Tie2-Cre Transgenic Mice: A New Model for Endothelial Cell-Lineage Analysis *in Vivo*

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Endocardial cells are thought to contribute at least in part to the formation of the endocardial cushion mesenchyme. Here, we created *Tie2-Cre* transgenic mice, in which expression of Cre recombinase is driven by an endothelial-specific promoter/enhancer. To analyze the lineage of *Cre* expressing cells, we used *CAG-CAT-Z* transgenic mice, in which expression of *lacZ* is activated only after Cre-mediated recombination. We detected pan-endothelial expression of the *Cre* transgene in *Tie2-Cre;CAG-CAT-Z* double-transgenic mice. This expression pattern is almost identical to *Tie2-lacZ* transgenic mice. However, interestingly, we observed strong and uniform *lacZ* expression in mesenchymal cells of the atrioventricular canal of *Tie2-Cre;CAG-CAT-Z* double-transgenic mice. We also detected *lacZ* expression in the mesenchymal cells in part of the proximal cardiac outflow tract, but not in the mesenchymal cells of the distal outflow tract and branchial arch arteries. LacZ staining in *Tie2-Cre;CAG-CAT-Z* embryos is consistent with endocardial–mesenchymal transformation in the atrioventricular canal and outflow tract regions. Our observations are consistent with previously reported results from *Cx43-lacZ, Wnt1-Cre;R26R,* and *Pax3-Cre;R26R* transgenic mice, in which *lacZ* expression in the cardiac outflow tract identified contributions in part from the cardiac neural crest. *Tie2-Cre* transgenic mice are a new genetic tool for the analyses of endothelial cell-lineage and endothelial cell–specific gene targeting. © 2001 Academic Press

Key Words: endocardial cushion; atrioventricular canal; cardiac outflow tract; *Cre-loxP.*

INTRODUCTION

Formation of the endocardial cushion involves endocardial–mesenchymal transformation as well as contributions from the cardiac neural crest. The primary source of mesenchyme in the atrioventricular (AV) canal in the mouse and chick embryo is thought to be endocardial cells (Bolender and Markwald, 1979; Markwald *et al.,* 1979, 1977). In the mouse, at embryonic day (E) 9–9.5, the heart consists of two epithelial layers, the endocardium and the myocardium, which are separated by an acellular matrix (the cardiac jelly). Between E9.5 and E10.0, a subset of cardiac endothelial cells in the AV canal and outflow tract (OT) transforms from an epithelial to a mesenchymal morphology, migrates into the previously acellular cardiac jelly, and participates in the formation of the valves and

