

Endothelin receptor type A expression defines a distinct cardiac subdomain within the heart field and is later implicated in chamber myocardium formation

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SUMMARY

The avian and mammalian heart originates from two distinct embryonic regions: an early differentiating first heart field and a dorsomedially located second heart field. It remains largely unknown when and how these subdivisions of the heart field divide into regions with different fates. Here, we identify in the mouse a subpopulation of the first (crescent-forming) field marked by endothelin receptor type A (*Ednra*) gene expression, which contributes to chamber myocardium through a unique type of cell behavior. *Ednra-lacZ/EGFP*-expressing cells arise in the ventrocaudal inflow region of the early linear heart tube, converge to the midline, move anteriorly along the outer curvature and give rise to chamber myocardium mainly of the left ventricle and both atria. This movement was confirmed by fluorescent dye-labeling and transplantation experiments. The *Ednra-lacZ/EGFP*-expressing subpopulation is characterized by the presence of *Tbx5*-expressing cells. *Ednra*-null embryonic hearts often demonstrate hypoplasia of the ventricular wall, low mitotic activity and decreased *Tbx5* expression with reciprocal expansion of *Tbx2* expression. Conversely, endothelin 1 stimulates ERK phosphorylation and *Tbx5* expression in the early embryonic heart. These results indicate that early *Ednra* expression defines a subdomain of the first heart field contributing to chamber formation, in which endothelin 1/*Ednra* signaling is involved. The present finding provides an insight into how subpopulations within the crescent-forming (first) heart field contribute to the coordination of heart morphogenesis through spatiotemporally defined cell movements.

KEY WORDS: Cardiac development, Heart fields, Endothelin, Mouse

INTRODUCTION

The heart is the first functioning organ to develop during embryogenesis. Different sources of cell populations from the cardiogenic mesoderm and the cardiac neural crest coordinately contribute to the formation of elaborate cardiac structures, including the four specialized chambers, the valves and supporting tissues, and the conduction system (Buckingham et al., 2005; Cai et al., 2008; Kirby, 2007; Zhou et al., 2008). Myocardial progenitor cells first appear bilaterally in the anterior splanchnic mesoderm and move towards the midline to form the cardiac crescent and then the primary heart tube. Through subsequent growth by accretion of cells at the poles from a newly identified progenitor population called the second heart field (Cai et al., 2003; Galli et al., 2008; Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001), the heart tube loops and in subsequent steps is sculpted into a four-chambered heart in mammals and birds (Abu-Issa and Kirby, 2007; Buckingham et al., 2005; Laugwitz et al., 2008).

The distinction between these two heart fields is further supported by retrospective clonal analysis using recombinant *lacZ* labeling (Meilhac et al., 2004). This analysis, in embryonic day (E) 8.5 mouse embryos, revealed two distinct cell lineages with different patterns of regionalization in the heart tube. The first lineage contributed to all left ventricular myocardium, some of the right ventricular myocardium, the atrioventricular canal and both atria. The second lineage contributed to the outflow tract and all other myocardial regions except for the left ventricle. Notably, the second lineage appears to correspond roughly to the second heart field marked by *Islet1* (*Isl1* – Mouse Genome Informatics) expression in terms of regional contribution (Cai et al., 2003; Galli et al., 2008). The clonal analysis also revealed that the two cell lineages segregate from a common progenitor around the time of gastrulation (Meilhac et al., 2004). Based on these findings, the first lineage is postulated to segregate first from the common progenitor pool to form the heart tube (Buckingham et al., 2005).

Following their segregation, the heart fields are probably further regionalized and diversified into various cell types. However, it remains largely unknown when and how different subpopulations arise within these heart-forming fields and how they interact with each other to coordinate heart morphogenesis.

Endothelin 1 (*Edn1*)/endothelin receptor type A (*Ednra*) signaling is known to be involved in cardiovascular and craniofacial development (Clouthier et al., 1998; Kurihara et al., 1994; Ozeki et al., 2004; Sato et al., 2008a; Sato et al., 2008b). Mice deficient in *Edn1/Ednra* signaling exhibit aortic arch malformation and outflow anomalies, which are attributed to cardiac neural crest defects (Kurihara et al., 1995; Yanagisawa et al., 1998). Correspondingly, *Ednra* is expressed in cranial and

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cardiac neural crest-derived mesenchymal cells (Clouthier et al., 1998; Kurihara et al., 1995; Maemura et al., 1996; Yanagisawa et al., 1998).

Recently, we established mice in which *lacZ* (which encodes β -galactosidase) and *EGFP* (which encodes enhanced green fluorescent protein) were introduced into the *Ednra* locus to recapitulate its endogenous expression (Sato et al., 2008a). Consequently, *lacZ/EGFP* expression emerged in the developing heart with a characteristic pattern. We then focused on this expression, expecting that it might lead to the identification of a novel myocardial subpopulation. Here, we demonstrate that early *Ednra-lacZ/EGFP* expression marks a subpopulation of the heart field with distinct regional identity. *Ednra-lacZ/EGFP*-positive cells are first localized in the ventral inflow region, move anteriorly along the outer curvature following the formation of the heart tube, and give rise to chamber myocardium. Dye-labeling and transplantation experiments confirmed this movement and contribution to chamber formation. We also observed developmental abnormalities in *Ednra*-null embryonic hearts, indicating the involvement of *Edn1* as a mitotic factor in early cardiac development.

MATERIALS AND METHODS

Mice

Ednra^{lacZ/+} (*lacZ* knock-in) mice have been described previously (Sato et al., 2008a). To generate mice carrying the *Ednra^{EGFP}* (*EGFP* knock-in) allele, we performed Cre recombinase-mediated cassette exchange (RMCE) on *Ednra^{neo/+}* embryonic stem (ES) cells in which an exchangeable floxed site was introduced into the *Ednra* locus as described previously (Sato et al., 2008a) (see Fig. S1 in the supplementary material). Briefly, the *EGFP* cassette excised from the pEGFP-N3 expression vector (Clontech) was introduced into the knock-in vector p66-2272 containing multiple cloning sites between *lox66* and *lox2272* (Araki et al., 2002). The resultant plasmids were transfected into *Ednra^{neo/+}* ES cells with AxCANCre recombinant adenovirus expressing the recombinase Cre tagged with a nuclear localization signal under the control of the CAG promoter (Kanegae et al., 1995). Targeted ES clones were injected into ICR blastocysts to generate germline chimeras that were then crossed with ICR females. Mice were housed in an environmentally controlled room at 23±2°C, with a relative humidity of 50-60% and under a 12-hour light:12-hour dark cycle. Genotypes were determined by PCR on tail-tip or amnion DNA using primers specific for RMCE-mediated recombination. Embryonic ages were determined by timed mating with the day of the plug being E0.5. The number of somites was also used to estimate developmental stages from E7.8 to E8.5. All animal experiments were reviewed and approved by the University of Tokyo Animal Care and Use Committee.

β -Galactosidase staining

lacZ expression was detected by staining with X-Gal (5-bromo-4-chloro-3-indolyl β -D-galactoside) for β -galactosidase activity. Whole-mount and section staining were performed as described previously (Nagy et al., 2003).

Immunohistochemistry

Embryo cryosections (12 μ m) were immunostained using the following antibodies: rat monoclonal anti-GFP (Nacalai Tesque, Kyoto, Japan; 1:200), rabbit anti-GFP (Medical and Biological Laboratories, Nagoya, Japan; 1:250), rabbit anti-Nkx2.5 (Santa Cruz, 1:250), mouse monoclonal anti-Is11 (39.5D5; Developmental Studies Hybridoma Bank; 1:100), mouse monoclonal anti-myosin heavy chain (MHC) (MF20-c; Developmental Studies Hybridoma Bank; 1:100), rabbit anti-desmin (Progen Biotechnik, Heidelberg, Germany; 1:200), mouse monoclonal phycoerythrin (PE)-conjugated anti-CD31 (BD Pharmingen; 1:200), mouse monoclonal anti-BrdU (Calbiochem; 1:20) and rabbit anti-phosphohistone H3 (pHH3) (Ser10) (Upstate Biotechnology; 1:250). Signals were visualized with

horseradish peroxidase- or FITC-conjugated secondary antibodies specific for the appropriate species. Some sections were treated with biotin-conjugated secondary antibodies and visualized using the VECTASTAIN ABC System (Vector Laboratories), streptavidin-FITC (Dako; 1:200) or streptavidin-TRITC (1:200, Beckman Coulter). Nuclei were visualized with TO-PRO-3 (Molecular Probes).

In situ hybridization

Whole-mount in situ hybridization was performed as described previously (Wilkinson, 1992). Sections (12 μ m) were prepared from frozen embryos. Treatment for in situ hybridization was as described previously with minor modifications (Ishii et al., 1997). The *Ednra*, *Cited1*, *Tbx2* and *Bmp2* probes have been described previously (Kokubo et al., 2007; Sato et al., 2008a). Probes for *Nkx2.5* (*Nkx2-5* – Mouse Genome Informatics), *Mlc2a* (*Myl7* – Mouse Genome Informatics) and *Isl1* (GenBank accession numbers: NM_008700, NM_021459 and NM_022879, respectively) were prepared using RT-PCR. The *Tbx5* probe was obtained from V. E. Papaioannou (Chapman et al., 1996; Sato et al., 2008a). The *Cx40* (*Gja5* – Mouse Genome Informatics) probe was obtained from D. Gros (Delorme et al., 1997). The *ANF* (*Nppa* – Mouse Genome Informatics) probe was obtained from T. Watanabe (Koibuchi and Chin, 2007). The *Hand1* probe was from D. Srivastava (Srivastava et al., 1995).

Fluorescent dye labeling

Embryos from 6- or 7-somite stages were collected, transferred to DMEM/F12 containing 10% FCS, and injected with PKH67 (green) or PKH26 (red) fluorescent dyes (Sigma) using a needle drawn from glass capillary tubing in order to label cells in the *lacZ*- or *EGFP*-expressing or adjacent regions. Embryos were then placed in a 15-ml culture bottle containing 2 ml culture medium (50% rat serum plus 50% DMEM/F12) and rotated at 20 rpm at 37°C while being continuously supplied with a suitable concentration of O₂ (5 or 20%) and CO₂ (5%) balanced with N₂ for 30 hours. Labeled embryos were observed using a Leica MZFLIII stereomicroscope equipped with a Hamamatsu C4742-95 digital camera. Some embryos were fixed for X-gal staining. For whole-heart labeling, E8.25 (6- to 7-somite stage) embryos were incubated in DMEM/F12 with 1 μ M SYTO16 (Molecular Probes), which stains the nuclei of live cells, at 37°C in 5% CO₂ for 30 minutes, and the heart tubes were excised.

Transplantation and explant culture experiment

Cardiac inflow tissues corresponding to the *Ednra-lacZ/EGFP*-positive region were excised from E8.25 (5- to 7-somite stage) embryos and transplanted into the same regions of recipient embryos (without removing their own inflow regions) using fine glass and tungsten needles. The *Ednra-EGFP*-positive tail regions were also transplanted as a control experiment. For transplantation of SYTO16-labeled cells, heart tubes were cut into three parts (outflow, ventricular and inflow regions) and were transplanted into the inflow regions of recipient embryos. Embryos were then placed in a collagen-coated 3.5-cm dish containing 500 μ l α -MEM with 10% horse serum at 37°C in 5% CO₂ balanced with N₂ for 24 hours. Following culture, embryos were observed under a fluorescence microscope or fixed in 4% paraformaldehyde and subjected to immunostaining. For explant culture, dissected SYTO16-labeled tissues were placed onto collagen-coated dishes and were incubated in α -MEM with 10% horse serum at 37°C in 5% CO₂ for 24 hours.

