. (H) Schematic of *Nkx*2.5 expression in E8.0–E8.5 mouse embryos with transverse section in the pharyngeal region. *Nkx*2.5 is expressed in both the FHF-derived linear heart tube (red) and pharyngeal mesoderm SHF (green, notched arrow), as well as pharyngeal endoderm (green, unnotched arrow). OFT, outflow tract; RV, right ventricle; LV, left ventricle.

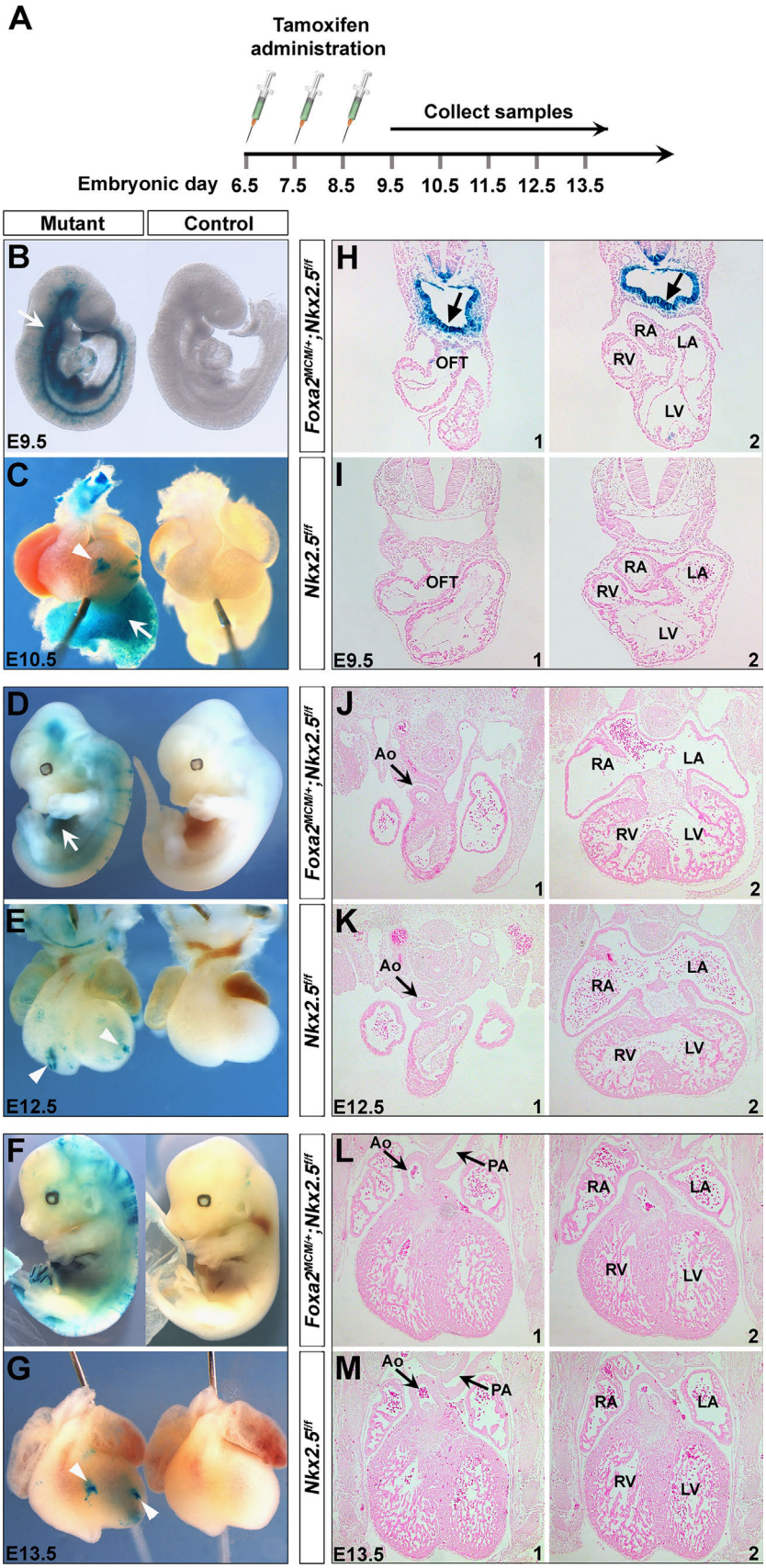

Fig. 2. Disruption of *Nkx*2.5 in the pharyngeal endoderm with *Foxa2*^{MerCreMer}. (A) Diagram of the time course of tamoxifen administration to pregnant females at E6.5, E7.5 and E8.5. Embryos were harvested at E9.5 (B), E10.5 (C), E12.5 (D) and E13.5 (F). (B–G) X-gal^{+} cells were detected in the pharyngeal endoderm (arrows in B and H) and endoderm-derived liver tissues (arrows in C and D) of mutant embryos (*Foxa2*$^{MerCreMer/+}$;*Nkx2.5*$^{f/f}$;*R26R*$^{LacZ/+}$). Control littermates (*Nkx2.5*$^{f/f}$;*R26R*$^{LacZ/+}$) were negative for X-gal staining (B–G). Scatted X-gal^{+} cells were detected in the mutant hearts (arrowheads in C, E and G) (Park et al., 2008). (H–M) Transverse sections of the embryos. Mutant hearts displayed indistinguishable morphology from their littermates. RA, right atrium; LA, left atrium; Ao, aorta; PA, pulmonary artery.