membranous septa (Kaufman, 1992; Runyan and Markwald, 1983). Neural crest cells do not appear to make a significant contribution to the AV canal, at least in the chick, based on studies which indicate that (1) neural crest cells have not yet migrated into the heart when the mesenchyme of the AV canal is developed (Noden *et al.,* 1995), and (2) AV septal defects are rarely seen after ablation of the premigratory cardiac neural crest (Nishibatake *et al.,* 1987). In contrast to mesenchyme in the AV canal, the origins of the mesenchyme in the OT are more controversial. Based on the experiments using chick-quail chimera embryos, the current belief is that two kinds of mesenchyme, cardiac neural crest-derived mesenchyme and non-neural crest-derived mesenchyme, play a role in outflow septation and remodeling. When the mesenchyme in the OT regions initially develops, nonneural crest cells are the primary source of the
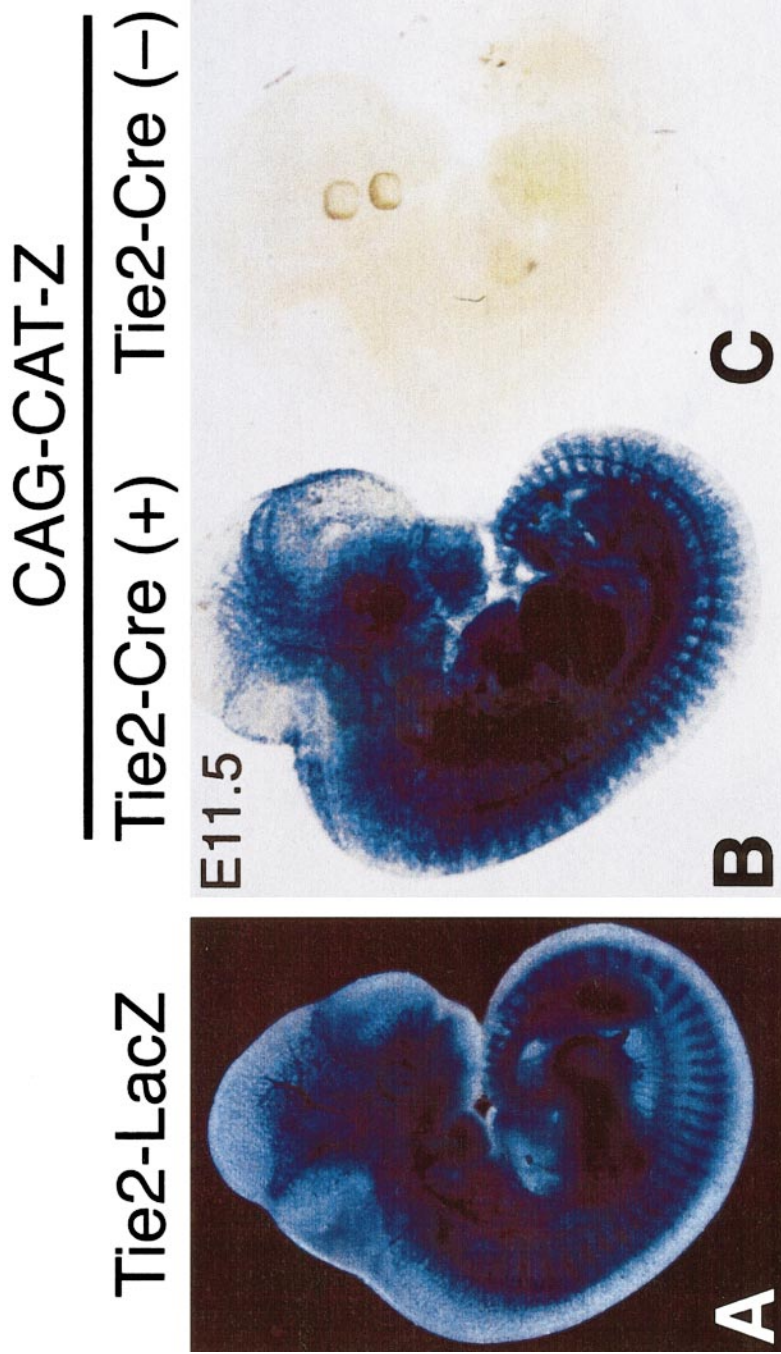
