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Efficient cre-mediated deletion in cardiac progenitor cells conferred by a 3'UTR-ires-Cre allele of the homeobox gene *Nkx2-5*

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ABSTRACT Conditional gene targeting and transgenic strategies utilizing Cre recombinase have been successfully applied to the analysis of development in mouse embryos. To create a conditional system applicable to heart progenitor cells, a *Cre recombinase* gene linked at its 5' end to an internal ribosome entry site (IRES) was inserted into the 3' untranslated region of the cardiac homeobox gene *Nkx2-5* using gene targeting. *Nkx2-5^{IRESCre}* mice were fully viable as homozygotes. We evaluated the efficacy of Cre-mediated deletion by crossing *Nkx2-5^{IRESCre}* mice with the Cre-dependent *R26R* and *Z/AP* reporter strains. Efficient deletion was observed in the cardiac crescent and heart tube in both strains. However, the *Z/AP* locus showed transient resistance to deletion in caudal heart progenitors. Such resistance was not evident at the *R26R* locus, suggesting that Cre-mediated deletion in myocardium may be locus-dependent. From cardiac crescent stages, deletion was seen not only in myocardium, but also endocardium, dorsal mesocardium and pericardial mesoderm. The Cre domain apparently includes cells dorsal to the heart that have been shown to constitute a secondary heart field, contributing myocardium to the outflow tract. Other sites of *Nkx2-5* expression, including pharyngeal endoderm and its derivatives, branchial arch epithelium, stomach, spleen, pancreas and liver, also showed efficient deletion. Our data suggest that the *Nkx2-5^{IRESCre}* strain will be useful for genetic dissection of the multiple tiers of lineage allocation to the forming heart as well as of molecular interactions within the heart fields and heart tube.

KEY WORDS: *Nkx2-5, Heart, Cre Recombinase, IRES, Conditional Gene Targeting*

Introduction

The heart is the first organ to form during embryonic development and its function is essential for viability of the conceptus beyond headfold stages (Icardo, 1997). Paired cardiac progenitor cell populations arise in nascent mesoderm during gastrulation and migrate to the anterior and anterior-lateral reaches of the embryo. Here they become specified under the influence of signals from the anterior pharyngeal endoderm (Nascone and Mercola, 1996) and key inductive roles for specific Wnt antagonists and members of the bone morphogenetic protein (BMP) family have been demonstrated (Marvin *et al.*, 2001; Schneider and Mercola, 2001; Schultheiss *et al.*, 1997). Committed myocardial and endocardial progenitors then converge on the ventral midline to form the heart tube (Icardo, 1997), which undergoes further complex morphogenesis and remodelling to create the four-chambered organ (Mjaatvedt *et al.*, 1999). Numerous "extra-cardiac" lineages also contribute to heart form and function (Kirby, 1999; Mikawa, 1999; Poelmann and Gittenberger-de Groot, 1999; Waldo *et al.*, 2001; Webb *et al.*, 1998).

The genetic pathways underlying heart development are now being dissected in different model systems and several transcription factors involved in differentiation and morphogenesis, including members of the homeodomain, GATA, MEF2, T-box and bHLH families, have been identified (Fishman and Olson, 1997). However, the patterning principles that guide heart development and chamber formation are understood in only scant detail. Furthermore, embryos lacking important cardiac regulatory genes often die early from patterning defects in the heart, negating full assessment of gene function at later times or in other organs. Other mutant

Abbreviations used in this paper: CMV, cytomegalovirus; IRES, Internal Ribosome Entry Site; LacZ, β-galactosidase; UTR, untranslated region.

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embryos may precociously die due to defects in non-cardiac tissues, precluding analysis of heart phenotypes. Thus, much is to be gained from the development of more specific and sophisticated genetic reagents.

Use of the bacteriophage P1 Cre recombinase system as a genetic tool has facilitated interrogation of developmental processes in several models (Nagy, 2000). Cre is a member of the integrase family of site-specific recombinases and recognises two inverted 34 bp sequences (*loxP* sites) catalysing strand exchange (Van Duyne, 2001). Using this system, genetic strategies in mice that seek to delete or activate target genes in specific cell lineages and/or in defined temporal windows have been developed (Muller, 1999; Nagy, 2000). For gene deletion, this requires intercrossing one strain of mice carrying the Cre recombinase gene expressed from a specific promoter, with another in which *loxP* sites have been inserted into innocuous positions flanking the target gene of interest. For transgenesis, a Cre-expressing strain is crossed with a strain which carries a silent transgene cassette separated from its promoter region by a foreign sequence flanked by *loxP* sites. Cre-mediated deletion then removes this sequence and activates the transgene. Cre functions efficiently in mammalian cells and many Cre strains have been created (Nagy and Mar, 2001), including examples expressing Cre under control of heart myofilament gene promoters (Hirota *et al.,* 1999; Minamino *et al.,* 2001; Miwa *et al.,* 2000; Sohal *et al.,* 2001).

To facilitate investigation of early cardiogenesis we have expressed Cre recombinase under control of the cardiac homeobox gene *Nkx2-5*, activated in heart progenitors at early crescent stages (Lints *et al.,* 1993). Using gene targeting, an *IRES-Cre* cassette was inserted into the 3' untranslated region of the *Nkx2-5* gene. In contrast to the embryonic lethality associated with *Nkx2-5* null mutations (Biben *et al.,* 2000; Lyons *et al.,* 1995; Tanaka *et al.,* 1999), *Nkx2-5*$^{IRESCre/IRESCre}$ homozygous mice were viable and healthy. Expression from the *Nkx2-5*^{IRESCre} allele was evaluated by crossing to *R26R* and *Z/AP* Cre-dependent reporter strains (Lobe *et al.,* 1999; Soriano, 1999), and efficient activation of reporter genes was observed in cell types known to express *Nkx2-5* and their progeny, including heart progenitors in the cardiac crescent and a population dorsal to the heart tube that may constitute a persisting heart progenitor field. These mice will be useful for deletion or activation of genes at the earliest phases of heart development, and complement existing strains expressing Cre from later times.