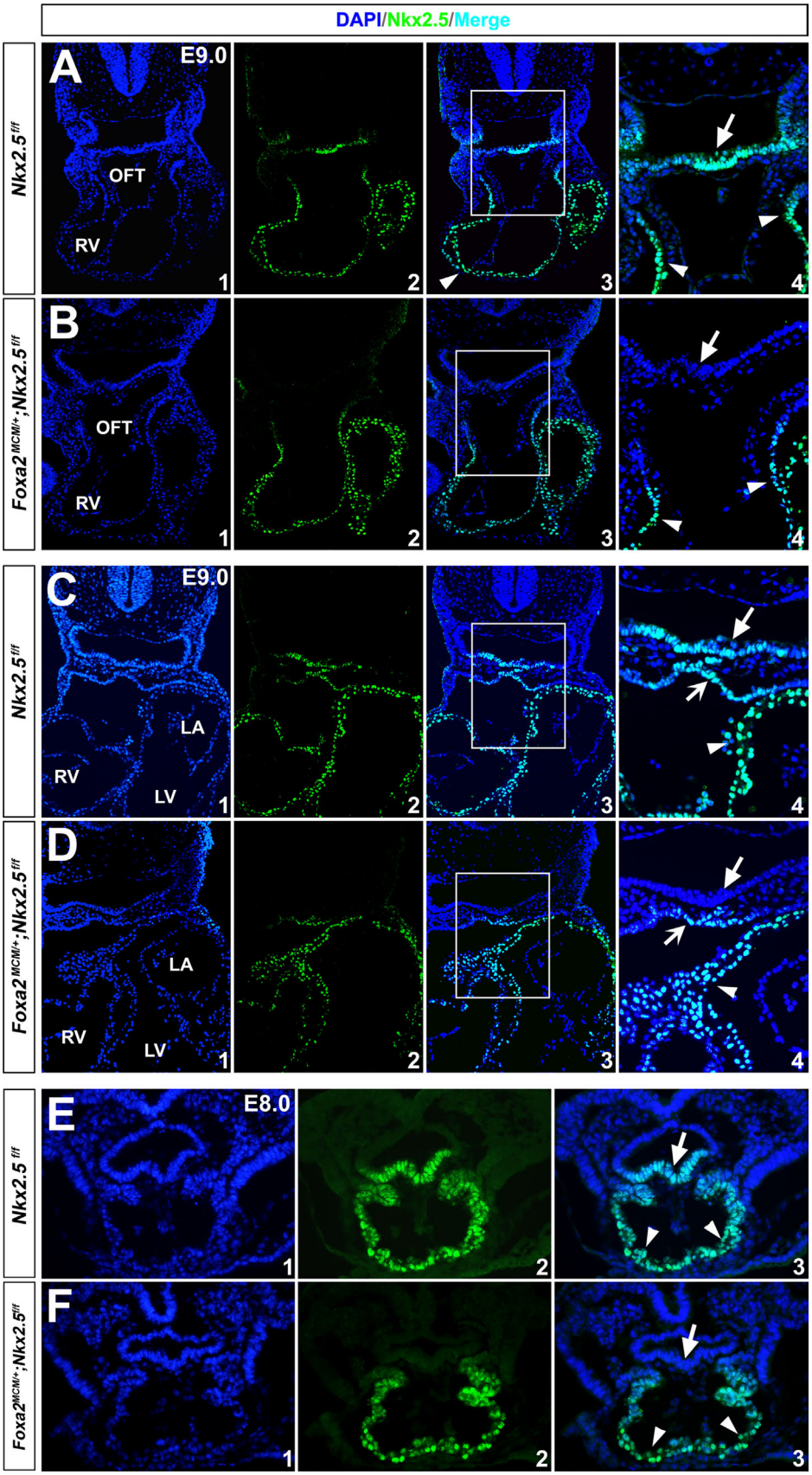

Fig. 3. Nkx2.5 protein is eliminated in the pharyngeal endoderm by *Foxa2*^{MerCreMer} with tamoxifen induction. Immunostaining of Nkx2.5 on transverse sections of mutant (*Foxa2*$^{MerCreMer/+}$;*Nkx2.5*$^{f/f}$;*R26R*$^{LacZ/+}$, B/D,F) and control embryos (*Nkx2.5*$^{f/f}$;*R26R*$^{LacZ/+}$, A/C,E) at E9.0 and E8.0. A/B, C/D and E/F are comparable sections of the control and mutant embryos at corresponding stages. (A,C,E) Consistent with RNA *in situ* hybridization, Nkx2.5 protein was detected in the myocardial wall (arrowheads), pharyngeal mesoderm (notched arrows) and underlying endodermal cells (unnotched arrows). (B,D,F) In the mutants, Nkx2.5 immunoreactivity was eliminated in the pharyngeal endoderm (unnotched arrows), but persisted normally in the heart tube (arrowheads) and pharyngeal mesoderm (notched arrows). A4/B4/C4/D4 are high magnification images for A3/B3/C3/D3 in the square areas, respectively.

littermate controls (Fig. 2C,E,G). Further histological analysis of the mutant hearts also supported these initial observations: no abnormalities were detected in outflow tract or right ventricle formation (Fig. 2H,I), aorta or pulmonary artery division (Fig. 2J–M), or chamber septation (Fig. 2L,M). These cardiac components are derived from, and regulated by, the SHF (Cai et al., 2003; Lin et al., 2006; Goddeeris et al., 2007; Dyer and Kirby, 2009; Hoffmann et al., 2009; Macatee et al., 2003; Park et al., 2006; Arnold et al., 2006; Ilagan et al., 2006; Zhang et al., 2006). As reported previously (Park et al., 2008), we detected a few scattered X-gal^{+} cells in the mutant hearts (arrowheads in Fig. 2C, E,G). This may be due to transient *Foxa2* expression in the anterior mesoderm at the late streak and early bud stage (Park et al., 2008; Tam and Steiner, 1999). These scattered recombination events had no detectable effect on the cardiac development of mutant embryos.

To address Nkx2.5 function at the molecular level in *Foxa2*$^{MerCreMer/+}$;*Nkx2.5*$^{f/f}$ mutants, we examined expression of