FIG. 1. Whole-mount X-Gal staining of Tie2-LacZ embryos and CAG-CAT-Z embryos, with or without Tie2-Cre transgene. (A) Lateral view of whole-mount stained E11.5 embryo of Tie2-lacZ transgenic mouse as a reference. Tissue-specific expression of lacZ is uniformly observed in virtually all blood vessels. (B, C) Lateral view of E11.5 littermates from a CAG-CAT-Z × Tie2-Cre cross. An embryo positive for both transgenes (B) shows a staining pattern identical to the Tie2-lacZ embryo. An embryo positive for CAG-CAT-Z but negative for Cre transgene (C) shows no staining.
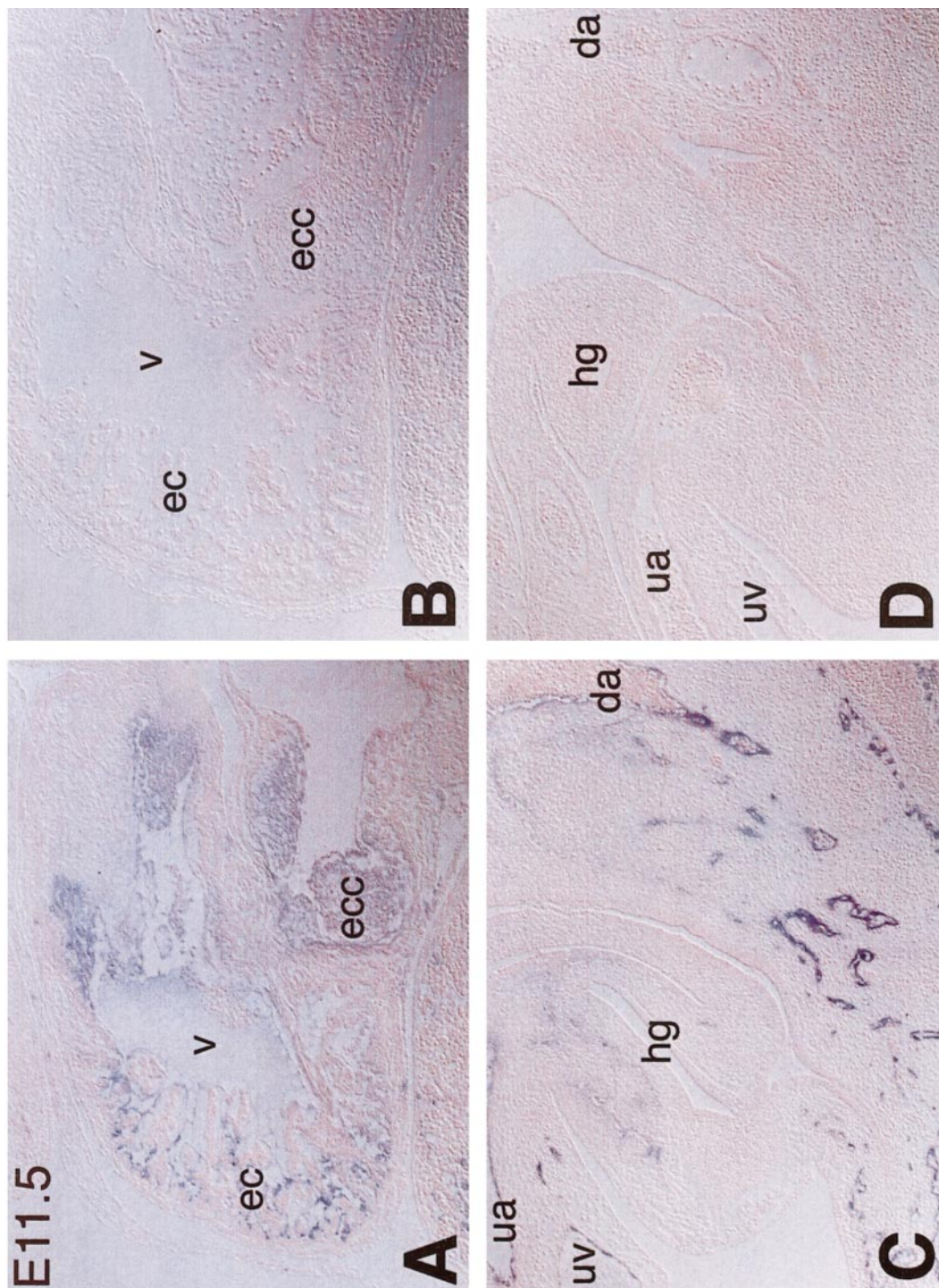
FIG. 2. Parasagittal sections of X-Gal stained CAG-CAT-Z embryos, with or without Tie2-Cre transgene. In E11.5 embryo positive for both transgenes (A, C), expression of lacZ is observed specifically in endocardium and endocardial cushion (A). Expression of lacZ is also detected in the endothelium of all small blood vessels as well as dorsal aorta and umbilical vessels (C). No ectopic expression outside the endothelial layer is detected. An embryo positive for CAG-CAT-Z but negative for Tie2-Cre (B, D) shows no staining. V, ventricle; ec, endocardium; ecc, endocardial cushion; ua, umbilical artery; uv, umbilical vein; hg, hindgut; da, dorsal aorta.