Results

Generation of Nkx2-5^{IRESCre} *Mice*

To express Cre recombinase under *Nkx2-5* cis-regulatory control, we devised a gene targeting strategy that generated a bi-cistronic Nkx2-5-Cre mRNA (Fig. 1A). In the targeting vector, a gene cassette (*IRES-Cre*) carrying an internal ribosome entry site linked to the gene encoding a nuclear-localising Cre recombinase was inserted into the 3' untranslated region (utr) of *Nkx2-5.* A hygromycin resistance gene cassette (*pgk-HYGRO-pA*) flanked by yeast flp recombinase target (FRT) sites was inserted downstream of *IRES-Cre*. Correct targeting occurred at a frequency of ~1 in 3 (36/96) hygromycin-resistant ES cell clones. Blastocyst injection of a single correctly targeted clone produced chimaeric animals that passed the modified allele through the germline, generating the strain *Nkx2-5*^{IRESCreHYGRO}. To remove the *pgk-HYGRO-pA* cassette, founders were crossed with transgenic mice expressing the Flp recombinase gene (*FLP1*) in germ cells (Dymecki, 1996). Mice testing positive for both the *FLP1* transgene and *Nkx2-5* modifications were bred to C57BL/6 mice and progeny lacking both *FLP1* and *pgk-HYGRO-pA* were identified. Founders of this new strain (*Nkx2-5*^{IRESCre}) were backcrossed onto C57BL/6 mice. Validation and genotyping of mice were by Southern analysis (Fig. 1B) or PCR (see Materials and Methods). Intercrossing revealed that *Nkx2-5*$^{IRESCre/IRESCre}$ homozygotes were fully viable, healthy and fertile over several generations. Furthermore, from litters produced by crossing heterozygous *Nkx2-5*$^{IRESCre/+}$ mice with heterozygotes for the null *Nkx2-5*^{GFP} allele (Biben *et al.,* 2000), 2/28

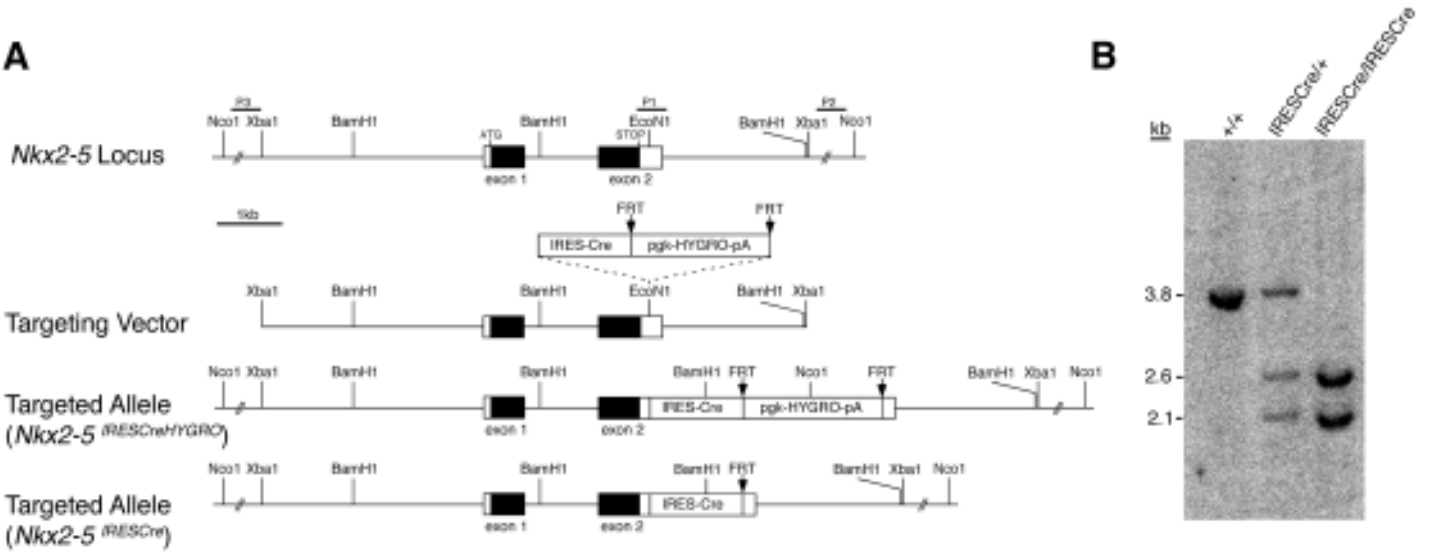

Fig. 1. Gene targeting strategy. (A) *Maps of the* Nkx2-5 *wildtype allele, gene targeting construct and resultant mutant alleles before* (Nkx2-5IRESCreHYGRO) *and after* (Nkx2-5IRESCre) *flp-mediated deletion. The* IRES-Cre *and* pgk-HYGRO-pA *cassettes are discussed in the text and Materials and Methods. P1-P3 refer to probes used in Southern analysis to validate targeted alleles. Note that P1 spans the* Eco*N1 insertion site for the* IRES-Cre *and* pgk-HYGRO-pA *cassettes. Coding exons 1 and 2 of the* Nkx2-5 *gene are boxed and protein coding regions are shaded. Several restriction endonuclease cleavage sites relevant to screening strategies are indicated. FRT indicates a flp recombinase target site.* **(B)** *Southern banding patterns of* Bam*HI-digested DNA from wildtype,* Nkx2-5IRESCre/+ *and* Nkx2-5IRESCre/IRESCre *mice using P1 as probe indicate a correctly targeted locus.*

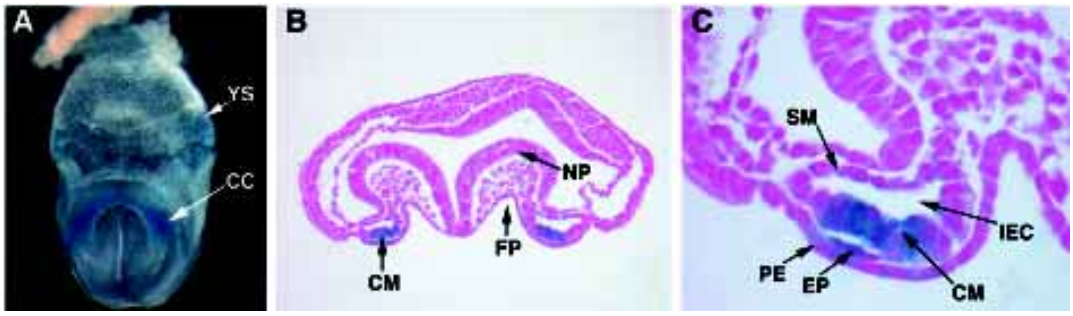

Fig. 2. Nkx2-5IRESCre expression in heart progenitor cells. (A) *E7.75-E8.0 embryo derived from a* Nkx2-5IRESCre/+ *x* R26R *cross stained in wholemount for LacZ. Note LacZ staining in the cardiac crescent and in a punctate pattern in the yolk sac.* **(B)** *Section of embryo shown in panel A midway through the wings of the cardiac crescent.* **(C)** *Higher power view of the cardiogenic region. Note strong expression in cardiogenic mesoderm and endocardial precursors, and weaker expression in somatic mesoderm and pharyngeal endoderm.* ***Abbreviations****: CC, cardiac crescent; CM, cardiogenic mesoderm; EP, endocardial precursors; FP, foregut pocket; IEC, intra-embryonic coelom; NP, neural plate; PE, pharyngeal endoderm; SM, somatic mesoderm; YS, yolk sac.*

Nkx2-5$^{IRESCre/GFP}$ neonates were recovered. While the yield of such heterozygotes appears less than Mendelian, the viability of some individuals and the full viability of *Nkx2-5*$^{IRESCre/IRESCre}$ homozygotes, suggest that the *Nkx2-5*^{IRESCre} allele is not strongly hypomorphic. Nevertheless, in the light of congenital heart defects conferred by heterozygous *Nkx2-5* mutations in both humans and mice (Biben *et al.*, 2000), further characterisation of this allele is clearly warranted.