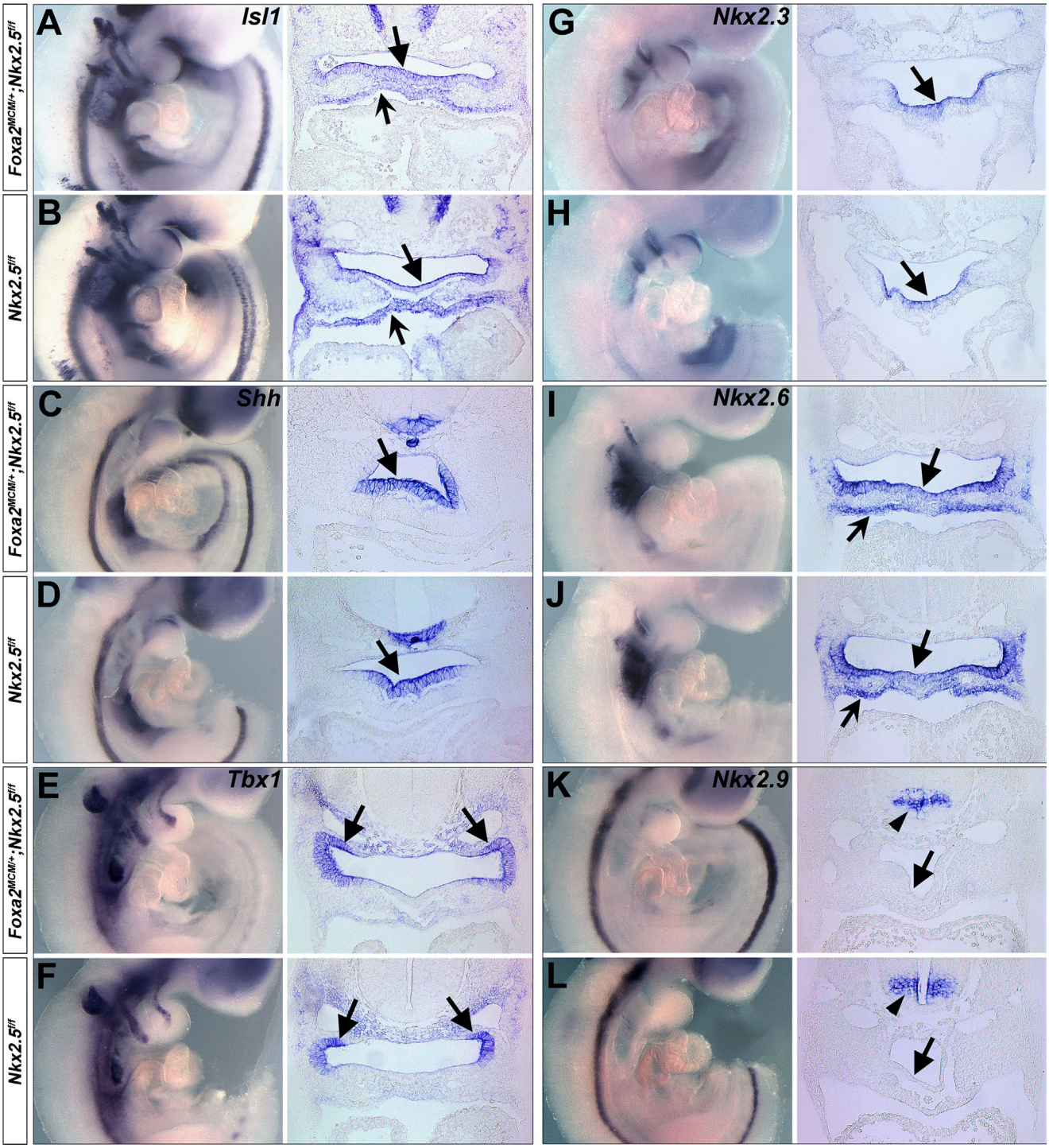

Fig. 4. Endodermal deletion of *Nkx*2.5 has little impact on gene expressions in the pharyngeal endoderm and mesoderm. (A–F) *Isl*1, *Shh* and *Tbx*1 are expressed in the pharyngeal endoderm (unnotched arrows) and/or mesoderm (notched arrows) (A,C,E), and their expression is unchanged in *Foxa2*$^{MerCreMer/+}$;*Nkx2.5*$^{f/f}$ embryos (B,D,F). (G–L) *Nkx*2.3 is expressed in the pharyngeal endoderm (G, unnotched arrows) and *Nkx*2.6 is expressed in both pharyngeal endoderm (I, unnotched arrows) and mesoderm (notched arrows). *Nkx*2.9 is expressed in the neural tube but not in the pharyngeal endoderm (K, unnotched arrows). Expression of these *Nk*-2 genes is normal in *Foxa2*$^{MerCreMer/+}$;*Nkx2.5*$^{f/f}$ embryos. H/J/L are corresponding controls.

genes in the pharyngeal endoderm and/or mesoderm with critical roles for SHF development, including *Isl*1 (Cai et al., 2003), *Shh* (Lin et al., 2006; Goddeeris et al., 2007; Dyer and Kirby, 2009; Hoffmann et al., 2009), *Tbx*1 (Arnold et al., 2006; Zhang et al., 2006, 2005), *Fgf*4/8/10 (Cai et al., 2003; Macatee et al., 2003; Park et al., 2006; Ilagan et al., 2006; Kelly and Buckingham, 2002; Golzio et al., 2012; Watanabe et al., 2010, 2012), *Bmp*4/7 (Cai et al., 2003; Wang et al., 2010; Kim et al., 2001) and *Foxa*2/*c*1/*c*2/*h*1 (Harrelson et al., 2012; Seo and Kume, 2006; von Both et al., 2004) at E9.5. RNA *in situ* hybridization revealed expression of these genes was in general unchanged in the mutants (Figs. 4A–F and S2, data not shown for *Foxc*1/*c*2/*h*1).

Mesodermal deletion of Nkx2.5 engenders similar cardiac defects to Nkx2.5 null mice

We next deleted Nkx2.5 function using *Mesp*1^{Cre} which is specifically expressed in nascent mesoderm during gastrulation (Saga et al., 1999, 2000). *Mesp*1^{Cre} progeny includes all the cells in the heart and the pharyngeal mesoderm, but no cells in the

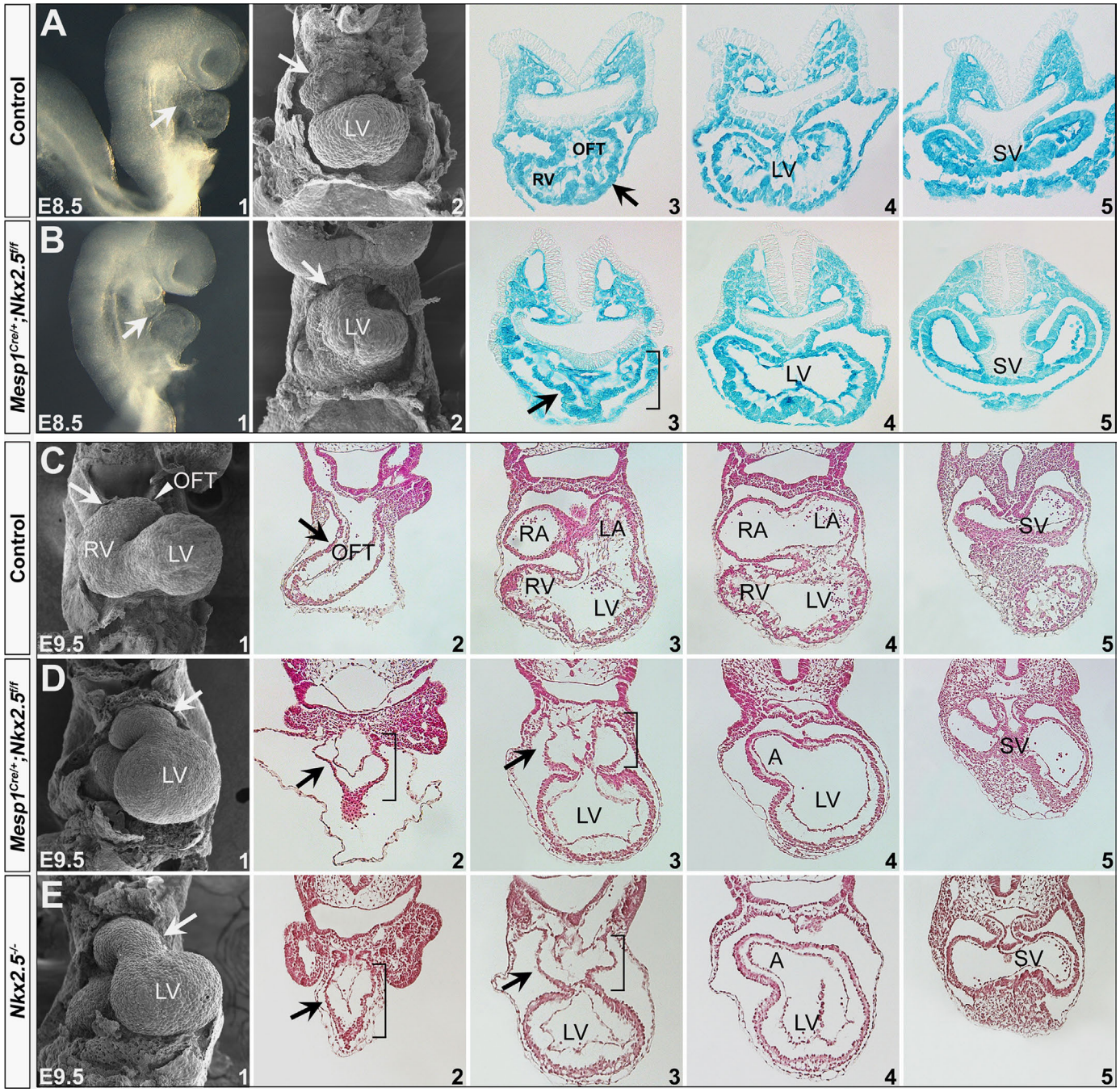

Fig. 5. Disruption of *Nkx*2.5 in the mesoderm with *Mesp*1^{Cre}. (A,B) Mouse embryos with mesoderm-specific deletion of *Nkx*2.5 have cardiac malformations at E8.5. A1/B1 are right lateral views and A2/B2 are frontal views with SEM. Cardiac OFT and RV were severely underdeveloped in the mutants (arrows in B1,2). Transverse sections of control (*Mesp*1$^{Cre/+}$;*Nkx*2.5$^{fl/+}$;*R26R*$^{LacZ/+}$) and mutant (*Mesp*1$^{Cre/+}$;*Nkx*2.5$^{fl/f}$;*R26R*$^{LacZ/+}$) stained with X-gal indicate *Mesp*1^{Cre} lineage includes mesoderm tissues (heart and pharyngeal mesoderm), but not ectoderm or endoderm (A3–5 and B3–5, and Fig. S3). OFT and RV defects were shown by transverse sections (arrow and bracket in A3/B3). (C–E) At E9.5, mutant hearts are unlooped and the OFT and RV are hypoplastic (D1). The general morphology of the mutant hearts is similar to that of *Nkx*2.5$^{-/-}$ hearts at E9.5 (E1). C2–5, D2–5 and E2–5 are transverse sections of the hearts. Brackets in D/E indicate hypoplastic OFT and RV.