BrdU labeling

Pregnant female mice at E9.5 were injected intraperitoneally with bromodeoxyuridine (BrdU; 0.2 mg/g body weight; Sigma). After 1 hour of BrdU exposure, embryos were harvested on ice-cold PBS to stop BrdU incorporation and were fixed in 4% paraformaldehyde for 1 hour. Cryosections were treated with 2 M HCl and subjected to immunostaining with anti-BrdU antibody. For each sample, two sequential transverse sections through the widest region of the left and right ventricles were taken and BrdU-positive nuclei were counted in the compact, trabecular and endocardial layers of the ventricular wall. The sections were then counterstained with Hematoxylin to visualize nuclei and the total of

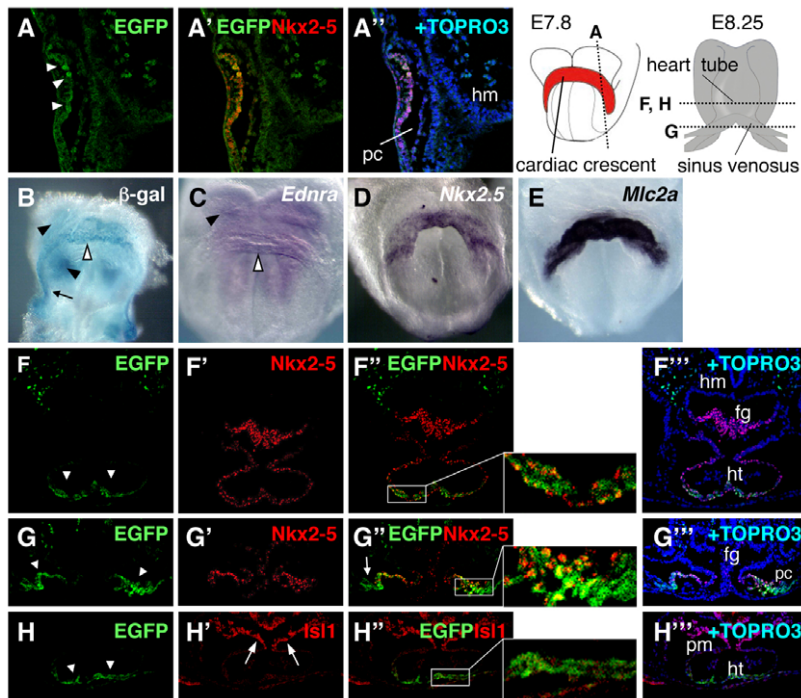


Fig. 1. *Ednra-lacZ/EGFP* expression in early developing hearts at the 1- to 4-somite stages. (A-A'') Sagittal sections of a 1-somite stage *Ednra-EGFP* mouse embryo immunostained for EGFP (A; green) and Nkx2.5 (A'; red), merged with TO-PRO-3 staining for nuclei (A''; blue). EGFP-positive cells were detected within the Nkx2.5-positive heart-forming region (white arrowheads). **(B)** Ventral view of a 3-somite stage *Ednra-lacZ* embryo stained for β -galactosidase activity. *lacZ*-expressing cells were detected within the cardiac crescent (white arrowhead), non-cardiogenic mesoderm (arrow) and head mesenchyme behind the crescent (black arrowheads). **(C-E)** Whole-mount in situ hybridization for *Ednra* (C), *Nkx2.5* (D) and *Mlc2a* (E) on a 3-somite stage embryo. White and black arrowheads indicate *Ednra* expression in the cardiac crescent and head mesenchyme, respectively. **(F-H'')** Transverse sections of 4-somite stage *Ednra-EGFP* embryos immunostained for EGFP (F,G,H,F',G',H'') and Nkx2.5 (F',G',H') or Isl1 (H',H'') with TO-PRO-3 staining (F'',G'',H''). The boxed regions in F',G',H'' are magnified in the panels to the right. EGFP-positive cells were detected within the Nkx2.5-positive heart tube (arrowheads) and Nkx2.5-negative lateral plate mesoderm (arrow in G'). EGFP expression did not overlap with Isl1 expression in the second heart field (arrows in H''). Planes of sections are indicated in the diagrams at the top. fg, foregut; hm, head mesenchyme; ht, heart tube; pc, pericardial coelom; pm, pharyngeal mesoderm.

number nuclei was counted in order to calculate the ratio of BrdU-positive to total nuclei. Statistical significance ($P < 0.05$) was determined using the paired *t*-test. Data are presented as mean \pm s.e.m.

Phosphorylation of ERK

Hearts were collected from E9.5 wild-type, *Ednra*^{+/EGFP} and *Ednra*^{EGFP/EGFP} embryos and lysed with lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, protease inhibitor cocktail (Roche), 1 mM sodium orthovanadate and 1 mM sodium fluoride]. The lysates were subjected to SDS-PAGE and immunoblotted using a mouse monoclonal antibody to phosphorylated ERK1/2 and a rabbit polyclonal antibody to total ERK1/2 (both Cell Signaling Technology). To evaluate the effect of Edn1, E9.5 wild-type hearts were incubated in serum-free DMEM with or without 10 μ M BQ123 (a cyclic peptide) for 3 hours then stimulated with 100 nM Edn1 for 5 minutes at 37°C. To evaluate the effect of BQ123 on basal ERK phosphorylation, E9.5 wild-type hearts were incubated in DMEM containing 10% fetal calf serum with or without 10 μ M BQ123 for 3 hours. Signal intensity was quantified with ImageJ software (NIH). One-way analysis of variance (ANOVA) with Tukey's test was applied for comparisons of phosphorylated ERK levels among genotypes.

Conventional RT-PCR

Hearts were collected from E8.25 and E9.5 embryos and sorted into EGFP-positive and EGFP-negative cells using a FACS VantageSE (BD Biosciences). Total RNA was extracted from sorted fractions with the use of Isogen (Nippon Gene, Tokyo, Japan), and 1 μ g samples were then reverse-transcribed using ReverTra Ace (Toyobo, Osaka, Japan) with oligo(dT) primer (Takara Bio, Shiga, Japan). The resulting cDNAs were amplified with Taq polymerase (Takara Bio) in a thermocycler. The sequences of the forward and reverse primers as well as the amplicon lengths are listed in Table S1 in the supplementary material. Custom primers were designed using Primer-BLAST online software (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Thermal cycling was performed for 25-30 cycles to maintain PCR conditions within the linear range of amplification before saturation was reached. Each cycle consisted of 30 seconds of denaturation at 94°C, 30 seconds of annealing at each annealing temperature (see Table S1 in the supplementary material) and 30 seconds of extension at 72°C. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as an internal control.

Quantitative real-time RT-PCR

Left ventricles were isolated from E9.5 embryos. To evaluate the effect of Edn1, isolated ventricles were cultured in DMEM plus 5% fetal calf serum with or without 100 nM Edn1 for 24 hours. Total RNA was extracted from a pool of seven samples for each genotype or culture condition. Quantification of the amount of each mRNA was performed by real-time RT-PCR analyses using a LightCycler (Roche) and Real-Time PCR Premix with SYBR Green (RBC Bioscience) following the manufacturer's protocol. The primers and reaction conditions are shown in Table S1 in the supplementary material. Thermal cycling was performed for 47 cycles after incubation at 96°C for 10 minutes in at least three separate runs. Each cycle consisted of 10 seconds of denaturation at 95°C, 10 seconds of annealing at each annealing temperature (see Table S1 in the supplementary material) and 11 seconds of extension at 72°C. The second-derivative maximum method was adopted to determine the crossing points automatically for individual samples, and relative amounts of mRNA were calculated based on the crossing-point analysis. Hypoxanthine phosphoribosyltransferase (*Hprt*) was used as an internal control. The result was expressed as a fold change relative to the control. The Mann-Whitney *U*-test was applied for comparisons of relative mRNA levels between genotypes or culture conditions.

RESULTS

Ednra-lacZ/EGFP expression defines a distinct subdomain within the cardiac crescent

We characterized marker gene expression in the embryonic heart of *Ednra*^{lacZ/+} and *Ednra*^{EGFP/+} mice. *Ednra-lacZ/EGFP*-positive cells were first detected in the crescent-forming cardiogenic mesoderm around the 1-somite stage (~E7.8) (Fig. 1A-A''). At the 1- to 3/4-somite stages (E7.8~E8.0), *Ednra-lacZ* signals coincided with detection of endogenous expression of *Ednra* (Fig. 1B,C) and were colocalized with *Nkx2.5* and *Mlc2a* (Fig. 1A',A'',D,E). Double immunostaining on sections revealed that *Ednra-EGFP* expression overlapped with *Nkx2.5* expression in the ventral region of the heart tube (Fig. 1F-F''). In the caudal region, *Ednra-lacZ/EGFP* expression extended to the *Nkx2.5*-negative lateral plate mesoderm (Fig. 1B,G-G'') and to the extra-embryonic mesoderm of the amnion along the border with the embryonic

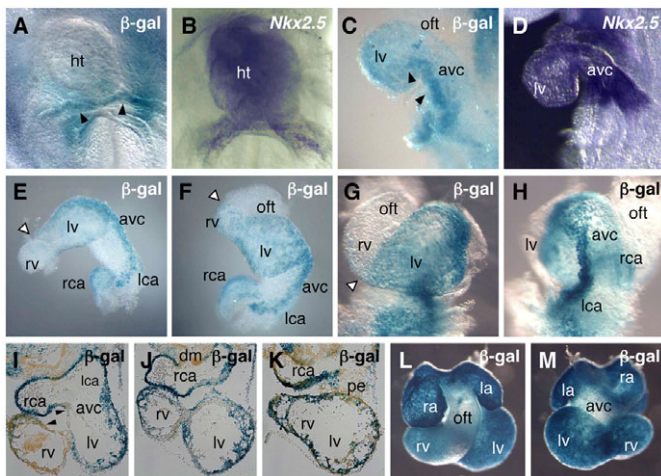


Fig. 2. β -Galactosidase staining and in situ hybridization for *Nkx2.5* on *Ednra-lacZ* hearts. (A-D) Hearts at 6/7- (A,B; ventral views) and 9/10- (C,D; left lateral views) somite stages, stained for β -galactosidase activity (A,C) or *Nkx2.5* expression (B,D). β -Galactosidase activity is most intense in the inflow region (arrowheads in A) and then extends towards the atrioventricular canal and left ventricle (arrowheads in C). (E-H) Dissected hearts at the 10- (E,F) and 13- (G,H) somite stages, stained for β -galactosidase activity and viewed from the inferior (E), ventral (F,G) and left-dorsal (H) sides. White arrowheads indicate boundaries of β -galactosidase expression. (I-K) Transverse sections of E9.5 *Ednra-lacZ* hearts stained for β -galactosidase activity. Sections are counterstained with Orange G. β -Galactosidase-positive cells contribute to all four chambers, but not to the atrioventricular canal and inner curvature. Arrowheads indicate expression boundaries along the inner curvature. Dorsal mesocardium and proepicardium were also negative for β -galactosidase activity. (L,M) Ventral (L) and dorsal (M) views of an E10.5 heart stained for β -galactosidase activity. avc, atrioventricular canal; dm, dorsal mesocardium; ht, heart tube; l/r(c/a), left/right (common) atrium; l/rv, left/right ventricle; offt, outflow tract; pe, proepicardium.

body. In contrast with its colocalization with *Nkx2.5*, *EGFP* expression never overlapped with *Isl1* expression, which marks the second heart field located behind the forming heart tube at this stage (Cai et al., 2003) (Fig. 1H-H'). These findings indicate that the *Ednra-lacZ/EGFP*-expressing cells might represent a myocardial subdomain within the first heart field.