Efficient Cre-mediated Deletion in Heart Progenitors

ROSA26 reporter (*R26R*) mice carry a Cre-dependent *LacZ* gene and can be used to assess the efficacy of Cre-expressing strains (Soriano, 1999). In embryonic progeny from crosses of *Nkx2-5*^{IRESCre} and *R26R* mice, extensive LacZ staining within the cardiac crescent was evident by E7.75-E8.0 (Fig. 2A). The strongest expression corresponded to the cardiogenic, splanchnic mesoderm (Fig. 2 B,C),

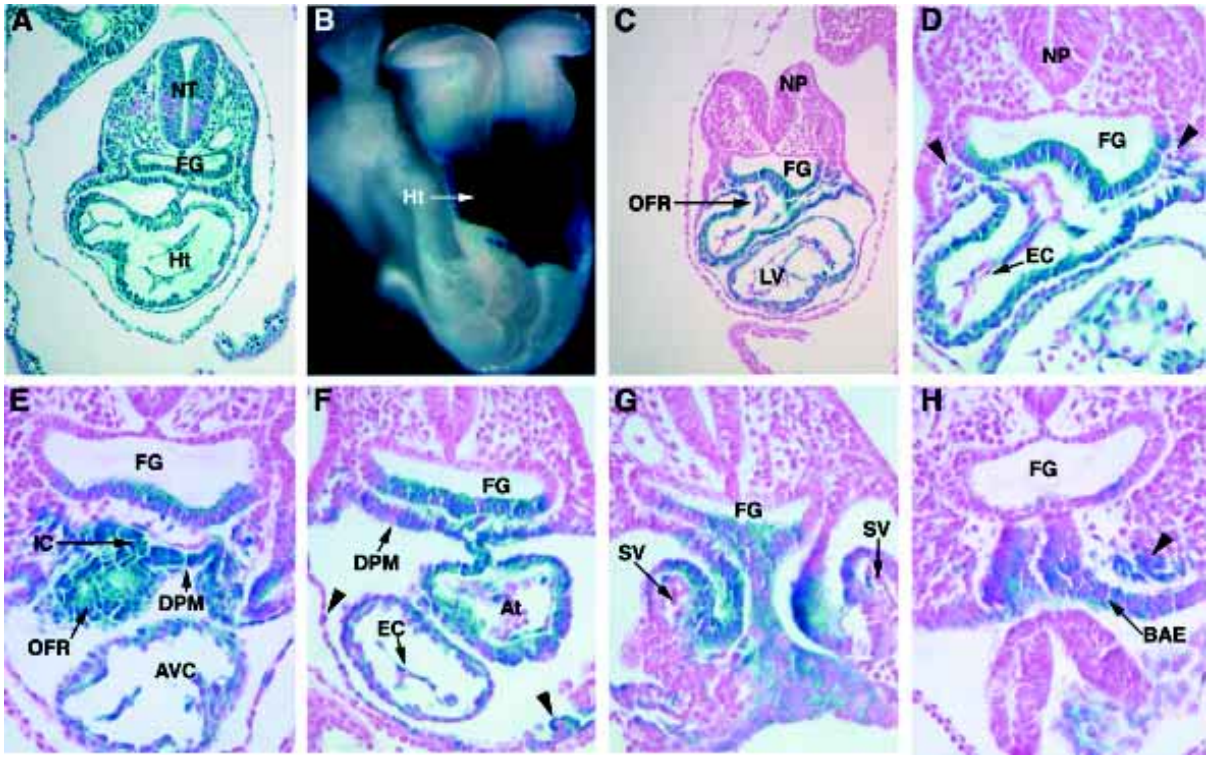

Fig. 3. Nkx2-5IRESCre expression at E8.5. (A) *LacZ-stained section of an E9.0 embryo derived from a cross between* R26R *mice and the* TgN[CMV-Cre]1Cgn *transgenic strain expressing Cre recombinase in the germline (Schwenk* et al., *1995). Note near-ubiquitous LacZ staining in heart and other tissues. (B-H) Wholemount (B) or sections (C-H) of a LacZ-stained E8.5 embryo derived from an* Nkx2-5IRESCre/+ *x* R26R *cross.* **(B)** *Strong LacZ expression throughout the cardiac and oral region.* **(C)** *Staining in the heart and pharyngeal (foregut) floor.* **(D)** *Mosaic staining in endocardial cells of the outflow region extending into the aortic arches and in continuity with clusters of LacZ-positive cells (arrowheads) in the forming first branchial arches.* **(E)** *Staining in the dorsal part (inner curvature) of the outflow region in continuity with cells of the dorsal pericardial mesoderm subjacent to the pharyngeal floor.* **(F)** *Staining in the atrium and dorsal pericardial mesoderm. Arrowheads point to LacZ-positive pericardial mesodermal cells. Pericardial mesoderm at this stage is only a single cell layer.* **(G)** *Staining in dorso-medial cells of the distal horns of the sinus venosus. Note that this section follows the floor of the foregut, which is also positive for LacZ.* **(H)** *Staining in ventro-medial cells of the forming first branchial arches. Arrowhead indicates a cluster of positive cells within arch mesenchyme, which are likely to be endothelial cell precursors.* Abbreviations*: At, atrium; AVC, atrioventricular canal; BAE, branchial arch epithelium; DPM, dorsal pericardial mesoderm; EC, endocardial cells; FG, foregut; Ht, heart; IC, inner curvature; LV, left ventricle; NP, neural plate; OFR, outflow region; SV, sinus venosus horns.*