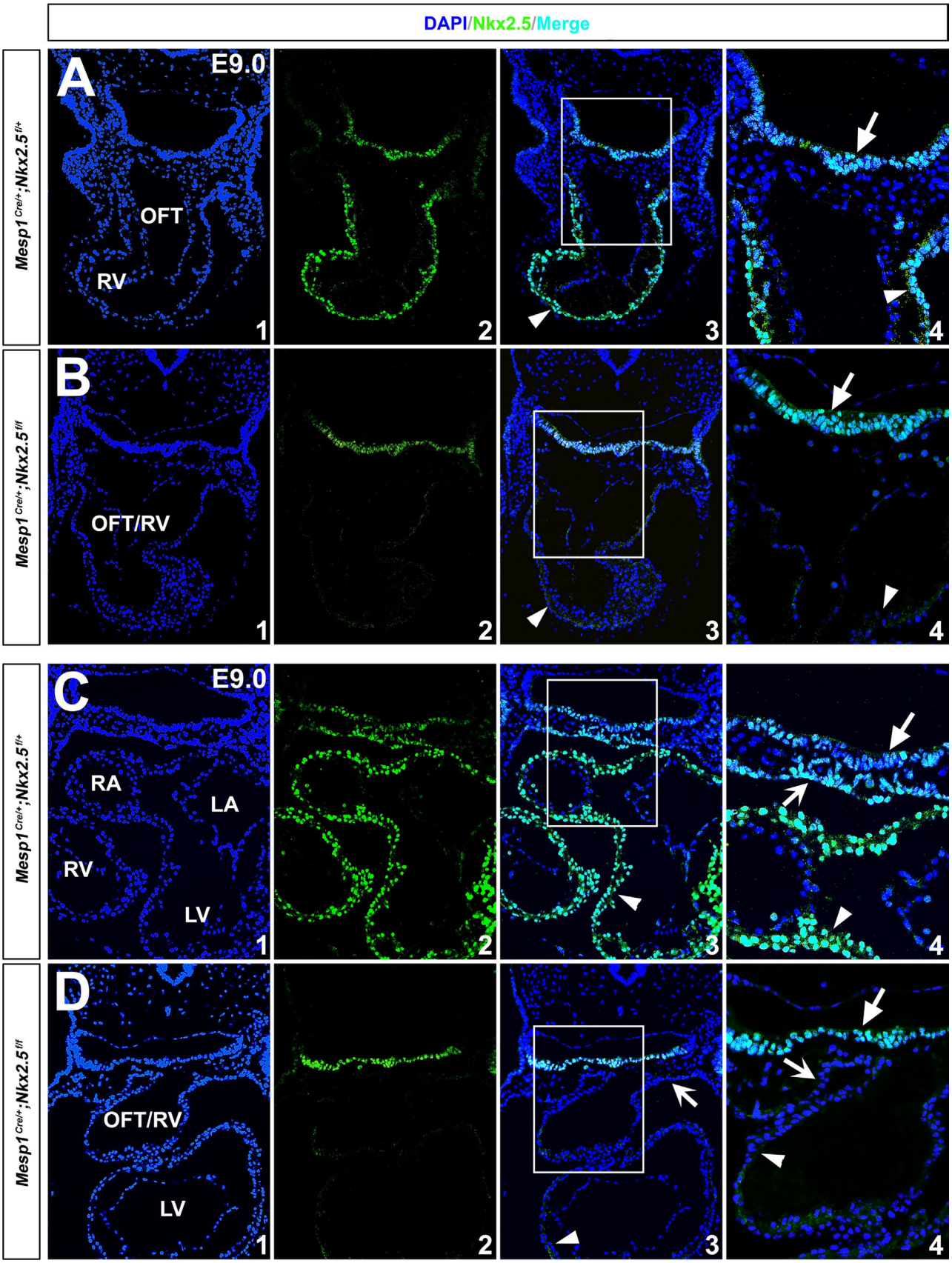

Fig. 6. Nkx2.5 expression is eliminated in the mesoderm by *Mesp1*^{Cre}. Nkx2.5 immunostaining on mutants (*Mesp1*$^{Cre/+}$;*Nkx2.5*$^{f/f}$;*R26R*$^{LacZ/+}$) and littermate controls (*Mesp1*$^{Cre/+}$;*Nkx2.5*$^{f/+}$;*R26R*$^{LacZ/+}$) at E9.0. (A,C) In controls, Nkx2.5 expression was detected in the myocardial wall (arrowheads), pharyngeal mesoderm (notched arrows) and endoderm cells (unnotched arrows). (B,D) In the mutants, Nkx2.5 expression was unaffected in the pharyngeal endoderm (unnotched arrows), but was absent in the heart tube (arrowheads) and pharyngeal mesoderm (notched arrows). A4/B4/C4/D4 are high magnification images for A3/B3/C3/D3 in the square areas, respectively.