***Ednra-lacZ/EGFP*-positive cells contribute to the chamber-forming myocardium**

During linear tube formation at the 6/7-somite stage (~E8.25), *Ednra-lacZ* expression was largely confined to the caudal inflow region corresponding to the venous pole (Fig. 2A,B). Subsequently, *Ednra-lacZ* expression was extended to the left lateral wall of the looping heart, forming an apparent trajectory along the outer curvature (Fig. 2C,D). At the 10- to 13-somite stage (~E8.5), β -galactosidase-positive cells were distributed from the venous pole to the left ventricle through a narrow region within the left lateral wall of the atrioventricular canal and atria (Fig. 2E-H). By contrast, the dorsal side of the heart, along the inner curvature and the outflow tract, lacked β -galactosidase expression (Fig. 2E-H). In the right ventricle, only the caudal (posterior) region was populated by β -galactosidase-positive cells (Fig. 2F,G). These patterns were almost identical to the endogenous *Ednra* expression revealed by in situ hybridization (see Fig. S2 in the supplementary material). At E9.5, β -galactosidase-positive cells were distributed in the common

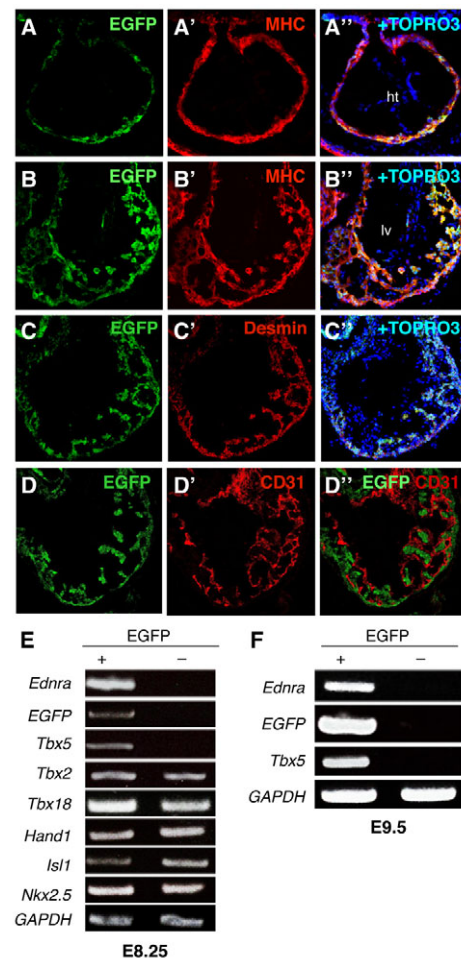


Fig. 3. Characterization of *Ednra-lacZ/EGFP*-expressing cells. (A-D') Co-immunostaining for EGFP and cardiac markers in early *Ednra-EGFP* hearts. Transverse sections of E8.25 (7-somite stage; A-A') and E9.5 (B-D') *Ednra-EGFP* embryos immunostained for EGFP (A-D, D') and myosin heavy chain (MHC; A', B'), desmin (C') or CD31 (D', D'') with TO-PRO-3 staining (A'', B'', C''). ht, heart tube; lv, left ventricle. (E, F) RT-PCR analysis of *Ednra-EGFP*-positive and -negative cells from E8.25 (E) and E9.5 (F) hearts.

atrium, left ventricle and a posterior part of the right ventricle (Fig. 2I-K). By contrast, the trabeculation-free region in the inner curvature, dorsal mesocardium and proepicardium lacked β -galactosidase expression (Fig. 2I-K). At E10.5, β -galactosidase was widely expressed in the four chambers, whereas the atrioventricular canal and outflow tract were mostly negative (Fig. 2L,M).

To confirm that *Ednra-lacZ/EGFP* expression marked the myocardial cell lineage, we examined myocardial marker expression. In the E8.25 heart tube and the E9.5 left ventricle, EGFP expression overlapped with myosin heavy chain expression (Fig. 3A,B). At E9.5, EGFP-expressing cells also expressed desmin (Fig. 3C), a marker for chamber-forming myocardium (Schaart et al., 1989). By contrast, EGFP expression did not overlap with CD31 expression, which marks endocardial cells (Fig. 3D). EGFP-expressing cells were further characterized by fluorescence-activated cell sorting (FACS) and RT-PCR. At E8.25 and E9.5, only EGFP-positive fractions expressed *Ednra* and *EGFP*. Remarkably, expression of *Tbx5*, a T-box transcription factor gene crucial for early heart development (Bruneau et al., 2001; Takeuchi et al.,

2003), was also only detected in EGFP-positive fractions (Fig. 3E,F). Section staining confirmed the presence of *Tbx5*-positive cells in the *Ednra*-EGFP-expressing region, particularly in the caudal inflow region (see Fig. S2 in the supplementary material). These results indicate that *Ednra-lacZ/EGFP* expression marks chamber-forming cardiomyocytes, including *Tbx5*-positive cells arising in the inflow region.

Dye injected into the ventral inflow region follows the subsequent distribution of the *Ednra-lacZ/EGFP*-positive subdomain

Extension of *Ednra-lacZ/EGFP* expression along the outer curvature raises the possibility that *Ednra-lacZ/EGFP*-expressing cells in the ventral inflow region move into the linear heart tube to give rise to chamber myocardium. However, it is also possible that *Ednra-lacZ/EGFP* expression is sequentially upregulated with different timing in cells already present in the heart tube. These possibilities are not mutually exclusive. To examine whether the upward cell movement contributes to the extension of *Ednra-lacZ/EGFP* expression, we injected PKH fluorescent dyes into the ventral inflow region and adjacent areas at the 6/7-somite stage and cultured the injected embryos for 30 hours. The injected areas, distribution patterns of labeled cells and summary of the results are shown in Fig. 4A. After 30 hours, labeled cells were found in the left lateral wall of the atrium and atrioventricular canal towards the left ventricle (I in Fig. 4A) in 13 out of 14 embryos in which dye was injected into the middle (a in Fig. 4A; $n=6$) or left (b in Fig. 4A; $n=8$) portion of the ventral inflow region (Fig. 4B-C'). Labeled cells were also found in the left ventricle in three embryos in which dye was injected into the middle region (Fig. 4B'). These results support the possibility of cell movement. Interestingly, five out of six embryos in which dye was injected into the right portion of the ventral inflow region (c in Fig. 4A) also had labeled cells in the contralateral left wall of the atrium and atrioventricular canal after 30 hours (Fig. 4D,D'). The one remaining embryo showed labeled cells in the future right atrium (Fig. 4E,E'). Thus, dye-injection experiments indicate that *lacZ/EGFP*-positive cells on both sides of the inflow region at the 6/7-somite stage contribute to the cell population extending to the left ventricle.

By contrast, only two out of 13 embryos in which dye was injected into the adjacent *lacZ/EGFP*-negative region revealed labeled cells in the atrial wall (Fig. 4A; d-g). In one of those embryos, labeled cells appeared in the left dorsocranial wall 30 hours after injection into the *lacZ/EGFP*-negative region dorsomedially adjacent to the left *lacZ/EGFP*-positive region (Fig. 4; f→III); in the other, labeled cells were detected in the right lateral wall after injection into the right *lacZ/EGFP*-negative region (Fig. 4; g→II). These distribution patterns are consistent with the previous report that cells in the right and left posterior second heart field contribute to the corresponding right and left wall of the atrium (Galli et al., 2008).

When transplanted, the *Ednra-lacZ/EGFP*-positive inflow region contributes to left ventricular and atrial myocardium

To confirm the movement from the ventral inflow region to the developing left ventricle and atrium, we transplanted, at E8.25 (5- to 7-somite stage), the *Ednra-lacZ/EGFP*-positive inflow region into the same region of wild-type embryos (Fig. 5A). After 24 hours, *Ednra*-EGFP-positive cells were detected in the left ventricle in five out of eight recipient embryos transplanted with the *Ednra*^{EGFP/+} inflow region (Fig. 5B-B'). By contrast, no EGFP signals were

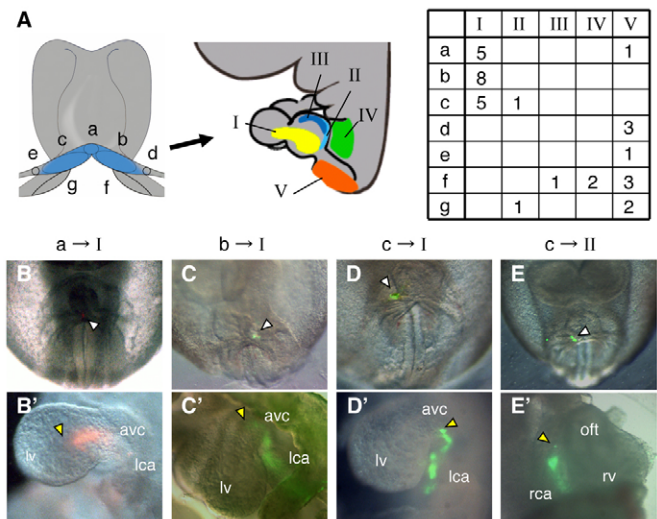


Fig. 4. PKH fluorescent dye injection into the inflow region.

(A) Schematics indicating areas of injection (left) and distribution after 30 hours (middle). Table shows the number of embryos observed with dye present in each of the regions I-V following injection at each of the sites a-g. a/b/c, middle/left/right areas within the *Ednra-lacZ/EGFP*-positive region (blue); d/e, areas lateral to b/c; f/g, left/right areas dorsomedial to the *Ednra-lacZ/EGFP*-positive region; I, left lateral wall of the inflow tract; II, right common atrial wall; III, dorsocranial wall of the left common atrium; IV, region around the pharyngeal mesoderm; V, region around the lateral mesoderm. (B-E') Representative results. Dye was injected at the sites indicated (white arrowheads in B-E) and traced after 30 hours (B'-E'). Yellow arrowheads indicate anterior boundaries of dye fluorescence. avc, atrioventricular canal; lca, left common atrium; lv, left ventricle; oft, outflow tract; rca, right common atrium.

detected when *Ednra*-EGFP-positive tails from *Ednra*^{EGFP/+} embryos were transplanted ($n=7$; Fig. 5C-C'). No fluorescent signals were observed in the left ventricle of wild-type recipients transplanted with a wild-type inflow region ($n=7$; Fig. 5D-D'). *Ednra*^{EGFP/+} recipients transplanted with the *Ednra*^{EGFP/+} inflow region exhibited strong endogenous EGFP signals in the ventricle, as expected ($n=6$; Fig. 5E-E').

To verify further the regional specificity with respect to movement, we transplanted different regions of the E8.25 heart tube, labeled with SYTO16 fluorescent dye, into the inflow region of the heart at the same developmental stage (Fig. 5F). When SYTO16-labeled cells were transplanted into the outflow or ventricular region, no upward movement was detected after 24 hours ($n=5$ for each; Fig. 5G-G'',H-H''). By contrast, SYTO16-labeled cells were found to distribute along the outer curvature towards the left ventricle after 24 hours in all five embryos transplanted with cells in the inflow region (Fig. 5I-I''). These results strongly support the movement and contribution of *Ednra-lacZ/EGFP*-positive inflow cells to the developing left ventricle.