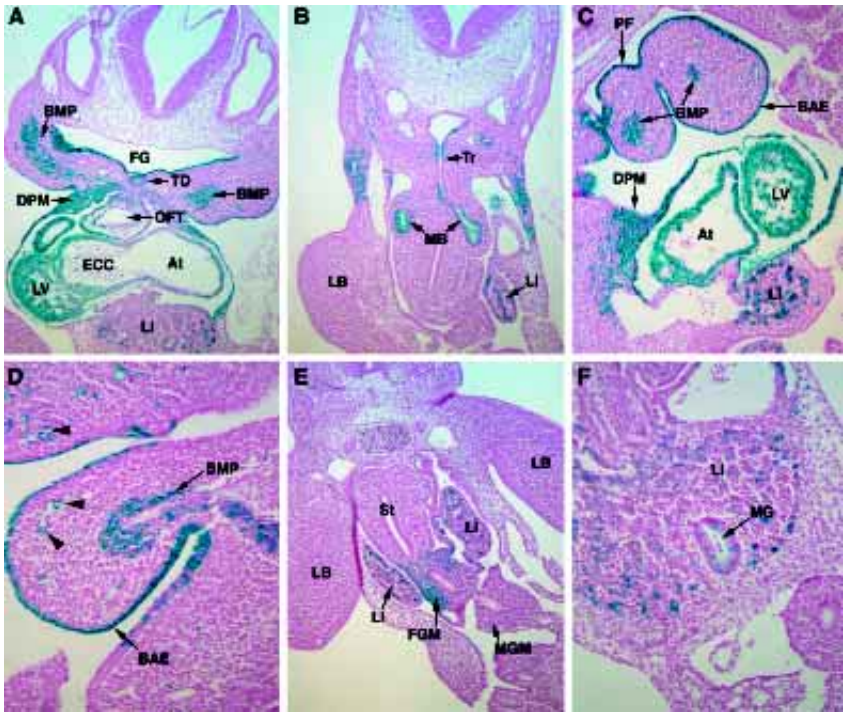

Fig. 4. Nkx2-5IRESCre expression in E10.5 embryos. *Sections shown are from E10.5 embryos derived from a* Nkx2-5IRESCre/+ *x* R26R *cross stained for LacZ in wholemount.* **(A)** *Transverse section showing staining in heart, liver, dorsal pericardial mesoderm and branchial myogenic plates.* **(B)** *Oblique transverse section showing staining in trachea, major bronchi and liver.* **(C)** *Parasagittal section showing staining in heart, dorsal pericardial mesoderm, liver, pharyngeal floor endoderm in continuity with branchial arch epithelium, and branchial myogenic plates.* **(D)** *LacZ expression in endothelial cells lining small vessels of the branchial arches (arrowheads), branchial arch epithelium and branchial myogenic plates surrounding a nerve.* **(E)** *Oblique transverse section showing staining in foregut and midgut mesoderm, and liver.* **(F)** *Staining in liver endoderm and mosaic staining in midgut endodermal epithelium. Abbreviations: At, atrium; BAE, branchial arch epithelium; BMP, branchial myogenic plates; DPM, dorsal pericardial mesoderm; ECC, endocardial cushions; FG, foregut; FGM, foregut mesoderm; LB, limb bud; Li, liver; LV, left ventricle; MB, major bronchi; MG, midgut epithelium; MGM, midgut mesoderm; OFT, outflow tract; PF, pharyngeal floor; St, stomach; TD, thyroid diverticulum; Tr, trachea.*

extending caudally to the transition point between the intra-embryonic coelom and exocoelom. Anteriorly, expression in the cardiogenic plate was weaker but detectable (Fig. 2A and not shown). As expected from the known localisation of *Nkx2-5* transcripts (Lints *et al.,* 1993), expression was detected in future pharyngeal endoderm subjacent to cardiogenic mesoderm. LacZ staining was also detected in the somatic mesodermal layer and its junctional region with splanchnic mesoderm (Fig. 2B), cells that give rise to pericardial mesoderm and dorsal mesocardium, respectively. Endocardial cell precursors located between the cardiogenic mesoderm and subjacent endoderm were also positive (Fig. 2B).

Nkx2-5^{IRESCre} *Expression at E8.5*

To confirm the effectiveness of *R26R* as a Cre reporter in the heart tube and surrounding tissues, we crossed *R26R* mice with transgenic mice expressing Cre recombinase in the germline (Schwenk *et al.,* 1995). Wholemount staining and sectioning of E8.5-E9.0 embryonic progeny showed that β-galactosidase (LacZ) activity was virtually ubiquitous, with only occasional patches of cells remaining unstained (Fig. 3A) (Soriano, 1999). In progeny of crosses between *Nkx2-5^{IRESCre}* and *R26R* mice, LacZ expression at E8.5 was highly specific to the heart region (Fig. 3B). Sections revealed staining throughout the myocardium of the heart, indicating efficient Cre-mediated deletion in this tissue (Fig. 3C). Staining in myocardium was contiguous with that in dorsal mesocardium at the inner curvature and pericardial mesoderm (Fig. 3 C-F), confirming expression in the somatic mesodermal layer at E7.75-E8.0 (Fig. 2 B,C). Peri-pharyngeal cells dorsal to the heart tube in the outflow region have been suggested to contain precursors of the definitive outflow tract (Waldo *et al.,* 2001) and sections showed that this region was also positive for LacZ (Fig. 3E). LacZ staining appeared throughout the common atrium and proximal horns of the sinus venosus of the E8.5 heart (Fig. 3F), although in distal sinus horns staining was evident only in dorso-medial cells (Fig. 3G). Endothelial cells throughout the heart, including those lining the endocardial cushions of the atrioventricular canal and outflow region, were positive although mosaic (Fig. 3 C,D). Endothelial expression in the outflow region extended into the forming first aortic arch arteries (Fig. 3D). This arterial endothelial staining was in turn linked to clusters of positive cells within the mesenchyme of the forming branchial arches (Fig. 3D), suggesting that such clusters are also endothelial precursors. Indeed, both large and small vessels within more mature arches contained endothelial cells staining positive for LacZ (Fig. 4D). Staining was also evident in the pharyngeal floor endoderm (Fig. 3 C-G), and in ventro-medial epithelium of the forming first branchial arch (Fig. 3H).

Evolving Features of Nkx2-5^{IRESCre} *expression at E10.5-E16.5*

LacZ expression was evident in virtually all myocardial and pericardial mesodermal cells at E10.5-E12.5 (Figs. 4A and 5A), although endocardial expression remained mosaic. The LacZ-positive dorsal cell population in continuity with myocardium had expanded to encompass many cell layers (Fig. 4 A,C). These dorsal cells were evident along the full anterior/posterior extent of the heart from the outflow tract (Fig. 4A) to the level of the atrium (Fig. 4C), and at E12.5 included dorsal mediastinal mesenchyme (Webb *et al.,* 1998), a mesenchymal population which lies in association with the atrial septum primum and pulmonary mesoderm (Fig. 5A). The pulmonary vein, which drains into the left atrium, arises within this mesenchyme (Fig. 5B). The atrial septum primum was positive for LacZ throughout (Fig. 5B).

Pharyngeal floor endoderm and its derivatives, including thyroid diverticulum (Fig. 4A), and major bronchi of the lungs (Fig. 4 A,B), were LacZ-positive at E10.5. At E12.5-E16.5, essentially all lung epithelium was stained (Fig. 5E). In contrast, esophagus contained only a few positive cells, consistent with its derivation from the non-staining dorsal region of the foregut (not shown).