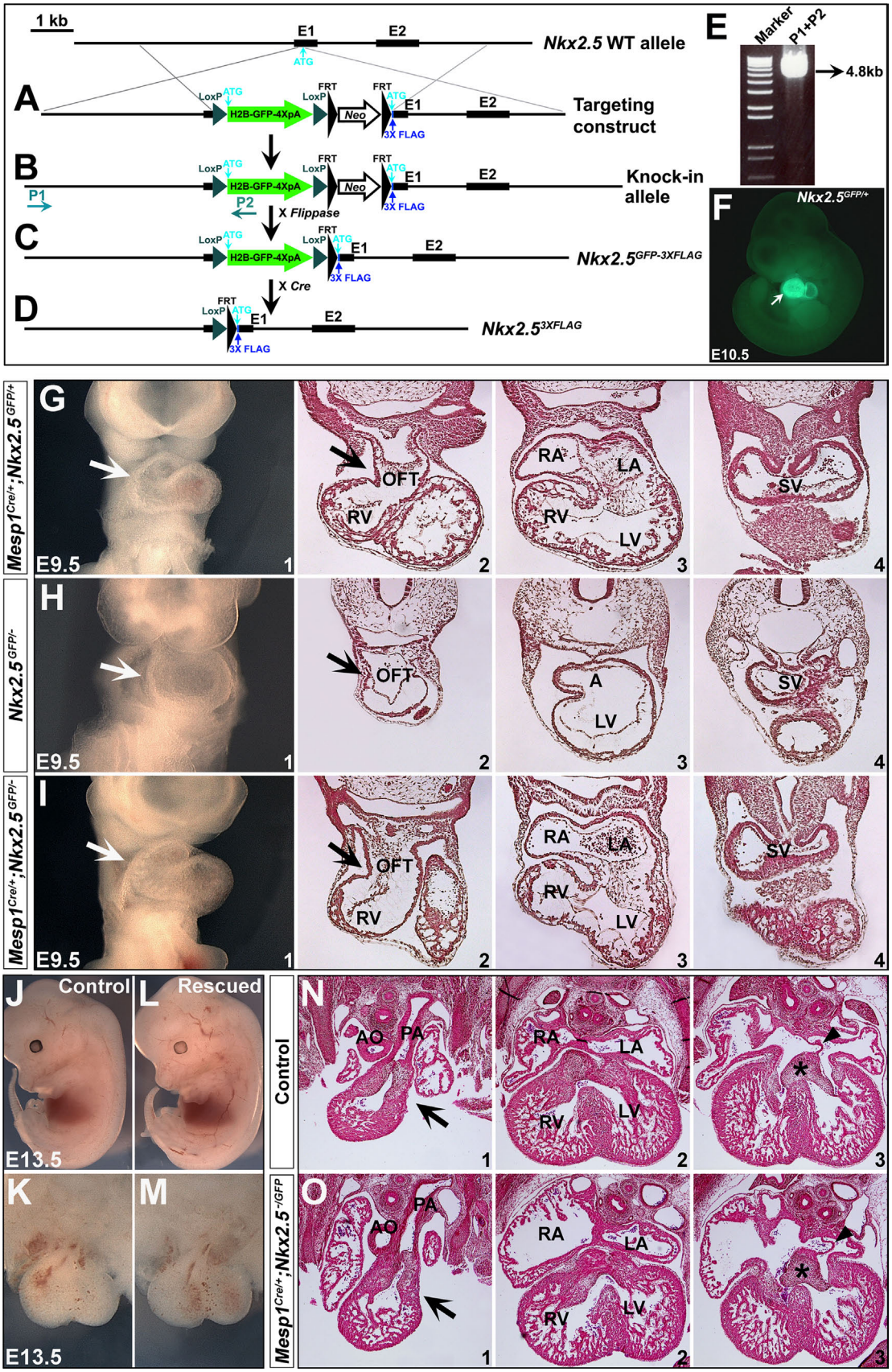

Fig. 7. Normal SHF development in the *Nkx*2.5 null mice after re-expressing of *Nkx*2.5 in the mesoderm. (A–F) Generation of $Nkx2.5^{H2B\text{-}GFP\text{-}3XFLAG}$ knock-in mice. An *H2B-GFP-Neo-3XFLAG* cassette (*loxP-H2B-GFP-4XpolyA-loxP-FRT-Neo-FRT-3XFLAG*) was inserted into *Nkx*2.5 exon 1 (6 bp upstream of ATG, *Neo* cassette is flanked by two *FRT* sites). *3XFLAG* has a Kozak sequence at the 5′ end. Long-range PCR was performed to screen ES cells with a primer external to 5′ targeting construct arm (P1) and a reverse primer within *H2B-GFP* cassette (P2) (A,B). Targeted ES clones were identified with a recombinant band of 4.8 kb further confirmed by DNA sequencing (E). $Nkx2.5^{H2B\text{-}GFP\text{-}Neo\text{-}3XFLAG/+}$ mice derived from the positive ES cells were crossed to *Flippase* (Rodriguez et al., 2000) to remove *Neo* (C, $Nkx2.5^{H2B\text{-}GFP\text{-}3XFLAG/+}$, also denoted as $Nkx2.5^{GFP/+}$). These mice were further crossed to Protamine-Cre to obtain $Nkx2.5^{3XFLAG/+}$ animals (D). GFP expression in $Nkx2.5^{GFP/+}$ embryo mirrors *Nkx*2.5 expression in the heart at E10.5 (arrow in F). (G,J,K,N) At E9.5 and E13.5, control mice ($Mesp1^{Cre/+}$;$Nkx2.5^{GFP/+}$) displayed normal heart development. (H) $Nkx2.5^{GFP/-}$ is null for Nkx2.5. (I,L,M,O) $Mesp1^{Cre/+}$;$Nkx2.5^{GFP/-}$ mouse hearts developed normally at E9.5 and E13.5. G1/H1/I1 are frontal views (E9.5) and G2–4/H2–4/I2–4 are transverse sections of the embryos at comparable locations. J/L are embryos at E13.5 in left lateral view. K/M are front views of hearts from J/L, respectively. N1–3/O1–3 are transverse sections of hearts (K/M) at comparable locations.

endoderm or ectoderm (Fig. S3 and Ref. Saga et al. (1999)). We crossed *Mesp1*$^{Cre/+}$;*Nkx2.5*$^{fl/+}$ mice to *Nkx2.5*$^{fl/f}$;*R26R*$^{LacZ/LacZ}$ and found all of the mutant embryos (*Mesp1*$^{Cre/+}$;*Nkx2.5*$^{fl/f}$;*R26R*$^{LacZ/+}$) had severely misshaped hearts as early as E8.5 (Fig. 5A1,B1). We examined the mutant hearts with SEM which revealed perturbation of outflow tract and right ventricle formation at E8.5 (arrows in Fig. 5A2,B2). Transverse sections of the mutant embryos also showed underdeveloped, shortened outflow tracts and right ventricles (Fig. 5A3–5,B3–5 and bracket in B3). X-gal staining indicated mesoderm-specific deletion in both controls and mutants (Fig. 5A3–5,B3–5). We further performed immunostaining and found Nkx2.5 protein was specifically eliminated in the mesoderm (heart, pharyngeal mesoderm), but was normal in the pharyngeal endoderm in the mutants at E9.0 (Fig. 6).

We compared the general morphology of mesoderm mutants (*Mesp1*$^{Cre/+}$;*Nkx2.5*$^{fl/f}$) to *Nkx2.5*$^{-/-}$ embryos at E9.5. Both mutant classes had hearts that did not loop with indistinguishable malformed shape in outflow tract and right ventricle (Fig. 5D,E). Compared with controls (Fig. 5C), *Nkx2.5*$^{-/-}$ and *Mesp1*$^{Cre/+}$;*Nkx2.5*$^{fl/f}$ embryos had a hypoplastic outflow tract and right ventricle, indicating perturbed SHF formation. Moreover, *Mesp1*$^{Cre/+}$;*Nkx2.5*$^{fl/f}$ mice did not survive beyond E10.5, as seen in *Nkx2.5*$^{-/-}$ embryos (Lyons et al., 1995; Tanaka et al., 1999). These observations suggest a pivotal and decisive role of mesodermal Nkx2.5 for early heart development.