As indicated by β -galactosidase staining, *Ednra-lacZ/EGFP* was widely expressed in the four chambers after E9.5, raising the possibility that many *Ednra*-negative cells in the early heart tube might become *Ednra*-positive at later stages. To test this idea, we performed explant culture of the ventricular and inflow regions of the E8.25 heart tube separately. At the start of culture, EGFP signals were clearly detectable in the inflow region, but were very low in the ventricle (see Fig. S3 in the supplementary material). After 24 hours, ventricular and inflow regions both demonstrated

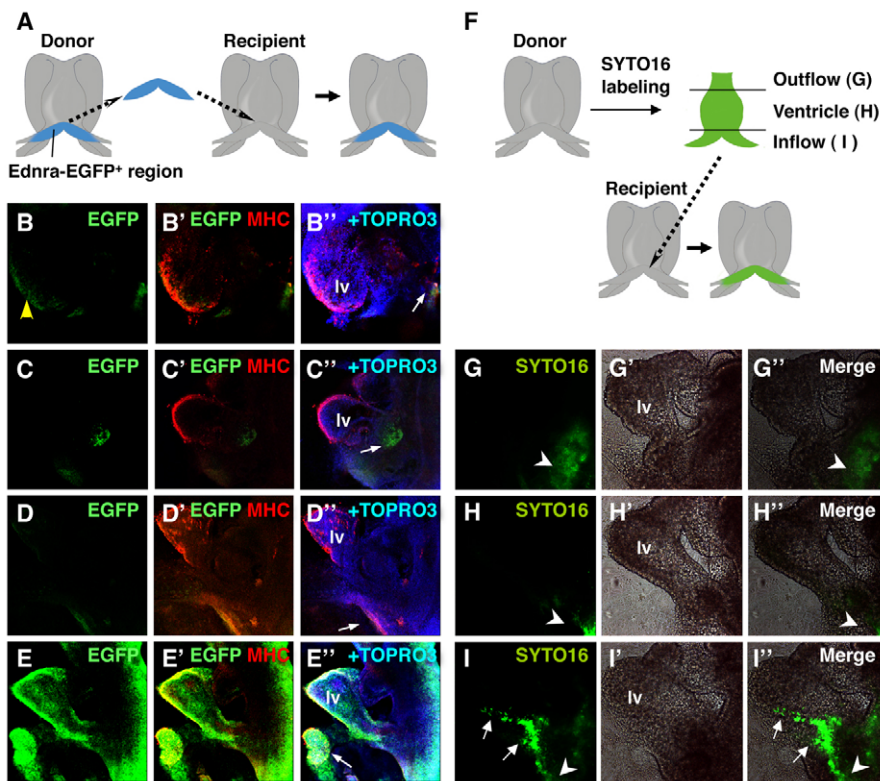


Fig. 5. Transplantation of the ventral inflow region. (A) Schematic indicating transplantation of the *Ednra*-EGFP-positive ventral inflow region. (B-E'') Representative results 24 hours after transplantation of the inflow region (B-B'', D-E'') or the tail region (C-C'') from *Ednra*^{EGFP/+} (B-C'', E-E'') or wild-type (D-D'') embryos into the same region of wild-type (B-D'') or *Ednra*^{EGFP/+} (E-E'') embryos. White arrows, transplants; yellow arrowhead, EGFP signals in the left ventricle (lv). (F) Schematic indicating transplantation of SYTO16-labeled heart regions. (G-I'') Representative results 24 hours after transplantation of the outflow (G-G''), ventricular (H-H'') and inflow (I-I'') regions. SYTO16 signals (G, H, I) and optical images (G'', H'', I'') are superimposed in G'', H'' and I''. Arrowheads, areas of transplantation; arrows, SYTO16-labeled cells along the outer curvature towards the left ventricle (lv).

intense EGFP signals (see Fig. S3 in the supplementary material). These results suggest that tube-forming cells that are *Ednra* negative at early stages might start to express *Ednra* later.

Defects in ventricular chamber formation in *Ednra*-null mice

In addition to outflow abnormalities as a result of cardiac neural crest defects, some *Edn1*-null embryos from *Edn* antagonist-treated pregnant mice exhibited ventricular hypoplasia (Kurihara et al., 1995), indicating involvement of *Edn* signaling in cardiac chamber development. To investigate this further, we revisited the *Ednra*-null phenotype.

At E9.5, when cardiac neural crest cells are not yet seen in the conus arteriosus (Jiang et al., 2000), *Ednra*^{lacZ/EGFP} (*Ednra*-null) embryos were obtained at the expected Mendelian ratio (49:102:56 wild-type:heterozygous:null). Of the 43 *Ednra*^{lacZ/EGFP} embryos examined, 26 (60%) showed normally developed hearts (Fig. 6A,B). By contrast, 17 *Ednra*^{lacZ/EGFP} embryos (40%) showed a gourd-shaped heart with disproportionate chamber sizes (Fig. 6C). These morphological changes indicate that *Ednra*-mediated signals might be involved in normal growth during chamber formation. To further characterize the *Ednra*-null phenotype, we compared the distribution of β -galactosidase-positive cells in the hearts of *Ednra*^{lacZ/+} and *Ednra*^{lacZ/EGFP} embryos at E9.5. β -galactosidase expression in the caudal (posterior) ventricular wall tended to distribute from left to right to a lesser extent in *Ednra*^{lacZ/EGFP} embryos than in *Ednra*^{lacZ/+} littermates (see Fig. S4 in the supplementary material). Histological examination revealed that *Ednra*^{lacZ/EGFP} embryos often had a poorly developed ventricular wall with a low distribution of β -galactosidase-positive cells (Fig. 6D-G).

Next, we performed BrdU labeling to examine whether the *Ednra*-null phenotype was associated with changes in proliferation. The number of proliferative cells incorporating BrdU was significantly decreased in the compact and trabecular layers of the

ventricular wall of *Ednra*-null embryos compared with those of heterozygous *Ednra*^{+/-EGFP} embryos (Fig. 6H-M). Decreased BrdU uptake in the right ventricle could possibly be explained by low proliferation rates of β -galactosidase-positive cells populated in the caudal (posterior) region (Fig. 2F,G). Indeed, in the right ventricular region, the number of BrdU-labeled cells with a low β -galactosidase-positive population was similar in *Ednra*-null and heterozygous embryos (data not shown). To confirm this, we double stained serial ventricular sections for β -galactosidase activity and phosphohistone H3 (pHH3), a marker of mitosis (Cimini et al., 2003), and counted pHH3-positive cells separately in populations positive and negative for β -galactosidase. The mitotic frequency of β -galactosidase-positive, but not β -galactosidase-negative, cells was decreased in the left and right ventricles of the *Ednra*-null heart (Fig. 6N). Decreased BrdU incorporation in the endocardium of the *Ednra*^{lacZ/EGFP} right ventricle is likely to be an indirect effect because the endocardial layer does not express *Ednra*-lacZ. We did not observe differences in the proportion of apoptotic cells between *Ednra*-null and heterozygous or wild-type embryos (data not shown).

Edn1 has been reported to act as a mitogen on cardiomyocytes by stimulating ERK phosphorylation (Sugden, 2003). We therefore examined ERK phosphorylation in E9.5 wild-type, *Ednra*^{+/-EGFP} and *Ednra*^{lacZ/EGFP} hearts. ERK phosphorylation levels were decreased in proportion to the number of *Ednra*-null alleles (Fig. 7A,B). In E9.5 hearts in vitro, stimulation of ERK phosphorylation by *Edn1* was abolished by the *Ednra* antagonist BQ123 (Fig. 7C). Basal ERK phosphorylation was also decreased by BQ123 (Fig. 7D). Taken together, these results suggest that the *Ednra* signal is involved in myocardial development as a mitogenic factor at early stages. By contrast, no differences in ERK phosphorylation were observed at E10.5 (data not shown), indicating that the negative effect of the *Ednra*-null mutation on myocardial proliferation might be overcome by other factors at later stages.

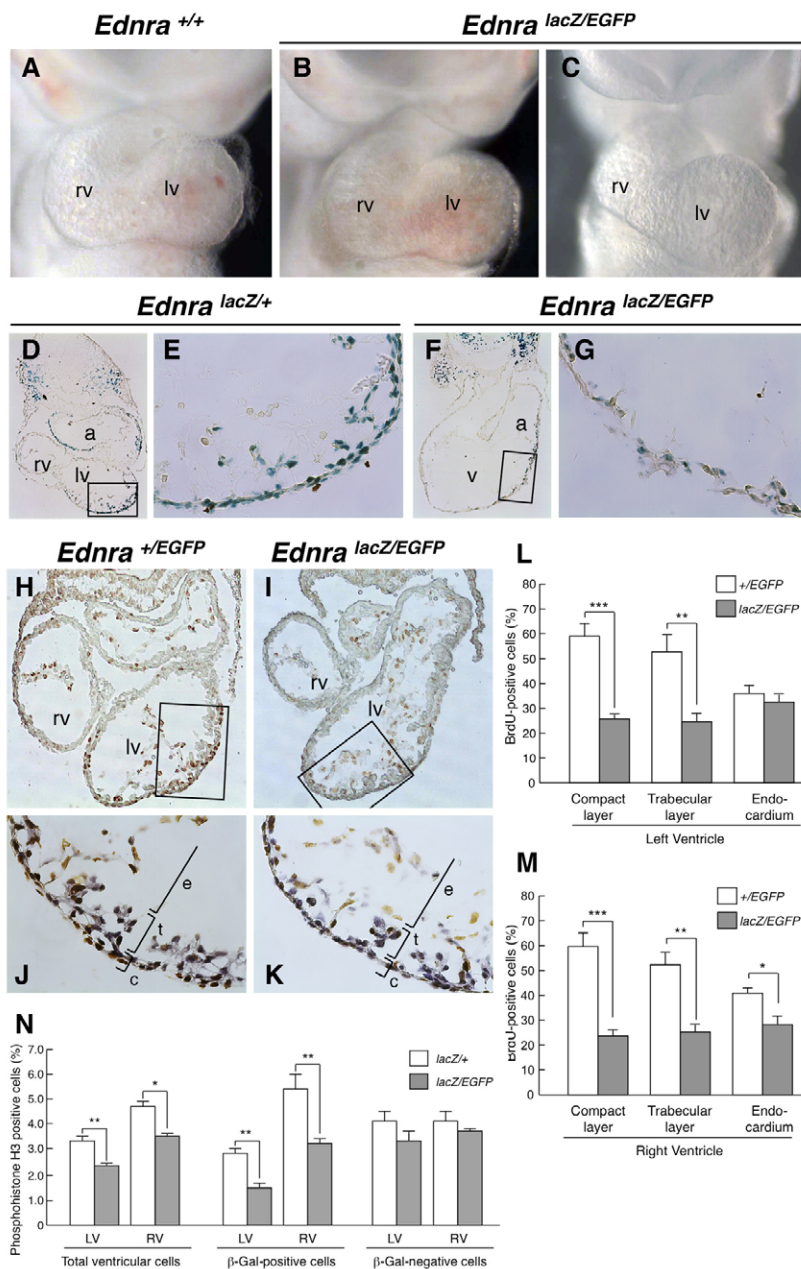


Fig. 6. Morphological, histological and cell proliferation analysis of *Ednra*-null hearts.

(A-C) Representative wild-type (A) and *Ednra*^{lacZ/EGFP} (*Ednra*-null) (B,C) hearts at E9.5. *Ednra*^{lacZ/EGFP} embryo hearts appear normal (B) or gourd-shaped with disproportionate chamber sizes (C). (D-G) Representative transverse sections of E9.25 *Ednra*^{lacZ/+} (D,E) and *Ednra*^{lacZ/EGFP} (F,G) hearts stained for β-galactosidase activity. The boxed areas in D and F are magnified in E and G, respectively. The *Ednra*^{lacZ/EGFP} heart is defective in ventricular wall formation and distribution of β-galactosidase-positive cells. (H-K) Representative transverse sections of BrdU-labeled (brown) *Ednra*^{+EGFP} (H,I) and *Ednra*^{lacZ/EGFP} (J,K) hearts at E9.5. The boxed areas in H and I are magnified in J and K, respectively, after co-staining with Hematoxylin to visualize nuclei (purple). (L,M) Quantification of cell proliferation indicated by the ratio of BrdU-positive cells to total cell number in different compartments of the left (L) and right (M) ventricular wall of E9.5 *Ednra*^{+EGFP} and *Ednra*^{lacZ/EGFP} hearts ($n=3$ for each). (N) Quantification of the percentage of phosphohistone H3-positive cells in the left (LV) and right (RV) ventricles of E9.5 *Ednra*^{lacZ/+} and *Ednra*^{lacZ/EGFP} hearts ($n=3$ for each) with respect to the numbers of β-galactosidase-positive, β-galactosidase-negative and total cells in three serial sections. Data are presented as mean±s.e.m. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. a, atrium; c, compact layer; e, endocardium; lv, left ventricle; rv, right ventricle; t, trabecular layer; v, ventricle.