In the region of the foregut/midgut junction, both mesoderm and endoderm were positive (Fig. 4 E,F). The positive endodermal population in this region was restricted to the liver diverticulum, pancreatic buds and immediate vicinity. Although LacZ staining in the gut epithelium itself appeared mosaic at E10.5 (Fig. 4F), virtually all of the liver parenchyma at E10.5-E12.5 appeared to be stained (Figs. 4 E,F and 5C). Staining of pancreatic acini at E16.5 was also evident although mosaic (Fig. 5F). The positive mesodermal population in the foregut/midgut junctional region extended from the inferior aspect of the stomach, presumably including the pyloric region known to express *Nkx2-5* (Lints *et al.,* 1993), to the duodenal mesoderm surrounding the liver diverticulum (Fig. 4E). At E16.5, all intestinal mesodermal lineages in the proximal duodenum, including lamina propria, submucosa and smooth muscle layers, showed LacZ staining (Fig. 5D). At E12.5, mesodermal staining associated with the stomach extended into the dorsal mesogastrium to include precursors of the spleen stroma and at least some of the pancreatic stroma (Fig. 5C).

LacZ expression in branchial arch epithelium at E10.5 was contiguous with that in pharyngeal floor endoderm and encompassed much of the surface of the forming arches (Fig. 4 A,C,D), except their most lateral aspects (Fig. 4A). At late foetal stages, epithelial staining was highly restricted to the jaw and neck region (Fig. 5G), and included dividing and differentiating keratinocytes of the skin (Fig. 5H), and potentially their stem cell compartment (Watt, 2001). Epithelial components of vibrissae and teeth were also positive (Fig. 5I). Muscle precursor populations located centrally within the branchial arches also stained for LacZ (Fig. 4 A,C,D), as did their muscle descendants in the head and neck region at later stages (Fig. 5H). LacZ staining in these populations appeared consistently mosaic (Fig. 4D).

Analysis of Cre-mediated Deletion in Heart using Z/AP Reporter Mice

Sensitivity to Cre-mediated deletion has been shown to be locus-dependent (Vooijs *et al.,* 2000). Thus, a single Cre reporter strain may not necessarily represent the benchmark for other loci. We examined recombination within heart progenitors and the heart tube using *Z/AP* reporter mice, which carry a constitutively-expressed *LacZ* transgene flanked by *loxP* sites, linked to a silent human placental alkaline phosphatase (hPAP) gene (*ALPP*) that is activated upon Cre-mediated deletion of *LacZ* (Lobe *et al.,* 1999). Embryos derived from crosses between *Nkx2-5*$^{IRESCre/+}$ and *Z/AP* mice were stained in wholemount for hPAP activity. Strong staining was seen in the cardiac crescent at E7.75-E8.0 (Fig. 6A), in the linear heart tube at E8.25 (Fig. 6B), and in the cardiac region at later stages (Fig. 6C). Sections at E10.5 and E12.5 revealed strong hPAP staining in myocardium and weaker staining in other *Nkx2-*

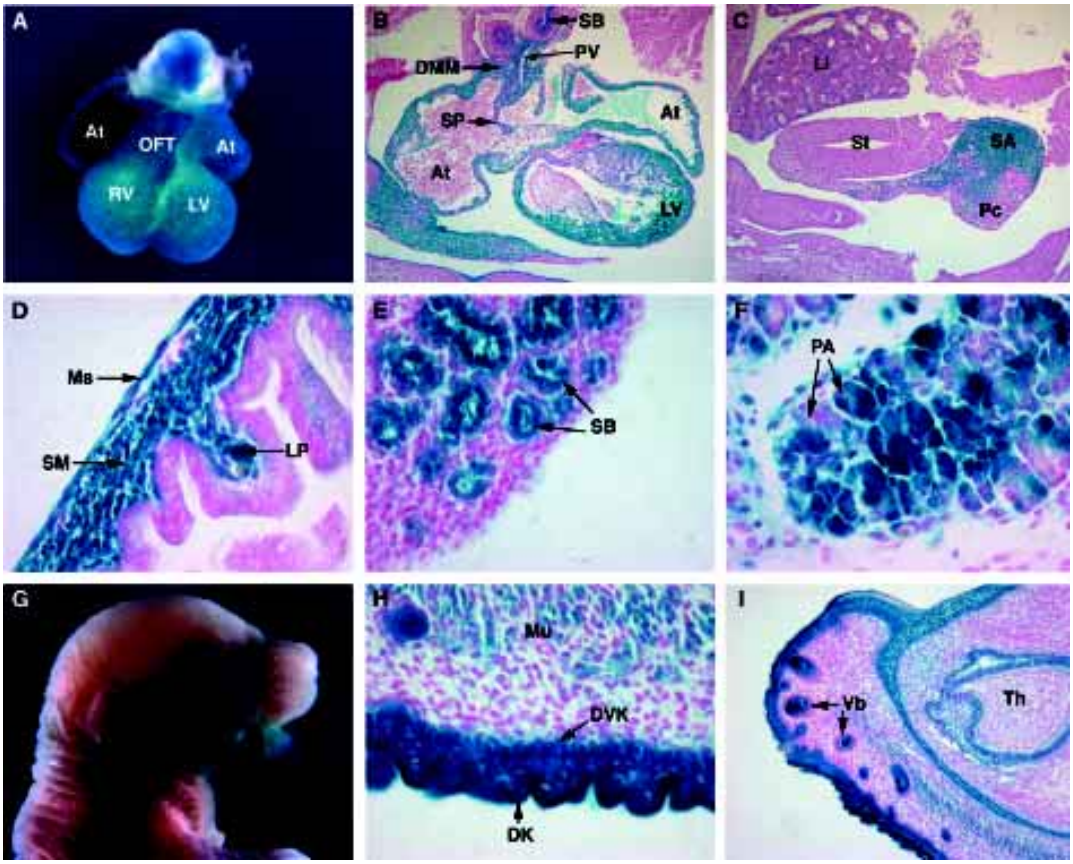

Fig. 5. Nkx2-5IRESCre expression from E12.5. *All embryos shown were derived from* Nkx2.5IRESCre/+ *x* R26R *crosses. LacZ staining is illustrated.* **(A)** *Dissected E12.5 heart showing staining in all chambers.* **(B)** *Transverse section of an E12.5 embryo showing LacZ-positive atrial myocardium and developing atrial septum primum. Note forming pulmonary vein arising within LacZ-positive dorsal mediastinal mesenchyme at the base of the atrial septum in continuity with mesenchyme surrounding the lung bronchi.* **(C)** *Section of E12.5 foregut region showing staining within the dorsal mesogastrium of the stomach embracing the anlage of the spleen and dorsal pancreatic bud.* **(D)** *Section of E16.5 intestine from the anterior duodenum showing staining in all mesodermal lineages.* **(E)** *Section of E16.5 lung showing staining in epithelia of the secondary bronchi.* **(F)** *Section of E16.5 pancreas showing mosaic expression of LacZ in acini.* **(G)** *E16.5 embryo stained in wholemount showing highly-restricted LacZ expression in epidermis in the lower head and neck region.* **(H)** *Sagittal section of an E16.5 embryo showing staining in dividing and differentiating keratinocytes of the skin.* **(I)** *Section of an E16.5 embryo showing staining in the epithelial component of vibrissae and in a tooth anlagen. Abbreviations: At, atrium; DVK, dividing keratinocytes; DK, differentiating keratinocytes; DMM, dorsal mediastinal mesenchyme; Li, liver; LP, lamina propria; LV, left ventricle; Mc, muscularis; Mu, muscle; OFT, outflow tract; PA, pancreatic acini; Pc, dorsal pancreatic bud; PV, pulmonary vein; RV, right ventricle; SB, secondary bronchi of lung buds; SA, spleen anlagen; SM, submucosa; SP, atrial septum primum; St, stomach; Th, tooth anlagen; Vb, vibrissae.*