Re-expression of Nkx2.5 in the mesoderm rescues Nkx2.5 null SHF defects

To test whether *Nkx2.5* expression in the pharyngeal mesoderm is sufficient for early SHF development, we generated a conditional Nkx2.5-expressing mouse model *Nkx2.5*$^{H2B\text{-}GFP\text{-}3XFLAG}$ (denoted as *Nkx2.5*$^{GFP/+}$) by inserting a *loxP-H2B-GFP-4XpolyA-loxP-3XFLAG*

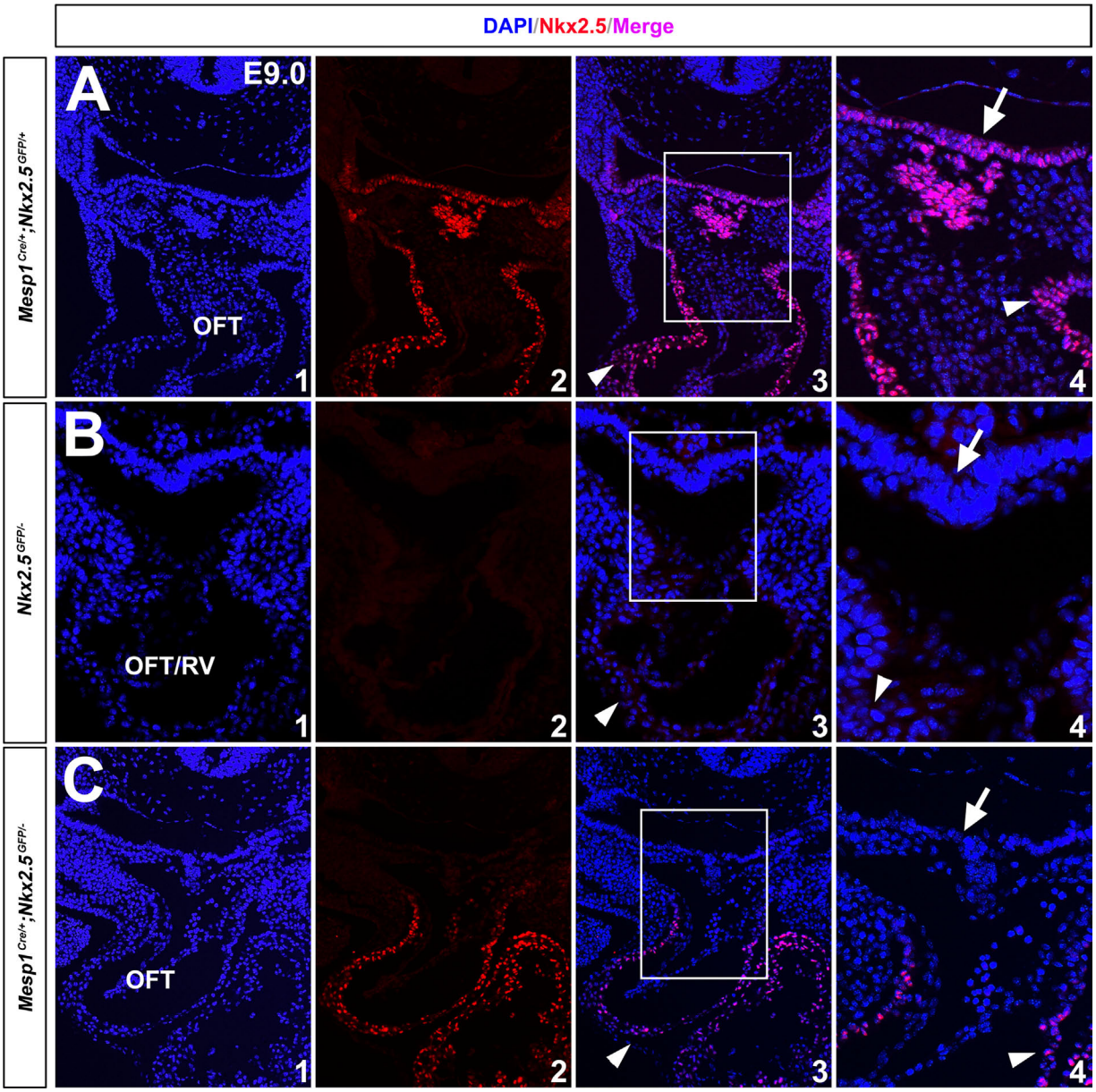

Fig. 8. Re-expression of Nkx2.5 in the mesoderm of *Mesp1*$^{Cre/+}$;*Nkx2.5*$^{GFP/-}$ mice. (A) Normal expression of Nkx2.5 in the pharyngeal endoderm (unnotched arrows) and mesoderm/heart (arrowheads) on the control mice (*Mesp1*$^{Cre/+}$;*Nkx2.5*$^{GFP/+}$) at E9.0. (B) Nkx2.5 was not detected in *Nkx2.5*$^{GFP/-}$ embryos. (C) Nkx2.5 is produced in the mesoderm/heart (arrowheads), but not endoderm (unnotched arrows), on *Mesp1*$^{Cre/+}$;*Nkx2.5*$^{GFP/-}$ mice. A4/B4/C4 are high magnification images for A3/B3/C3 in the square areas, respectively.

cassette into the start codon of *Nkx*2.5 through gene targeting (Fig. 7A–C). The 3*XFLAG* codons are in frame with *Nkx*2.5 cDNA sequences. In mice bearing this allele, GFP expression is under control of endogenous *Nkx*2.5 regulatory elements (Fig. 7F). The 3XFLAG-Nkx2.5 fusion protein is produced when the *H2B-GFP-4X polyA* cassette is removed (by Cre excision, Fig. 7D). We crossed $Nkx2.5^{GFP/+}$ to Protamine-Cre mice and $Nkx2.5^{3XFLAG/+}$ animals were obtained. Further intercross of $Nkx2.5^{3XFLAG/+}$ showed that $Nkx2.5^{3XFLAG/3XFLAG}$ homozygous mice were viable and normal as their wild-type littermates from embryonic stages to adulthood (data not shown), suggesting 3XFLAG-Nkx2.5 fusion protein retains Nkx2.5 activity.

We crossed $Mesp1^{Cre/+}$;$Nkx2.5^{+/-}$ mice to $Nkx2.5^{GFP/+}$ mice and $Nkx2.5^{GFP/-}$ embryos were obtained (Fig. 7H). They displayed malformed hearts as $Nkx2.5^{-/-}$ at E9.5 (Fig. 5E), indicating $Nkx2.5^{GFP}$ is a functional null allele of *Nkx*2.5. $Mesp1^{Cre/+}$;$Nkx2.5^{GFP/+}$ hearts developed normally. As discussed above, the $Nkx2.5^{GFP}$ allele in $Mesp1^{Cre/+}$;$Nkx2.5^{GFP/-}$ mice will generate 3XFLAG-Nkx2.5 fusion protein competent to wild-type Nkx2.5 in the mesoderm. Intriguingly, $Mesp1^{Cre/+}$;$Nkx2.5^{GFP/-}$ mice restored normal development in the heart at E9.5–E13.5 (Fig. 7I,L,M). Immunostaining confirmed Nkx2.5 was only detected in the mesodermal lineages (Fig. 8C, arrowhead), but not in the pharyngeal endoderm (Fig. 8C4, unnotched arrow) of these embryos. Of note, mice with genotype $Mesp1^{Cre/+}$;$Nkx2.5^{GFP/GFP}$ can survive to birth with normal morphology (data not shown). These results indicate that mesodermal Nkx2.5 expression is sufficient for early SHF development.

Based on these results, we reason that Nkx2.5-regulated paracrine signals from the pharyngeal endoderm, if any, have minimal or no effect on the development of SHF. Mesodermal deletion of *Nkx*2.5 caused virtually identical cardiac phenotypes to $Nkx2.5^{-/-}$ hearts, and that re-expression of Nkx2.5 in the mesoderm rescued $Nkx2.5^{GFP/-}$ cardiac defects, conclusively demonstrated that mesodermal, but not endodermal, *Nkx*2.5 expression provides the requisite signals for regulating early SHF development.