Changes in T-box transcription factor gene expression in *Ednra*-null hearts

To examine whether the *Ednra*-null mutation affected gene expression in the early developing heart, we performed in situ hybridization for several marker genes on E9.5 *Ednra*^{+EGFP} and *Ednra*^{lacZ/EGFP} hearts. *Ednra*^{lacZ/EGFP} left ventricles showed decreased expression of *Tbx5* and its downstream gene *Cx40* (Fig. 8A,B), whereas the expression of other chamber myocardium markers such as *ANF*, *Hand1*, *Cited1* and *Mlc2a* was not affected (Fig. 8C-F). Decreased mRNA levels of *Tbx5* and *Cx40*, but not *Hand1*, were also confirmed by quantitative RT-PCR (Fig. 8G). Furthermore, *Tbx5* mRNA levels were increased by Edn1 in excised E9.5 heart explants (Fig. 8H).

The effect of the *Ednra*-null mutation on gene expression patterns was examined further by whole-mount in situ hybridization. In E8.25 *Ednra*^{+EGFP} and *Ednra*^{lacZ/EGFP} hearts, *Tbx5* expression in the inflow region had a similar pattern to that

of *Ednra* (Fig. 9A). At E9.5, *Tbx5* expression was expanded anteriorly towards the left ventricle in *Ednra*^{+EGFP} embryos (Fig. 9B), as described previously (Bruneau et al., 1999). This anterior expansion of *Tbx5* expression was decreased in *Ednra*^{lacZ/EGFP} hearts, whereas *Tbx5* expression in the posterior (inflow) region was similar to that of *Ednra*^{+EGFP} embryos (Fig. 9C). By contrast, the *Tbx2*-expressing region, which normally corresponds to the atrioventricular canal (Aanhaanen et al., 2009), was reciprocally expanded in *Ednra*^{lacZ/EGFP} hearts (Fig. 9D,E). *Bmp2* expression in the atrioventricular canal was similar in *Ednra*^{+EGFP} and *Ednra*^{lacZ/EGFP} hearts (Fig. 9F,G), indicating that the expansion of *Tbx2* expression was independent of *Bmp2*, an inducer of *Tbx2* in the atrioventricular canal (Kokubo et al., 2007; Yamada et al., 2000). These results indicate that Edn1/*Ednra* signaling might be involved in the regulation of T-box transcription factor gene expression in early developing hearts.

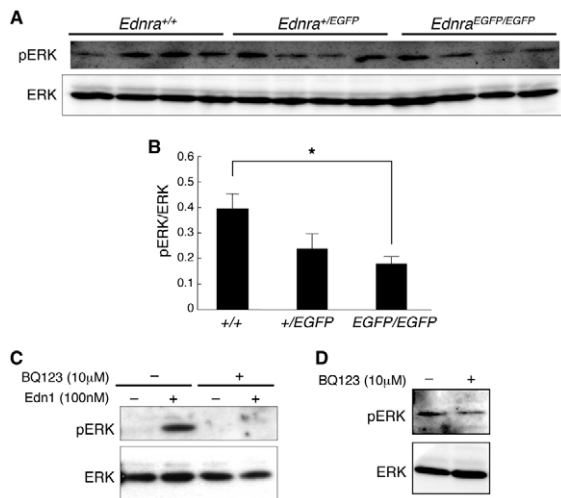


Fig. 7. Analysis of ERK phosphorylation. (A) Western blot of individual hearts excised from E9.5 wild-type, *Ednra*^{+EGFP} and *Ednra*^{EGFP/EGFP} embryos using antibodies to phosphorylated (pERK) and total ERK1/2 (ERK). (B) Quantification of the ratio of phosphorylated to total ERK protein levels in A, normalized to control (wild-type) samples ($n=5$ per group). Data are presented as mean \pm s.e.m. * $P<0.05$. (C) Edn1 stimulates ERK phosphorylation in isolated E9.5 hearts. Edn1-induced ERK phosphorylation is completely abolished by the *Ednra* antagonist BQ123. (D) BQ123 downregulates basal ERK phosphorylation in isolated E9.5 hearts.

DISCUSSION

Regionalization of the first heart field and contribution to chamber formation

Here, we identified an *Ednra-lacZ/EGFP*-expressing cell population that is first detected in the cardiac crescent. At the early heart tube-forming stage, these cells are present in the ventral inflow region. Subsequently, dye-labeling experiments indicate that they move upward along the outer curvature between the 6/7-somite and 9/10-somite stages. This timing corresponds to the start of looping just after the formation of the linear heart tube (Abu-Issa and Kirby, 2007), indicating that the linear heart tube is mainly composed of *Ednra*-negative cells and that the *Ednra*-positive cells are recruited into the looping heart tube. This upward cell movement was confirmed and was shown to be specific for cells in the inflow region by dye-labeling and transplantation experiments. These findings suggest that *Ednra*-positive cells arising from the crescent-forming first heart field are a distinct subpopulation and contribute to chamber formation in a manner different from that of the early tube-forming cells.

de la Cruz and colleagues have performed extensive *in vivo* labeling experiments in the chick and showed that the inflow region between the interventricular grooves and the caudal end of the linear heart tube contributes to the trabeculated portion of the left ventricle (de la Cruz et al., 1989). The present study identified cells in the ventral wall within this inflow region as a subpopulation with a distinct gene expression signature and cell movement; cells at both sides converge to the midline and move upward along the outer curvature. In the chick embryo, the outer curvature is formed by the ventral seam after fusion of the bilateral cardiogenic fields (Abu-Issa and Kirby, 2008). Although in the chick embryo, unlike in the mouse, the bilateral heart fields remain separate until the tube-forming stage without

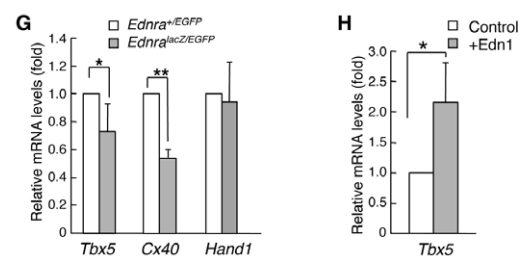
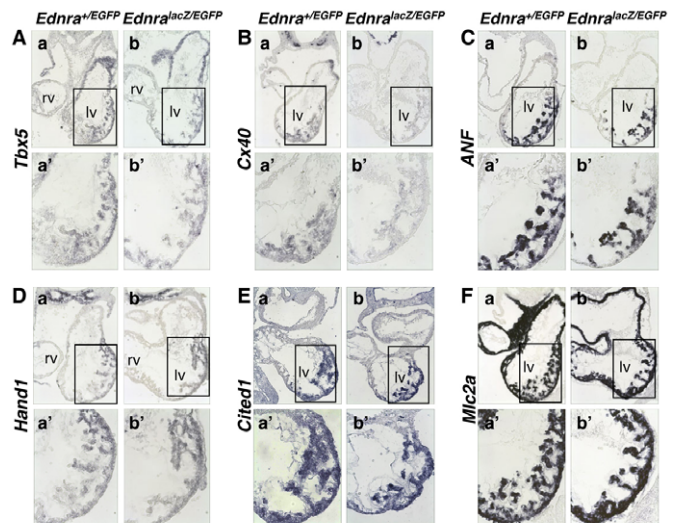


Fig. 8. Gene expression analysis of E9.5 *Ednra*-null hearts. (A-F) *In situ* hybridization of *Ednra*^{+EGFP} (a,a') and *Ednra*^{lacZ/EGFP} (b,b') hearts for *Tbx5* (A), *Cx40* (B), *ANF* (C), *Hand1* (D), *Cited1* (E) and *Mlc2a* (F). a' and b' are magnifications of the regions indicated in a and b, respectively. lv, left ventricle; rv, right ventricle. (G,H) Real-time RT-PCR analysis. (G) *Tbx5* and *Cx40*, but not *Hand1*, expression levels are lower in *Ednra*^{lacZ/EGFP} hearts than in *Ednra*^{+EGFP} hearts ($n=5$ per group). (H) In E9.5 wild-type ventricles, *Tbx5* expression is upregulated by stimulation with 100 nM Edn1 for 24 hours ($n=4$ per group). Data are presented as mean \pm s.e.m. * $P<0.05$, ** $P<0.01$.

forming the cardiac crescent, the mode of outer curvature formation by midline convergence of bilateral cells might be common to chick and mouse embryos.

The *Ednra-lacZ/EGFP*-expressing cell population is characterized by the presence of cells that express *Tbx5*, which is expressed in a posterior-to-anterior gradient in the inflow region and is important for left ventricular identity (Hoogaars et al., 2007). The *Ednra*-expressing region is distinct from the second heart field, marked by *Isl1* expression. However, *Isl1* expression was detected in both *Ednra-EGFP*-positive and -negative cells at E8.25 in our FACS and RT-PCR experiment. Recently, van den Berg et al. reported that, in chick, an *Isl1*-positive proliferating center caudodorsal to the inflow tract provides cells to the venous and arterial poles of the elongating heart tube (van den Berg et al., 2009). Apparently, *Ednra-lacZ/EGFP*-positive cells are distinct from this population because they are localized to the ventral region of the inflow and are *Isl1*-negative at the crescent/tube-forming stages. However, *Ednra-lacZ/EGFP*-positive cells might also be derived from the *Isl1*-positive pool as *Isl1* is initially expressed in all cardiogenic mesoderm and is downregulated on differentiation (Prall et al., 2007; Yuan and Schoenwolf, 2000). At later stages, many atrial cells express *Ednra-lacZ/EGFP*, suggesting that the second heart field-derived cells may also start to express this gene

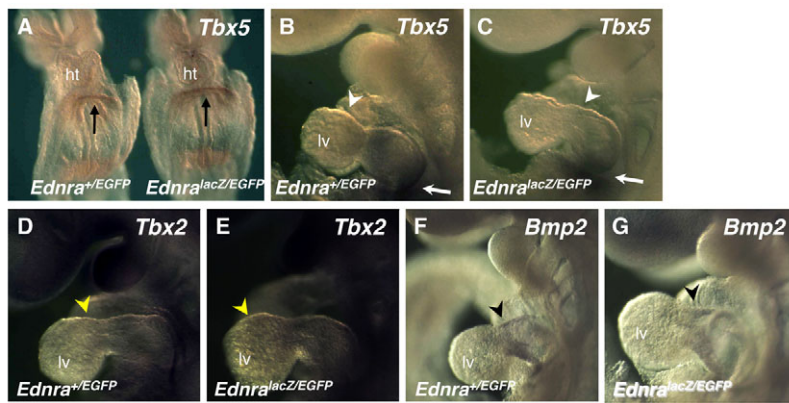


Fig. 9. Changes in *Tbx5* and *Tbx2* expression patterns in *Ednra*-null hearts. (A) In situ hybridization of E8.25 *Ednra*^{+EGFP} and *Ednra*^{lacZ/EGFP} hearts. *Tbx5* expression in the inflow region was similar in both hearts (arrows). (B-G) In situ hybridization of E9.5 *Ednra*^{+EGFP} (B,D,F) and *Ednra*^{lacZ/EGFP} (C,E,G) hearts for *Tbx5* (B,C), *Tbx2* (D,E) and *Bmp2* (F,G). Anterior expansion of *Tbx5* expression (white arrowheads) was decreased in *Ednra*^{lacZ/EGFP} hearts, whereas *Tbx5* expression in the posterior (inflow) region (arrows) was similar. By contrast, the *Tbx2*-expressing region (yellow arrowheads) was expanded in *Ednra*^{lacZ/EGFP} hearts. *Bmp2* expression in the atrioventricular canal (black arrowheads) was similar in *Ednra*^{+EGFP} and *Ednra*^{lacZ/EGFP} hearts. ht, heart tube; lv, left ventricle.

at a later time than do first heart field-derived cells. Thus, *Isl1*-positive cells may eventually express *Ednra*, but the timing might be different between cardiac regions. Indeed, explant culture experiments revealed that *Ednra*-negative cells in the early heart tube might become *Ednra*-positive at later stages, indicating that later *Ednra*-positive cardiomyocytes are derived both from early *Ednra*-positive inflow cells and from tube-forming cells that are *Ednra*-negative at early stages.