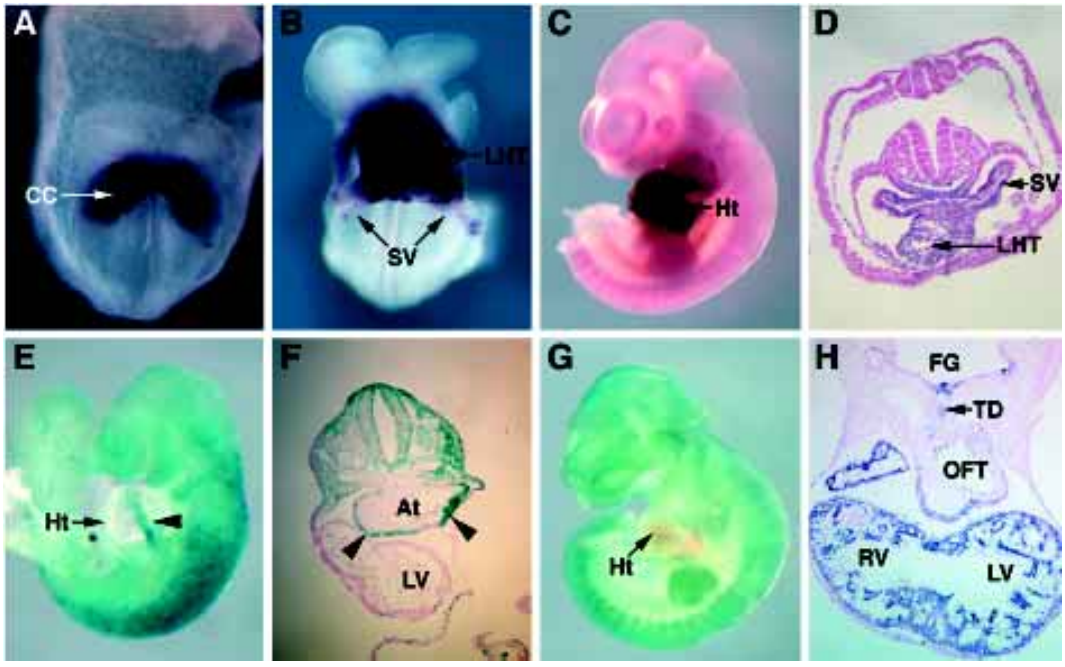

Fig. 6. Analysis of Nkx2-5IRESCre expression using Z/AP reporter mice. *All panels (except D) show embryos resulting from* Nkx2-5IRESCre *x* Z/AP *crosses.* **(A)** *E7.75-E8.0 embryo showing hPAP staining in the cardiac crescent.* **(B)** *E8.0 embryo showing hPAP staining in the linear heart tube. Note that the caudal aspects of the sinoatrial region appear unstained.* **(C)** *E11.5 embryo showing hPAP staining in the heart region.* **(D)** *Section of an E8.0 embryo derived from an* Nkx2-5IRESCre/+ *x* R26R *cross, showing LacZ staining in the linear heart tube and sinoatrium. Note extensive staining in horns of the sinus venosus.* **(E)** *E9.0 embryo showing persistent LacZ staining in a stripe along the left side of the heart.* **(F)** *Section of an E9.0 embryo showing LacZ staining within myocardium of the left and right sides of the common atrium (arrowheads).* **(G)** *E10.0 embryo showing LacZ staining broadly distributed throughout the embryo, but largely absent from the heart.* **(H)** *Transverse section of an E11.5 embryo showing strong alkaline phosphatase staining in the myocardium, and weaker staining in foregut floor endoderm and thyroid diverticulum. Abbreviations: At, atrium; CC, cardiac crescent; FG, foregut; Ht, heart; LHT, linear heart tube; LV, left ventricle; OFT, outflow tract; RV, right ventricle; SV, sinus venosus; TD, thyroid diverticulum.*

5-positive tissues, as described above for crosses with *R26R* mice (Fig. 6H). The differential hPAP staining intensity presumably reflects the variable tissue activity of the cytomegalovirus (CMV) promoter, which drives hPAP expression in *Z/AP* mice. Indeed, a similarly differential pattern was observed using LacZ as the CMV readout in undeleted mice.

In *Z/AP* crosses, we noted that hPAP staining within the wings of the cardiac crescent at E7.75-E8.0 and in the sinoatrial region of the linear heart tube at E8.25 (Fig. 6 A,B) did not appear to extend as far caudally when compared to equivalent stage embryos processed for detection of *Nkx2-5* transcripts by *in situ* hybridization (Lints *et al.,* 1993) or of Cre-activated LacZ activity in the *R26R* strain (Fig. 6D). We stained E8.5-E9.0 embryos from *Nkx2-5*$^{IRESCre/+}$ x *Z/AP* crosses for LacZ activity, expressed from the undeleted *Z/AP* allele, and a prominent stripe of expression was consistently evident in the heart along the left sinoatrial wall extending into the atrioventricular canal and ventricle (Fig. 6E). Sections confirmed LacZ expression in myocardium of both the left and right sides of the common atrium, and left side of the atrioventricular canal and ventricle, as well as complete absence of expression from other regions of the heart (Fig. 6F). The effect was much reduced or absent in hearts at E10.0 (Fig. 6G). Thus, while Cre-mediated deletion of the *Z/AP* allele is generally efficient in myocardium, certain caudal cells are transiently resistant to deletion.