Nkx2.5 is a homolog of *Drosophila* tinman and belongs to Nk-2 class of homeobox genes (Harvey, 1996). In vertebrates, several *Nk*-2 homologs, including *Nkx*2.3 (Lee et al., 1996), *Nkx*2.6/2.8 (Brand et al., 1997; Boettger et al., 1997; Reecy et al., 1997; Biben et al., 1998), *Nkx*2.7 (Lee et al., 1996), and *Nkx*2.9 (Pabst et al., 1998; Newman and Krieg, 1998) are expressed in the pharyngeal endoderm and mesoderm, with a pattern that overlaps both temporally and spatially with *Nkx*2.5 during early cardiogenesis. In *Xenopus*, Nkx2.5 and Nkx2.3 regulate heart formation in a functionally redundant manner (Fu et al., 1998). Zebrafish Nkx2.5 and Nkx2.7 also function redundantly to control cardiac morphogenesis (Tu et al., 2009; Targoff et al., 2013). In mice, $Nkx2.5^{-/-}$;$Nkx2.6^{-/-}$ double mutant has severely disrupted pharyngeal endoderm formation (Tanaka et al., 2001), whereas $Nkx2.5^{-/-}$ or $Nkx2.6^{-/-}$ single mutation displayed normal pharynx development (Tanaka et al., 2001, 2000). Our observation that endodermal deletion of *Nkx*2.5 permitted normal cardiac formation may indicate a redundant activity of Nkx2.5 with other Nk-2 family members in the pharyngeal endoderm for SHF development. In examining $Foxa2^{MerCreMer/+}$;$Nkx2.5^{f/f}$ embryos, we detected normal *Nkx*2.3 and *Nkx*2.6 pharyngeal endodermal expression (Fig. 4G–J). *Nkx*2.7 ortholog was not present in mice, and *Nkx*2.9 was only expressed in the neural tube (Fig. 4K,L). We speculate the overlapping endodermal expression of *Nkx*2.3 and *Nkx*2.6 may compensate *Nkx*2.5 loss in the pharyngeal endoderm in $Foxa2^{MerCreMer/+}$;$Nkx2.5^{f/f}$ embryos. In the future, it will be of interest to investigate the compound mutations of *Nkx*2.5 with other *Nk*-2 genes in the pharyngeal endoderm, to understand whether Nk-2 family genes act redundantly to regulate SHF formation through the endoderm.

Acknowledgments

The authors thank Drs. Yumiko Saga (National Institute of Genetics, Japan) and Ken Chien (MGH, Boston, USA) for their generosity in providing the $Mesp1^{Cre/+}$ and $Nkx2.5^{flox/flox}$ mice, respectively. We are also very grateful to Dr. Bruce Gelb for critical reading of this manuscript, and Dr. Kevin Kelly in the Transgenic Core of Mount Sinai for generating mouse models. A.N.K is supported by an NIH T32 training grant. C.L.C. is supported by grants from the NIH/NHLBI (1R01HL095810 and 1K02HL094688), the American Heart Association (0855808D) and the March of Dimes Foundation (5-FY07-642).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.02.023.

References

Abu-Issa, R., Kirby, M.L., 2007. Heart field: from mesoderm to heart tube. Annu. Rev. Cell Dev. Biol. 23, 45–68.
Alsan, B.H., Schultheiss, T.M., 2002. Regulation of avian cardiogenesis by Fgf8 signaling. Development 129, 1935–1943.
Arnold, J.S., et al., 2006. Inactivation of Tbx1 in the pharyngeal endoderm results in 22q11DS malformations. Development 133, 977–987.
Biben, C., Hatzistavrou, T., Harvey, R.P., 1998. Expression of NK-2 class homeobox gene Nkx2-6 in foregut endoderm and heart. Mech. Dev. 73, 125–127.
Boettger, T., Stein, S., Kessel, M., 1997. The chicken NKX2.8 homeobox gene: a novel member of the NK-2 gene family. Dev. Genes Evol. 207, 65–70.
von Both, I., et al., 2004. Foxh1 is essential for development of the anterior heart field. Dev. Cell 7, 331–345.
Brand, T., 2003. Heart development: molecular insights into cardiac specification and early morphogenesis. Dev. Biol. 258, 1–19.
Brand, T., Andree, B., Schneider, A., Buchberger, A., Arnold, H.H., 1997. Chicken NKx2-8, a novel homeobox gene expressed during early heart and foregut development. Mech. Dev. 64, 53–59.
Buckingham, M., Meilhac, S., Zaffran, S., 2005. Building the mammalian heart from two sources of myocardial cells. Nat. Rev. Genet. 6, 826–835.
Cai, C.L., et al., 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.
Dyer, L.A., Kirby, M.L., 2009. Sonic hedgehog maintains proliferation in secondary heart field progenitors and is required for normal arterial pole formation. Dev. Biol. 330, 305–317.
Fu, Y., Yan, W., Mohun, T.J., Evans, S.M., 1998. Vertebrate tinman homologues XNkx2-3 and XNkx2-5 are required for heart formation in a functionally redundant manner. Development 125, 4439–4449.
Goddeeris, M.M., Schwartz, R., Klingensmith, J., Meyers, E.N., 2007. Independent requirements for Hedgehog signaling by both the anterior heart field and neural crest cells for outflow tract development. Development 134, 1593–1604.
Golzio, C. et al., 2012. ISL1 directly regulates FGF10 transcription during human cardiac outflow formation. PLoS One 7, e30677.
Harrelson, Z., Kaestner, K.H., Evans, S.M., 2012. Foxa2 mediates critical functions of prechordal plate in patterning and morphogenesis and is cell autonomously required for early ventral endoderm morphogenesis. Biol. Open 1, 173–181.
Harvey, R.P., 1996. NK-2 homeobox genes and heart development. Dev. Biol. 178, 203–216.
Harvey, R.P., et al., 2002. Homeodomain factor Nkx2-5 in heart development and disease. Cold Spring Harb. Symp. Quant. Biol. 67, 107–114.
Hoffmann, A.D., Peterson, M.A., Friedland-Little, J.M., Anderson, S.A., Moskowitz, I. P., 2009. Sonic hedgehog is required in pulmonary endoderm for atrial septation. Development 136, 1761–1770.
Ilagan, R., et al., 2006. Fgf8 is required for anterior heart field development. Development 133, 2435–2445.
Kasahara, H., Bartunkova, S., Schinke, M., Tanaka, M., Izumo, S., 1998. Cardiac and extracardiac expression of Csx/Nkx2.5 homeodomain protein. Circ. Res. 82, 936–946.
Kelly, R.G., Buckingham, M.E., 2002. The anterior heart-forming field: voyage to the arterial pole of the heart. Trends Genet. 18, 210–216.
Kelly, R.G., Brown, N.A., Buckingham, M.E., 2001. The arterial pole of the mouse heart forms from Fgf10-expressing cells in pharyngeal mesoderm. Dev. Cell 1, 435–440.
Kim, R.Y., Robertson, E.J., Solloway, M.J., 2001. Bmp6 and Bmp7 are required for cushion formation and septation in the developing mouse heart. Dev. Biol. 235, 449–466.
Komuro, I., Izumo, S., 1993. Csx: a murine homeobox-containing gene specifically expressed in the developing heart. Proc. Natl. Acad. Sci. USA 90, 8145–8149.