According to the ballooning model for chamber formation, which is now widely accepted, the ventricular chambers bulge from the outer curvature of the looped heart (Christoffels et al., 2000). The developing chambers show high proliferative activity and are characterized by the upregulation of chamber-specific myocardial genes. A two-step model has been proposed for this process: the first step is the formation of a primary heart tube and the second step involves localized chamber differentiation in the ventral side (outer curvature) of the heart tube while primary myocardium is continuously recruited at arterial and venous poles of the tube (Christoffels et al., 2000; Moorman and Christoffels, 2003). However, it was not clear when and how chamber-forming cells in the outer curvature are specified. The present study suggests that *Ednra*-positive inflow cells might constitute part of the outer curvature by upward movement and contribute to chamber formation.

Dye-labeling experiments indicated that *Ednra*-positive cells along the outer curvature are derived from the bilateral inflow region. This implies that cells of both sides meet in the midline and distribute mainly to the left lateral wall as a mixed population. Dye labeling also demonstrated a contribution of *Ednra*-positive cells to the right atrial myocardium, although less frequently than to the left atrium. Galli et al. have demonstrated that the left and right sides of the posterior regions of the second heart field contribute to the left and right atrium, respectively (Galli et al., 2008). Thus, the atrial myocardium seems to be derived from at least two different cell sources in a different manner.

Role of endothelin signaling in early heart development

The present study characterizes further the *Ednra*-null phenotype in the early embryonic heart. Ventricular hypoplasia and associated decreased proliferation rates in *Ednra*-null hearts at E9.5 indicate that *Ednra*-mediated signals are involved in myocardial growth and ventricular formation. Around this stage, *Edn1* is secreted by the endothelia of the outflow tract and adjacent vessels (Kurihara et al., 1995) and might act on *Ednra*-expressing cells arising from the inflow tract in a paracrine manner.

The present result appears to be contradictory to a previous report in which a cardiomyocyte-specific *Ednra*-knockout resulted in no detectable phenotype (Kedzierski et al., 2003). This discrepancy might be explained by the time lag between the start of *Ednra* expression (~E7.8) and myosin heavy chain promoter-driven Cre activation (E8.5-E9.5) (Eckardt et al., 2006; Niu et al., 2005), which might permit *Ednra* expression at early stages. Although it is still possible that the phenotype we describe is an indirect effect of the *Ednra*-null phenotype in other tissues, decreased mitotic frequency in β -galactosidase-positive cells, but not in β -galactosidase-negative cells, of the *Ednra*-null heart supports the possibility that the phenotype is the result of a direct effect on early cardiomyocytes through the *Ednra* signaling pathway.

The *Edn1/Ednra* signal is known to induce hypertrophic growth of cardiac myocytes through G_q/G_{11} -mediated activation of the ERK pathway (Sugden, 2003). Consistently, ERK phosphorylation tended to decrease in E9.5 *Ednra*-null hearts and was stimulated by *Edn1* treatment. G_q/G_{11} -deficient embryos have severe myocardial hypoplasia in both the compact and trabecular layers, which might account for the mid-gestation lethality of these embryos (Offermanns et al., 1998). These findings lead us to speculate that *Edn1/Ednra* might be involved in the activation of a G_q/G_{11} -mediated mitotic pathway crucial for early myocardial development.

In addition, *Tbx5* and *Cx40* expression was downregulated in *Ednra*-null hearts and upregulated by stimulation with *Edn1*. Recent studies have implicated *Tbx5* in the regulation of myocardial growth and proliferation (Georges et al., 2008; Goetz et al., 2006) and expression of *Tbx5* is affected by growth factors (Georges et al., 2008). Given these findings, it would be interesting to investigate further the relationship between *Edn1/Ednra* signaling and *Tbx5*-dependent myocardial growth/proliferation and chamber specification. Conversely, *Tbx2* expression was expanded towards the left ventricle without changes in *Bmp2* expression in *Ednra*-null hearts. Recently, Aanhaanen et al. reported that *Tbx2*-expressing cells arising in the bilateral limbs of the crescent contribute to the atrioventricular canal and, subsequently, to the *Tbx2*-negative left ventricle, particularly to the basal free wall where *Tbx2* expression is lost (Aanhaanen et al., 2009). In *Tbx2*-null hearts, the atrioventricular canal differentiates prematurely to chamber myocardium and proliferates at increased rates similar to that of chamber myocardium, indicating that *Tbx2* might regulate the timing of chamber myocardial differentiation of *Tbx2*-expressing cells allocated to the left ventricular free wall (Aanhaanen et al., 2009). Considering that the *Ednra-lacZ/EGFP*-

positive cell population of the E8.25 heart expresses both *Tbx5* and *Tbx2*, these results raise the possibility that *Edn1/Ednra* signaling might be involved in chamber formation through the regulation of T-box transcription factor gene expression.

Relationship between cell populations expressing *Ednra* at early and late stages in cardiac development

The present study has revealed that *Ednra-lacZ/EGFP*-expressing cells represent a distinct subset of the first heart field and of the inflow region of the heart, contributing to chamber myocardial formation. Identification of this population has revealed novel aspects of early cell behavior contributing to heart morphogenesis. It has also shown an expansion of *Ednra* expression within chamber-forming cardiomyocytes and implicates the *Ednra* signal as a mitotic factor and potential regulator of T-box transcription factor gene expression in early cardiac development. The present experiments, investigating cellular distribution/movement and gene expression profiles, indicate that the early *Ednra*-expressing cells contribute partly to the late *Ednra*-expressing population together with cardiomyocytes, which start to express *Ednra* around E9.5 or later. These findings might provide new insight into the understanding of normal cardiac development, which is relevant to the pathogenesis of congenital heart diseases involving abnormalities of chamber morphogenesis.

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Competing interests statement

The authors declare no competing financial interests.

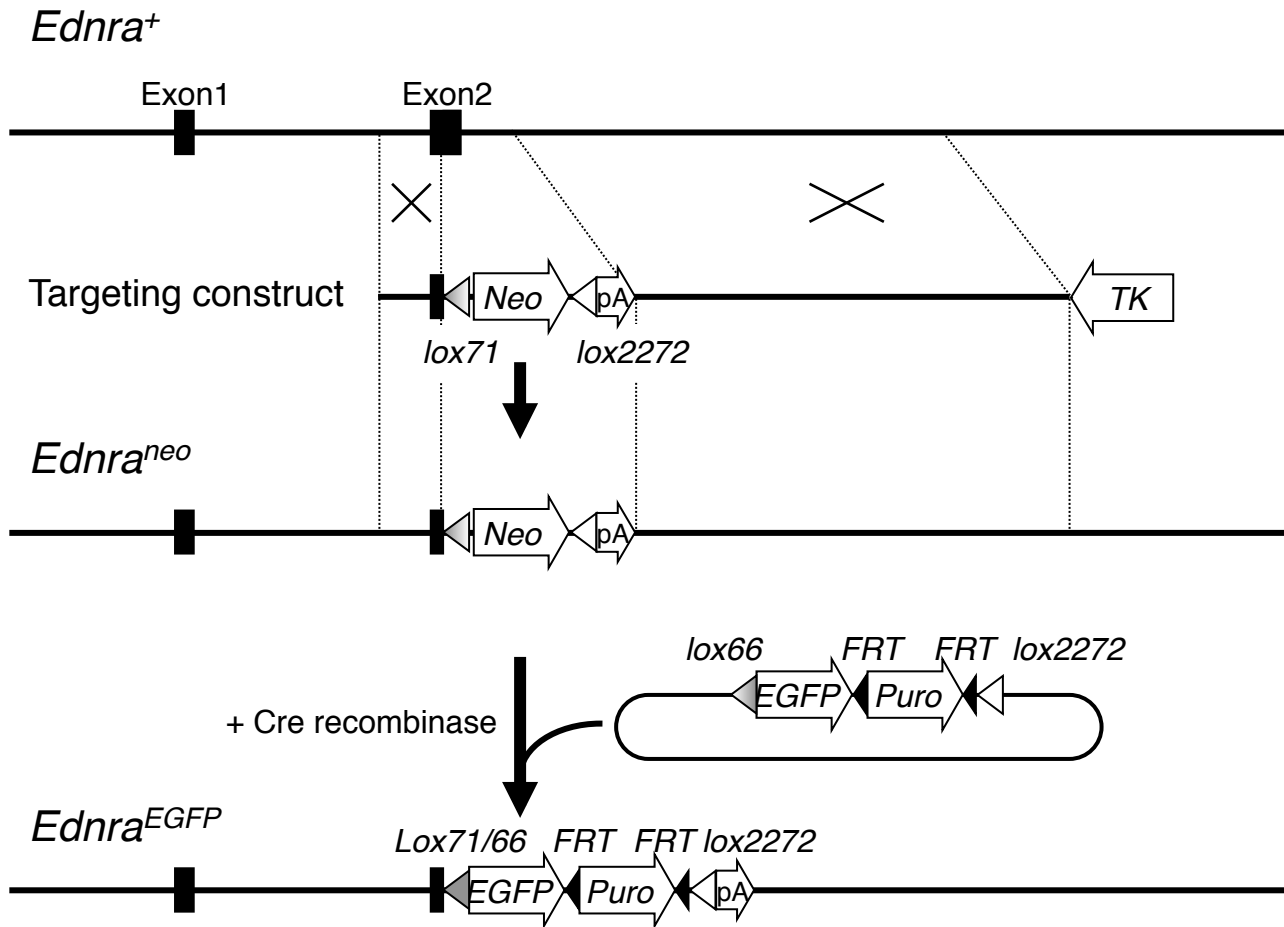
Supplementary material

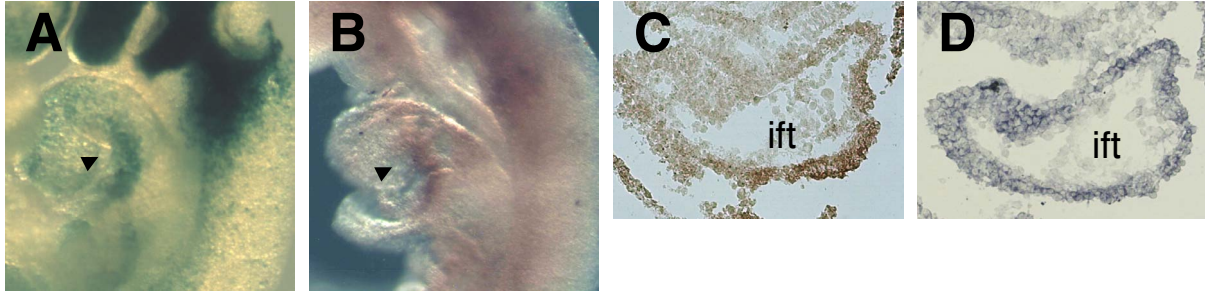
Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.054015/-/DC1>