Cre-mediated Deletion in Yolk Sac Endothelial Cells

One extra-embryonic site of Cre expression was detected. In E7.5 embryos derived from crosses of *Nkx2-5*$^{IRESCre/+}$ and *R26R* mice, there were a few LacZ-positive cells in the yolk sac as well as the cardiac crescent (not shown). Yolk sac staining was more extensive at E7.75-E8.0 (Fig. 2A) and widespread thereafter (Fig. 7 A,B), although the fraction of yolk sac cells showing LacZ positivity varied considerably between individual embryos. Sec-

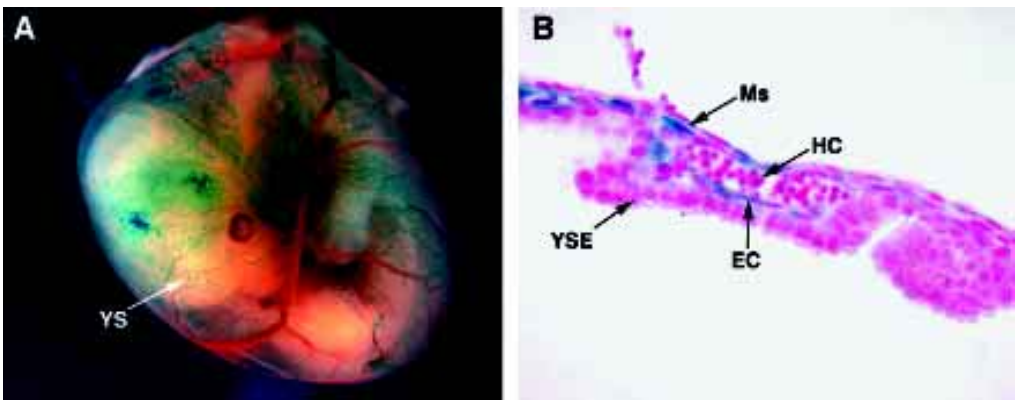

Fig. 7. Nkx2-5IRESCre expression in yolk sac mesoderm. **(A)** *Wholemount LacZ staining of E12.5 embryo derived from a* Nkx2-5IRESCre/+ *x* R26R *cross. Note widespread LacZ staining in yolk sac vasculature.* **(B)** *Section of an E12.5 yolk sac showing LacZ expression predominantly in vascular endothelial and hematopoietic cells. Hematopoietic cells are weakly stained at this stage due to their small cytoplasmic volume. Abbreviations: EC, endothelial cells; HC, hematopoietic cells; Ms, mesothelium; YS, yolk sac; YSE, yolk sac endoderm.*

tions of E12.5 embryos showed staining predominantly in both endothelial and hematopoietic cells (Fig. 7B).

Discussion

We have created a mouse strain (*Nkx2-5^{IRESCre}*) expressing Cre recombinase in heart progenitors. An *IRES-Cre* cassette was inserted into the 3' untranslated region of the homeobox gene *Nkx2-5*, bringing Cre expression directly under *Nkx2-5* cis-regulatory control via production of an Nkx2-5-Cre bicistronic mRNA. Expression from the *Nkx2-5^{IRESCre}* allele was evaluated by crossing Cre mice to the Cre-dependent *R26R* and *Z/AP* reporter strains. As well as detecting persistent sites of Cre expression, reporter strains of this nature provide a permanent lineage record of sites having transiently expressed Cre at earlier times. In *Nkx2-5$^{IRESCre/+}$* x *R26R* crosses, efficient Cre-mediated deletion was evident in the cardiac crescent from around E7.75 and in the heart tube thereafter. Although *Nkx2-5* transcripts have been detected only in the myocardium of the heart (Lints *et al.,* 1993), Cre-mediated LacZ expression from E7.75-E8.0 was seen in both myocardial and endocardial cells. This suggests that Cre is transiently expressed in early endocardial cells or their precursors, and is consistent with the notion that at least a subset of those cells derive from a common myocardial/endocardial precursor (Linask and Lash, 1993).

Expression in the endocardium was mosaic. This may be because only a proportion of cells in this population expressed Cre at the threshold level necessary for Cre-mediated deletion. However, it may also relate to the apparent heterogeneous origins of endocardial cells during development. In early chick and quail hearts, endocardium is heterogeneous for the marker JB3 (fibrillin-2), and only JB3$^+$ cells undergo epithelial-mesenchymal transformation during endocardial cushion formation (Mjaatvedt *et al.,* 1999). It has been suggested that JB3$^+$ cells derive from the cardiogenic mesoderm, while a class of JB3$^-$ endothelial cells arising within non-cardiac tissues migrates into the heart precursor region. In the light of these studies it will be interesting to map the evolving spatial distribution of LacZ-staining endocardial cells in *Nkx2-5$^{IRESCre/+}$* x *R26R* embryos. However, our initial observations did not suggest a clear segregation of stained and unstained cells. In the outflow region of E8.5 hearts, LacZ-positive endocardial cells were contiguous with similarly positive endothelial cells within the first pair of branchial arch arteries, and these cells were in turn contiguous with clusters of positive cells within branchial arch mesenchyme. These latter cells appear to be precursors of endothelial cells of the branchial arch vascular plexus.

Cre-mediated LacZ activation at E7.75-E8.0 was also seen in the somatic mesodermal layer and its junctional region with splanchnic mesoderm, which give rise to pericardial mesoderm and the dorsal mesocardium, respectively. This is consistent with previous data showing localisation of *Nkx2-5* transcripts to cardiac crescent mesoderm prior to intra-embryonic coelom formation and separation of splanchnic and somatic layers (Harvey *et al.,* 1999), and to dorsal pericardial mesoderm and dorsal mesocardium at later stages (Waldo *et al.,* 2001). An important aspect of this pattern relates to the heart progenitor fields. In *Xenopus*, cells destined to form the dorsal mesocardium and pericardium are part of the primary heart field and display regulative behaviour if definitive heart progenitors are removed (Raffin *et al.,* 2000). In the chick, these cells lie outside of what is regarded as the primary heart field, but are recruited to form the definitive outflow tract myocardium after a primary heart tube has been created (Waldo *et al.,* 2001). As such, they are regarded as constituting a secondary heart field. The dorsal LacZ-positive cells observed in our mice included this secondary heart field. Furthermore, they were contiguous along the dorsal aspect of the heart tube with mesodermal cells (dorsal mediastinal mesenchyme) that lie in contact with the inflow region of the heart and which are known to be drawn into the atria during their development (Webb *et al.,* 1998).

In addition to the cardiac region, a number of other sites of LacZ expression were evident, virtually all of which could be anticipated from previous studies on *Nkx2-5* expression. For example, pharyngeal floor endoderm, thyroid diverticulum, spleen and the pyloric region of the stomach are all known sites of *Nkx2-5* expression during organogenesis (Lints *et al.,* 1993). Similarly, LacZ expression in liver parenchyma and branchial myogenic plates from E10.5 correlates with detection of Nkx2-5 protein in liver, and head and neck muscles at E15.5 (Kasahara *et al.,* 1998). Expression in lung epithelium presumably reflects transient expression in its precursors located within foregut endoderm at earlier times. Likewise, mosaic expression in pancreatic acini is likely to arise because transient expression around the liver diverticulum partially overlaps with the precursor domains of the dorsal and ventral pancreatic buds. Expression in proximal duodenal mesoderm originates within the transient zone of expression on each side of the foregut/midgut junction.