Lee, K.H., Xu, Q., Breitbart, R.E., 1996. A new tinman-related gene, nkx2.7, anticipates the expression of nkx2.5 and nkx2.3 in zebrafish heart and pharyngeal endoderm. Dev. Biol. 180, 722–731.
Lin, L., Bu, L., Cai, C.L., Zhang, X., Evans, S., 2006. Isl1 is upstream of sonic hedgehog in a pathway required for cardiac morphogenesis. Dev. Biol. 295, 756–763.
Lints, T.J., Parsons, L.M., Hartley, L., Lyons, I., Harvey, R.P., 1993. Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. Development 119, 969.
Lough, J., Sugi, Y., 2000. Endoderm and heart development. Dev. Dyn. 217, 327–342.
Lyons, I., et al., 1995. Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5. Genes Dev. 9, 1654–1666.
Macatee, T.L., et al., 2003. Ablation of specific expression domains reveals discrete functions of ectoderm- and endoderm-derived FGF8 during cardiovascular and pharyngeal development. Development 130, 6361–6374.
Madabhushi, M., Lacy, E., 2011. Anterior visceral endoderm directs ventral morphogenesis and placement of head and heart via BMP2 expression. Dev. Cell 21, 907–919.
Marvin, M.J., Di Rocco, G., Gardiner, A., Bush, S.M., Lassar, A.B., 2001. Inhibition of Wnt activity induces heart formation from posterior mesoderm. Genes Dev. 15, 316–327.
Mjaatvedt, C.H., et al., 2001. The outflow tract of the heart is recruited from a novel heart-forming field. Dev. Biol. 238, 97–109.
Nakajima, Y., et al., 2009. Heart development before beating. Anat. Sci. Int. 84, 67–76.
Nascone, N., Mercola, M., 1995. An inductive role for the endoderm in Xenopus cardiogenesis. Development 121, 515–523.
Newman, C.S., Krieg, P.A., 1998. Tinman-related genes expressed during heart development in Xenopus. Dev. Genet. 22, 230–238.
Pabst, O., Herbrand, H., Arnold, H.H., 1998. Nkx2-9 is a novel homeobox transcription factor which demarcates ventral domains in the developing mouse CNS. Mech. Dev. 73, 85–93.
Park, E.J., et al., 2006. Required, tissue-specific roles for Fgf8 in outflow tract formation and remodeling. Development 133, 2419–2433.
Park, E.J., et al., 2008. System for tamoxifen-inducible expression of cre-recombinase from the Foxa2 locus in mice. Dev. Dyn. 237, 447–453.
Pashmforoush, M., et al., 2004. Nkx2-5 pathways and congenital heart disease; loss of ventricular myocyte lineage specification leads to progressive cardiomyopathy and complete heart block. Cell 117, 373–386.
Prall, O.W., et al., 2007. An Nkx2-5/Bmp2/Smad1 negative feedback loop controls heart progenitor specification and proliferation. Cell 128, 947–959.
Reecy, J.M., et al., 1997. Chicken Nkx-2.8: a novel homeobox gene expressed in early heart progenitor cells and pharyngeal pouch-2 and -3 endoderm. Dev. Biol. 188, 295–311.
Rodriguez, C.I., et al., 2000. High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. Nat. Genet. 25, 139–140.
Saga, Y., et al., 1999. MesP1 is expressed in the heart precursor cells and required for the formation of a single heart tube. Development 126, 3437–3447.
Saga, Y., Kitajima, S., Miyagawa-Tomita, S., 2000. Mesp1 expression is the earliest sign of cardiovascular development. Trends Cardiovasc. Med. 10, 345–352.
Schneider, V.A., Mercola, M., 2001. Wnt antagonism initiates cardiogenesis in *Xenopus laevis*. Genes Dev. 15, 304–315.
Schott, J.J., et al., 1998. Congenital heart disease caused by mutations in the transcription factor NKX2-5. Science 281, 108–111.
Schultheiss, T.M., Xydas, S., Lassar, A.B., 1995. Induction of avian cardiac myogenesis by anterior endoderm. Development 121, 4203–4214.
Searcy, R.D., Vincent, E.B., Liberatore, C.M., Yutzey, K.E., 1998. A GATA-dependent nkx-2.5 regulatory element activates early cardiac gene expression in transgenic mice. Development 125, 4461–4470.
Seo, S., Kume, T., 2006. Forkhead transcription factors, Foxc1 and Foxc2, are required for the morphogenesis of the cardiac outflow tract. Dev. Biol. 296, 421–436.
Soriano, P., 1999. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat. Genet. 21, 70–71.
Tam, P.P., Steiner, K.A., 1999. Anterior patterning by synergistic activity of the early gastrula organizer and the anterior germ layer tissues of the mouse embryo. Development 126, 5171–5179.
Tanaka, M., Chen, Z., Bartunkova, S., Yamasaki, N., Izumo, S., 1999. The cardiac homeobox gene Csx/Nkx2.5 lies genetically upstream of multiple genes essential for heart development. Development 126, 1269–1280.
Tanaka, M., Yamasaki, N., Izumo, S., 2000. Phenotypic characterization of the murine Nkx2.6 homeobox gene by gene targeting. Mol. Cell. Biol. 20, 2874–2879.
Tanaka, M., Schinke, M., Liao, H.S., Yamasaki, N., Izumo, S., 2001. Nkx2.5 and Nkx2.6, homologs of *Drosophila* tinman, are required for development of the pharynx. Mol. Cell. Biol. 21, 4391–4398.
Targoff, K.L., et al., 2013. Nkx genes are essential for maintenance of ventricular identity. Development 140, 4203–4213.
Tu, C.T., Yang, T.C., Tsai, H.J., 2009. Nkx2.7 and Nkx2.5 function redundantly and are required for cardiac morphogenesis of zebrafish embryos. PLoS One 4, e4249.
Tzahor, E., Lassar, A.B., 2001. Wnt signals from the neural tube block ectopic cardiogenesis. Genes Dev. 15, 255–260.
Waldo, K.L., et al., 2001. Conotruncal myocardium arises from a secondary heart field. Development 128, 3179–3188.
Wang, J., et al., 2010. Bmp signaling regulates myocardial differentiation from cardiac progenitors through a MicroRNA-mediated mechanism. Dev. Cell 19, 903–912.
Watanabe, Y., et al., 2012. Fibroblast growth factor 10 gene regulation in the second heart field by Tbx1, Nkx2-5, and Islet1 reveals a genetic switch for down-regulation in the myocardium. Proc. Natl. Acad. Sci. USA 109, 18273–18280.
Watanabe, Y., et al., 2010. Role of mesodermal FGF8 and FGF10 overlaps in the development of the arterial pole of the heart and pharyngeal arch arteries. Circ. Res. 106, 495–503.
Wilkinson, D.G., 1992. In Situ Hybridization: A Practical Approach. Oxford University Press, New York.
Zhang, Z., et al., 2005. Tbx1 expression in pharyngeal epithelia is necessary for pharyngeal arch artery development. Development 132, 5307–5315.
Zhang, Z., Huynh, T., Baldini, A., 2006. Mesodermal expression of Tbx1 is necessary and sufficient for pharyngeal arch and cardiac outflow tract development. Development 133, 3587–3595.