References

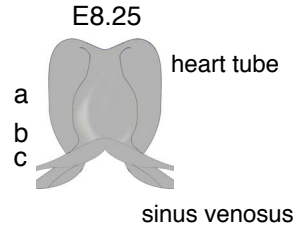
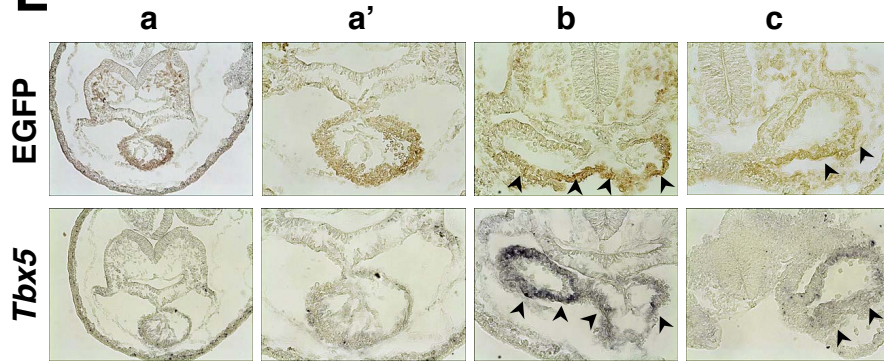
- Aanhaanen, W. T., Brons, J. F., Dominguez, J. N., Rana, M. S., Norden, J., Airik, R., Wakker, V., de Gier-de Vries, C., Brown, N. A., Kispert, A. et al. (2009). The *Tbx2*⁺ primary myocardium of the atrioventricular canal forms the atrioventricular node and the base of the left ventricle. *Circ. Res.* **104**, 1267-1274.
- Abu-Issa, R. and Kirby, M. L. (2007). Heart field: from mesoderm to heart tube. *Annu. Rev. Cell Dev. Biol.* **23**, 45-68.
- Abu-Issa, R. and Kirby, M. L. (2008). Patterning of the heart field in the chick. *Dev. Biol.* **319**, 223-233.
- Araki, K., Araki, M. and Yamamura, K. (2002). Site-directed integration of the cre gene mediated by Cre recombinase using a combination of mutant lox sites. *Nucleic Acids Res.* **30**, e103.
- Bruneau, B. G., Logan, M., Davis, N., Levi, T., Tabin, C. J., Seidman, J. G. and Seidman, C. E. (1999). Chamber-specific cardiac expression of *Tbx5* and heart defects in Holt-Oram syndrome. *Dev. Biol.* **211**, 100-108.
- Bruneau, B. G., Nemer, G., Schmitt, J. P., Charron, F., Robitaille, L., Caron, S., Conner, D. A., Gessler, M., Nemer, M., Seidman, C. E. et al. (2001). A murine model of Holt-Oram syndrome defines roles of the T-box transcription factor *Tbx5* in cardiogenesis and disease. *Cell* **106**, 709-721.
- Buckingham, M., Meilhac, S. and Zaffran, S. (2005). Building the mammalian heart from two sources of myocardial cells. *Nat. Rev. Genet.* **6**, 826-835.
- Cai, C. L., Liang, X., Shi, Y., Chu, P. H., Pfaff, S. L., Chen, J. and 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. et al. (2008). A myocardial lineage derives from *Tbx18* epicardial cells. *Nature* **454**, 104-108.
- Chapman, D. L., Garvey, N., Hancock, S., Alexiou, M., Agulnik, S. I., Gibson-Brown, J. J., Cebra-Thomas, J., Bollag, R. J., Silver, L. M. and Papaioannou, V. E. (1996). Expression of the T-box family genes, *Tbx1-Tbx5*, during early mouse development. *Dev. Dyn.* **206**, 379-390.
- Christoffels, V. M., Habets, P. E., Franco, D., Campione, M., de Jong, F., Lamers, W. H., Bao, Z. Z., Palmer, S., Biben, C., Harvey, R. P. et al. (2000). Chamber formation and morphogenesis in the developing mammalian heart. *Dev. Biol.* **223**, 266-278.
- Cimini, D., Mattiuzzo, M., Torosantucci, L. and Degrossi, F. (2003). Histone hyperacetylation in mitosis prevents sister chromatid separation and produces chromosome segregation defects. *Mol. Biol. Cell* **14**, 3821-3833.
- Clouthier, D. E., Hosoda, K., Richardson, J. A., Williams, S. C., Yanagisawa, H., Kuwaki, T., Kumada, M., Hammer, R. E. and Yanagisawa, M. (1998). Cranial and cardiac neural crest defects in endothelin-A receptor-deficient mice. *Development* **125**, 813-824.
- de la Cruz, M. V., Sanchez-Gomez, C. and Palomino, M. A. (1989). The primitive cardiac regions in the straight tube heart (Stage 9⁺) and their anatomical expression in the mature heart: an experimental study in the chick embryo. *J. Anat.* **165**, 121-131.
- Delorme, B., Dahl, E., Jarry-Guichard, T., Briand, J. P., Willecke, K., Gros, D. and Theveniau-Ruissy, M. (1997). Expression pattern of connexin gene products at the early developmental stages of the mouse cardiovascular system. *Circ. Res.* **81**, 423-437.
- Eckardt, D., Kirchhoff, S., Kim, J. S., Degen, J., Theis, M., Ott, T., Wiesmann, F., Doevendans, P. A., Lamers, W. H., de Bakker, J. M. et al. (2006). Cardiomyocyte-restricted deletion of connexin43 during mouse development. *J. Mol. Cell. Cardiol.* **41**, 963-971.
- Galli, D., Dominguez, J. N., Zaffran, S., Munk, A., Brown, N. A. and Buckingham, M. E. (2008). Atrial myocardium derives from the posterior region of the second heart field, which acquires left-right identity as *Pitx2c* is expressed. *Development* **135**, 1157-1167.
- Georges, R., Nemer, G., Morin, M., Lefebvre, C. and Nemer, M. (2008). Distinct expression and function of alternatively spliced *Tbx5* isoforms in cell growth and differentiation. *Mol. Cell. Biol.* **28**, 4052-4067.
- Goetz, S. C., Brown, D. D. and Conlon, F. L. (2006). *TBX5* is required for embryonic cardiac cell cycle progression. *Development* **133**, 2575-2584.
- Hoogaars, W. M., Barnett, P., Moorman, A. F. and Christoffels, V. M. (2007). T-box factors determine cardiac design. *Cell. Mol. Life Sci.* **64**, 646-660.
- Ishii, Y., Fukuda, K., Saiga, H., Matsushita, S. and Yasugi, S. (1997). Early specification of intestinal epithelium in the chicken embryo: a study on the localization and regulation of *CdxA* expression. *Dev. Growth Differ.* **39**, 643-653.
- Jiang, X., Rowitch, D. H., Soriano, P., McMahon, A. P. and Sucov, H. M. (2000). Fate of the mammalian cardiac neural crest. *Development* **127**, 1607-1616.
- Kanegae, Y., Lee, G., Sato, Y., Tanaka, M., Nakai, M., Sakaki, T., Sugano, S. and Saito, I. (1995). Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific Cre recombinase. *Nucleic Acids Res.* **23**, 3816-3821.
- Kedzierski, R. M., Grayburn, P. A., Kisanuki, Y. Y., Williams, C. S., Hammer, R. E., Richardson, J. A., Schneider, M. D. and Yanagisawa, M. (2003). Cardiomyocyte-specific endothelin A receptor knockout mice have normal cardiac function and an unaltered hypertrophic response to angiotensin II and isoproterenol. *Mol. Cell. Biol.* **23**, 8226-8232.
- Kelly, R. G., Brown, N. A. and Buckingham, M. E. (2001). The arterial pole of the mouse heart forms from *Fgf10*-expressing cells in pharyngeal mesoderm. *Dev. Cell* **1**, 435-440.
- Kirby, M. (2007). *Cardiac Development*. New York: Oxford University Press.
- Koibuchi, N. and Chin, M. T. (2007). *CHF1/Hey2* plays a pivotal role in left ventricular maturation through suppression of ectopic atrial gene expression. *Circ. Res.* **100**, 850-855.
- Kokubo, H., Tomita-Miyagawa, S., Hamada, Y. and Saga, Y. (2007). *Hes1* and *Hes2* regulate atrioventricular boundary formation in the developing heart through the repression of *Tbx2*. *Development* **134**, 747-755.
- Kurihara, Y., Kurihara, H., Suzuki, H., Kodama, T., Maemura, K., Nagai, R., Oda, H., Kuwaki, T., Cao, W. H., Kamada, N. et al. (1994). Elevated blood pressure and craniofacial abnormalities in mice deficient in endothelin-1. *Nature* **368**, 703-710.
- Kurihara, Y., Kurihara, H., Oda, H., Maemura, K., Nagai, R., Ishikawa, T. and Yazaki, Y. (1995). Aortic arch malformations and ventricular septal defect in mice deficient in endothelin-1. *J. Clin. Invest.* **96**, 293-300.
- Laugwitz, K. L., Moretti, A., Caron, L., Nakano, A. and Chien, K. R. (2008). *Isl1* cardiovascular progenitors: a single source for heart lineages? *Development* **135**, 193-205.
- Maemura, K., Kurihara, H., Kurihara, Y., Oda, H., Ishikawa, T., Copeland, N. G., Gilbert, D. J., Jenkins, N. A. and Yazaki, Y. (1996). Sequence analysis, chromosomal location, and developmental expression of the mouse preproendothelin-1 gene. *Genomics* **31**, 177-184.