A single novel site of Cre expression was detected, that in endothelial and hematopoietic cells within yolk sac mesoderm from E7.5-E8.0. This expression likely reflects transient expression of *Nkx2-5* within the common precursor of these cells, the hemangioblast, or indeed within its progenitors arising in posterior mesoderm at gastrulation. While *Nkx2-5* expression in yolk sac mesoderm has not been reported previously, the LacZ staining correlates with the yolk sac vascular defects seen in *Nkx2-5* knockout embryos (Tanaka *et al.,* 1999).

The utility of Cre strains for conditional gene targeting and ectopic transgene expression rests on the efficacy of Cre-mediated deletion in tissues of interest. At the *R26R* locus, the *Nkx2-5^{IRESCre}* allele conferred highly efficient Cre-mediated deletion within heart precursors and myocardium, pharyngeal floor endoderm and its derivatives, branchial arch epithelium, spleen, caudal stomach, liver and proximal intestinal mesoderm. LacZ expression at other sites, including branchial myogenic plates and pancreas, appeared mosaic, limiting the usefulness of this Cre allele for genetic manipulations in these tissues, particularly when applied to the deletion of genes with cell non-autonomous effects.

A further important complexity in the Cre/*loxP* system is the apparent resistance of some loci to Cre-mediated deletion (Vooijs *et al.,* 2000). In crosses with *Z/AP* reporter mice, we detected transient delay in Cre-mediated deletion within the caudal aspect of the linear heart tube and in the sinoatrial region of the looping heart. A strong stripe of LacZ expression was seen on the left side of the atrioventricular canal extending into the left ventricle. We can surmise from our crosses with *R26R* mice that active Cre recombinase is expressed in those cells. Furthermore, since the *CMV-LacZ* reporter gene is clearly functional, it is unlikely that the *Z/AP* locus is heterochromatic within the cells in question. It is possible therefore that the resistant cells represent a population of myocytes that transiently occupy a state in which Cre-mediated

deletion at the *Z/AP* locus is relatively more resistant. However, differences between myocytes based on the levels of Cre expression or on the stabilities of reporter proteins cannot be discounted at present.

Our data demonstrate Cre-mediated deletion in both myocardial and endocardial cells, and in both primary and secondary heart fields. Unlike strains in which Cre is expressed from myofilament gene promoters, *Nkx2-5^{IRESCre}* mice should be useful for genetic dissection of the multiple tiers of lineage allocation to the forming heart and molecular interactions within the heart fields and heart tube. The viability of *Nkx2-5$^{IRESCre/IRESCre}$* homozygotes has the additional benefit of allowing introduction of two Cre alleles into experimental crosses. Furthermore, the fact that some *Nkx2-5$^{IRESCre/GFP}$* mice are viable suggests that the *Nkx2-5^{IRESCre}* allele is not strongly hypomorphic, an important issue if this strain is to be used for deletion of genes that might interact with the *Nkx2-5* pathway.

Materials and Methods

Gene Targeting

Nkx2-5^{IRESCreHYGRO} and *Nkx2-5^{IRESCre}* mice (*Mus musculus*) were generated by gene targeting using W9.5 ES cells (129SvJ strain) and standard methods (Barnett and Koentgen, 2001). The targeting vector spanned an 8.5 kb *Xba*I fragment of the *Nkx2-5* gene and a cassette (*IRES-Cre*) comprising a picornavirus internal ribosome entry site (Wu *et al.,* 1994) linked to the 5' end of a Cre recombinase gene (Gu *et al.,* 1993) was inserted into an *Eco*NI site within the 3'utr in coding exon 2 (Fig. 1A). A hygromycin resistance gene cassette (*pgk-HYGRO-pA*) flanked by flp recombinase target (FRT) sites (Kilby *et al.,* 1993) and carrying phosphoglycerokinase gene promoter (*pgk*) and polyadenylation (*pA*) sequences (Stanley *et al.,* 2000) was inserted downstream of *IRES-Cre*. To remove the *pgk-HYGRO-pA* cassette, founders were crossed with transgenic mice (*B6;SJL-TgN(ACTFLPe)9205Dym*) expressing Flp recombinase (*FLP1*) in the germline (Dymecki, 1996). *FLP1* mice were originally established on a [C57BL/6 x SJL]F2 background and have since been backcrossed onto C57BL/6 mice for several generations. *Nkx2-5$^{IRESCre/+}$* used in this study had been backcrossed to C57BL/6 mice for 3-6 generations. Transgenic mice expressing Cre recombinase in the germline were of the strain *TgN[CMV-Cre]1Cgn* (Schwenk *et al.,* 1995). Genotyping of mice was performed on tail DNA by Southern analysis (Fig. 1B) and/or PCR. Southern blotting of *Nco*I or *Bam*HI-digested DNA was as described (Church and Gilbert, 1984) using probes P1-P3 indicated in Figure 1A. P1 was a 463 bp PCR-generated probe corresponding to nucleotides 1120-1583 of the *Nkx2-5* cDNA (Genbank 493581) (Lints *et al.,* 1993). P2 was a 669 bp *Sac*I/*Kpn*I genomic fragment from plasmid pgMlx^{129}-B2(383). P3 was a 250 bp *Bgl*II/*Xba*I genomic fragment from the plasmid pgNkx2-5X/X-2(999). PCR was performed using the following conditions: 30 cycles of 94°C, 30 secs; 60°C, 20 secs; 72°C, 60 secs; in the presence of 250μM dNTPs and 1unit of Taq polymerase in a 20μl reaction buffer as specified by the manufacturer (Roche). Oligonucleotides pairs were: *Nkx2-5* antisense (5'-ACG CAC TCA CTT TAA TGG GAA GAG-3') with either Cre-specific (5'-GAT GAC TCT GGT CAG AGA TAC CTG-3') or *Nkx2-5* sense (5'-GCC CTG TCC CTC GGA TTT CAC ACC-3'), generating products of 583 bp and 264 bp, respectively. The Institute for Laboratory Animal Research (http://www4.nas.edu/cls/ilarhome.nsf) designator for the *Nkx2-5^{IRESCre}* line is *Nkx2-5^{tm2Rph}*.

LacZ and Alkaline Phosphatase Assays

Expression from the *Nkx2-5^{IRESCre}* allele was monitored by crossing *Nkx2-5$^{IRESCre/+}$* or *Nkx2-5$^{IRESCre/IRESCre}$* mice to heterozygous mice of the ROSA26 reporter (R26R; *Gtrosa26^{tm1Sor}*) (Soriano, 1999) and the *Z/AP* reporter strains (*Tg(ACT*β-β*geo/ALPP)1Lbe*) (Lobe *et al.,* 1999). LacZ and alkaline phosphatase activities were assayed on wholemount embryos or on tissue cryostat sections as previously reported (Lobe *et al.,* 1999).

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