- Meilhac, S. M., Esner, M., Kelly, R. G., Nicolas, J. F. and Buckingham, M. E. (2004). The clonal origin of myocardial cells in different regions of the embryonic mouse heart. *Dev. Cell* **6**, 685-698.
- Mjaatvedt, C. H., Nakaoka, T., Moreno-Rodriguez, R., Norris, R. A., Kern, M. J., Eisenberg, C. A., Turner, D. and Markwald, R. R. (2001). The outflow tract of the heart is recruited from a novel heart-forming field. *Dev. Biol.* **238**, 97-109.
- Moorman, A. F. and Christoffels, V. M. (2003). Cardiac chamber formation: development, genes, and evolution. *Physiol. Rev.* **83**, 1223-1267.
- Nagy, A., Gertsenstein, M., Vintersten, K. and Behringer, R. (2003). *Manipulating the Mouse Embryo: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Niu, Z., Yu, W., Zhang, S. X., Barron, M., Belaguli, N. S., Schneider, M. D., Parmacek, M., Nordheim, A. and Schwartz, R. J. (2005). Conditional mutagenesis of the murine serum response factor gene blocks cardiogenesis and the transcription of downstream gene targets. *J. Biol. Chem.* **280**, 32531-32538.
- Offermanns, S., Zhao, L. P., Gohla, A., Sarosi, I., Simon, M. I. and Wilkie, T. M. (1998). Embryonic cardiomyocyte hypoplasia and craniofacial defects in G alpha q/G alpha 11-mutant mice. *EMBO J.* **17**, 4304-4312.
- Ozeki, H., Kurihara, Y., Tonami, K., Watatani, S. and Kurihara, H. (2004). Endothelin-1 regulates the dorsoventral branchial arch patterning in mice. *Mech. Dev.* **121**, 387-395.
- Prall, O. W., Menon, M. K., Solloway, M. J., Watanabe, Y., Zaffran, S., Bajolle, F., Biben, C., McBride, J. J., Robertson, B. R., Chaulet, H. et al. (2007). An Nkx2-5/Bmp2/Smad1 negative feedback loop controls heart progenitor specification and proliferation. *Cell* **128**, 947-959.
- Sato, T., Kawamura, Y., Asai, R., Amano, T., Uchijima, Y., Dettlaff-Swiercz, D. A., Offermanns, S., Kurihara, Y. and Kurihara, H. (2008a). Recombinase-mediated cassette exchange reveals the selective use of Gq/G11-dependent and -independent endothelin 1/endothelin type A receptor signaling in pharyngeal arch development. *Development* **135**, 755-765.
- Sato, T., Kurihara, Y., Asai, R., Kawamura, Y., Tonami, K., Uchijima, Y., Heude, E., Ekker, M., Levi, G. and Kurihara, H. (2008b). An endothelin-1 switch specifies maxillomandibular identity. *Proc. Natl. Acad. Sci. USA* **105**, 18806-18811.
- Schaart, G., Viebahn, C., Langmann, W. and Ramaekers, F. (1989). Desmin and titin expression in early postimplantation mouse embryos. *Development* **107**, 585-596.
- Srivastava, D., Cserjesi, P. and Olson, E. N. (1995). A subclass of bHLH proteins required for cardiac morphogenesis. *Science* **270**, 1995-1999.
- Sugden, P. H. (2003). An overview of endothelin signaling in the cardiac myocyte. *J. Mol. Cell. Cardiol.* **35**, 871-886.
- Takeuchi, J. K., Ohgi, M., Koshiba-Takeuchi, K., Shiratori, H., Sakaki, I., Ogura, K., Saijoh, Y. and Ogura, T. (2003). Tbx5 specifies the left/right ventricles and ventricular septum position during cardiogenesis. *Development* **130**, 5953-5964.
- van den Berg, G., Abu-Issa, R., de Boer, B. A., Hutson, M. R., de Boer, P. A., Soufan, A. T., Ruijter, J. M., Kirby, M. L., van den Hoff, M. J. and Moorman, A. F. (2009). A caudal proliferating growth center contributes to both poles of the forming heart tube. *Circ. Res.* **104**, 179-188.
- Waldo, K. L., Kumiski, D. H., Wallis, K. T., Stadt, H. A., Hutson, M. R., Platt, D. H. and Kirby, M. L. (2001). Conotruncal myocardium arises from a secondary heart field. *Development* **128**, 3179-3188.
- Wilkinson, D. (1992). *In Situ Hybridization: a Practical Approach*. Oxford, UK: IRL Press.
- Yamada, M., Revelli, J. P., Eichele, G., Barron, M. and Schwartz, R. J. (2000). Expression of chick Tbx-2, Tbx-3, and Tbx-5 genes during early heart development: evidence for BMP2 induction of Tbx2. *Dev. Biol.* **228**, 95-105.
- Yanagisawa, H., Hammer, R. E., Richardson, J. A., Williams, S. C., Clouthier, D. E. and Yanagisawa, M. (1998). Role of Endothelin-1/Endothelin-A receptor-mediated signaling pathway in the aortic arch patterning in mice. *J. Clin. Invest.* **102**, 22-33.
- Yuan, S. and Schoenwolf, G. C. (2000). Islet-1 marks the early heart rudiments and is asymmetrically expressed during early rotation of the foregut in the chick embryo. *Anat. Rec.* **260**, 204-207.
- Zhou, B., Ma, Q., Rajagopal, S., Wu, S. M., Domian, I., Rivera-Feliciano, J., Jiang, D., von Gise, A., Ikeda, S., Chien, K. R. et al. (2008). Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. *Nature* **454**, 109-113.

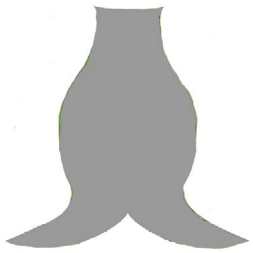




E



Heart tube

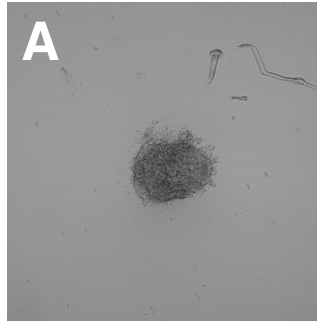


Ventricle

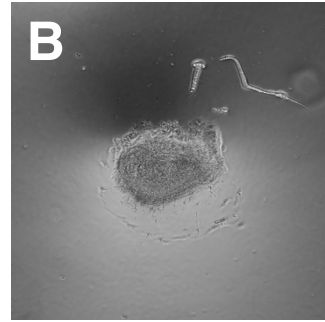


Inflow

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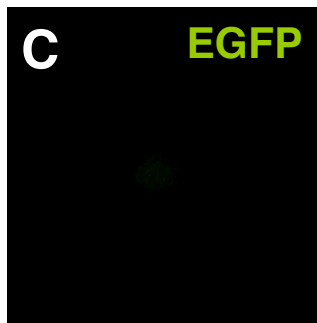


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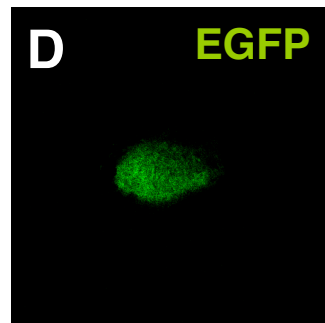
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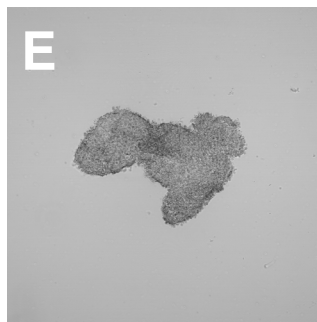


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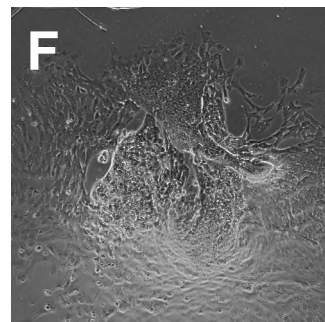
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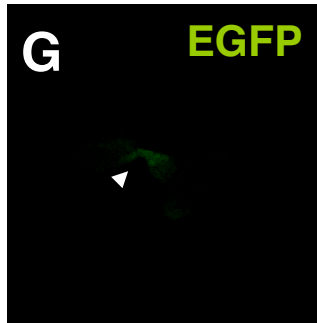


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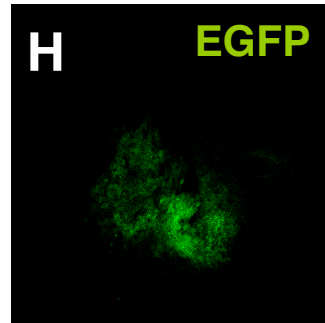
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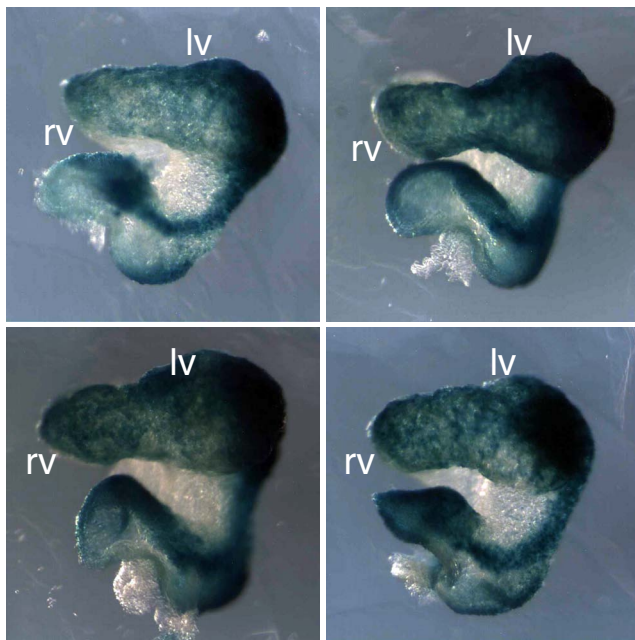


H

EGFP



A *Ednra* *lacZ/+*



B *Ednra* *lacZ/EGFP*

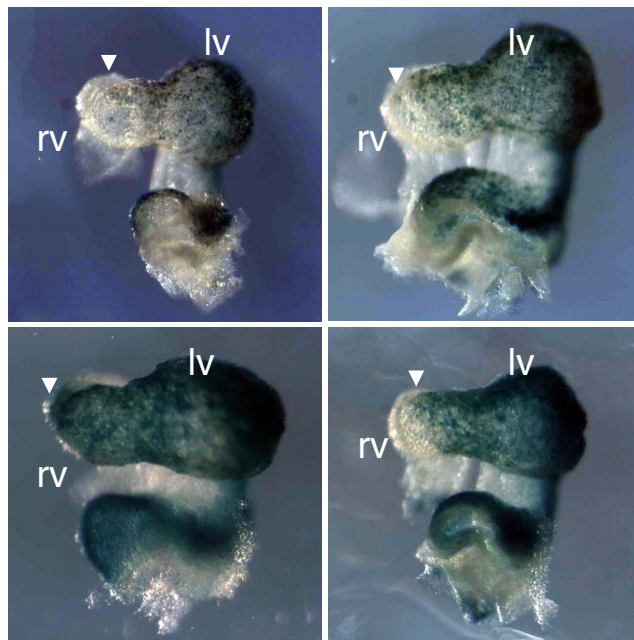


Table S1. Primers and reaction conditions for RT-PCR analysis

Gene	Accession number	Forward (5'-3')	Reverse (5'-3')	Amplicon length (bp)	Annealing temperature (°C)
Conventional RT-PCR					
<i>Cx40</i>	NM_008121	TGGGCAGTTGAACAGCAGCCAG	CCCAGGAAGCTCCAGTCACCCA	119	60
<i>Ednra</i>	NM_010332.2	ACGCTGGCCTTTTCG	CTGAGCAGTTCACACCGGTTCTTATC	603	61
<i>EGFP</i>	U57609.1	GACGTAAACGGCCACAAGTTCA	GAAGTCCAGCAGGACCATGTGATC	608	61
<i>Gapdh</i>	XR_030830.1	GGTGTGAACCACGAGAAATAT	AGATCCACGACGGACACATT	334	61
<i>Hand1</i>	NM_008213.2	GGAGTTGCCTCAGCAGCCCG	CCGTAGCCGCTGCGTCCTTT	200	60
<i>Isl1</i>	NM_021459.3	GCCTGCAGCCGACAGCTCAT	AGGTCCGCAAGGTGTGCAGC	271	60
<i>Nkx2.5</i>	NM_008700.2	CCCAGCCGCCCCACATTTT	GCGCCATCCGTCTCGGCTTT	132	60
<i>Tbx2</i>	NM_009324.2	CGAGATGCCTAAACGCATG	CAAGATGTCATTGGCTCGCAC	190	60
<i>Tbx5</i>	NM_011537.2	ACACATGGCCCAGCATGCCC	CCCGCACTGCCTGACCACAG	138	60
<i>Tbx18</i>	NM_023814.3	GGTATGTTTACCATAGCTCT	AAGGTGAGAGTCCGTAGTGAT	472	60
Real time PCR					
<i>Hand1</i>	NM_008213.2	GGAGTTGCCTCAGCAGCCCG	CCGTAGCCGCTGCGTCCTTT	200	61
<i>HPRT</i>	NM_013556.2	AGCGCAAGTTGAATCTGC	AGCGACAATCTACCAGAG	119	60
<i>Nkx2.5</i>	NM_008700.2	CCCAGCCGCCCCACATTTT	GCGCCATCCGTCTCGGCTTT	132	61
<i>Tbx5</i>	NM_011537.2	ACACATGGCCCAGCATGCCC	CCCGCACTGCCTGACCACAG	138	61