mesenchyme. In later stage embryos, in which the aortico-pulmonary (AP) septation complex starts to develop, cardiac neural crest cells are the primary component of the mesenchyme in the septation complex. However, significant regions in the conotruncal ridges are composed of nonneural crest-derived mesenchyme (Waldo *et al.,* 1998, 1999). The source of the nonneural crest-derived mesenchyme may be endocardial cells.

Study of the avian system has an advantage over the mammalian system because of the accessibility of developing embryos as well as availability of lineage-specific probes (Coffin and Poole, 1988; Crossin *et al.,* 1986; Pardanaud *et al.,* 1987; Wunsch *et al.,* 1994) and chick-quail chimera (Le Douarin, 1982). Although in vitro assays using mouse endocardial cushion explants can reveal molecular pathways involved in endocardial–mesenchymal transformation (Nakajima *et al.,* 1997; Yamamura *et al.,* 1997), it is essential to investigate the formation of the endocardial cushion in vivo. For this purpose, it is highly useful to establish mouse models in which we can dissect molecular mechanisms underlying lineage establishment with the aid of lineage-specific promoters/enhancers.

The *Cre/loxP* system can be used for lineage analyses in the mouse. Cre is the bacteriophage P1-derived recombinase which efficiently excises DNA flanked by two directly repeated *loxP* recognition sites (Sauer and Henderson, 1988; Sternberg and Hamilton, 1981). Temporal or spatial regulation of Cre-mediated recombination can be achieved by the use of tissue-specific and developmental stage-specific promoters, ligand-inducible promoters, or ligand-dependent Cre fusion proteins (Feil *et al.,* 1996; Gu *et al.,* 1994; Kühn *et al.,* 1995; Kulkarni *et al.,* 1999). Mice carrying reporter transgenes (e.g., *lacZ*) whose expression is controlled by a tissue-specific promoter will show the "current" expression of the transgene. In contrast, *Cre*-transgenic mice can be used to analyze the lineage history of the transgene expression when they also carry Cre-dependent reporter transgenes whose expression is activated only after Cre-mediated recombination (Araki *et al.,* 1995; Soriano, 1999).

Promoter and enhancer regions of the mouse *Tie2* gene drive transgene expression specifically in endothelial cells (Schlaeger *et al.,* 1997). The *Tie2* gene encodes an angiopoietin receptor, a member of the receptor tyrosine kinase family (Davis *et al.,* 1996; Sato *et al.,* 1993). *Tie2* expression is detected as the first endothelial cells arise, remains positive in endothelial cells throughout development, and is detectable in virtually all endothelial cells of adult tissues (Sato *et al.,* 1993; Schnürch and Risau, 1993; Wong *et al.,* 1997). *Tie2-lacZ* transgenic mice demonstrate a pan-endothelial-specific pattern of lacZ staining throughout embryogenesis and adulthood (Schlaeger *et al.,* 1997). Since this promoter/enhancer region of the mouse *Tie2* gene has the capacity to drive pan-endothelial cell transgene expression, we generated *Tie2-Cre* transgenic mice for endothelial cell-lineage analyses in mice and for endothelial-cell specific gene targeting. These mice provide new insight into endothelial–mesenchymal transformation in vivo in the mammalian developing heart.

MATERIALS AND METHODS

Construction of the Tie2-Cre Transgene

The *Cre* expression vector pBS185 (Gibco-BRL, Gaithersburg, MD) was digested with *Xho*I and *Hin*dIII to excise a 2.7-kb fragment consisting of *Cre* cDNA and *metallothionein-1* (*MT-1*) polyA signal sequence. The 2.7-kb fragment was blunt-ended with Klenow enzyme and ligated to the murine 2.1-kb *Tie2* promoter, pg50H1-2 (Schlaeger *et al.,* 1997). Then, the murine *Tie2* enhancer fragment extending from a *Nae*I site of exon 1, approximately 10 kb into the first intron and derived from pg50-2.11 (Schlaeger *et al.,* 1997), was ligated downstream of the *MT-1* polyA signal sequence. The resulting *Tie2-Cre* transgene is comprised of the *Tie2* promoter, *Cre* cDNA, *MT-1* polyA signal sequence, and *Tie2* intron 1 enhancer.

Production of Transgenic Mice

All procedures were approved by the Institutional Animal Care Research Advisory Committee at The University of Texas Southwestern Medical Center at Dallas. The *Tie2-Cre* transgene was excised from the plasmid vector backbone. Microinjection into fertilized C57BL6 $\times$ SJLF$_1$ oocytes and other surgical procedures were performed as described (Hofmann *et al.,* 1988). The genotypes of all offsprings were analyzed by PCR as well as dot blots on genomic DNA from tail biopsies using Nytlan membranes (Schleicher and Schuell, Keene, NH), which was hybridized with random-prime radiolabeled *Cre* cDNA probe. For PCR analysis, DNAs were amplified 35 cycles (94°C for 1 min, 60°C for 1.5 min, 72°C for 2 min) in a thermal cycler. The primers were 5′-CGCATAACCAGTGAAACAGCATTGC-3′ in *Cre* coding region and 5′-CCCTGTGCTCAGACAGAAATGAGA-3′ in the *Tie2* promoter region.

Detection of Transgene Expression by Reverse Transcriptase (RT)-PCR

Total RNA was extracted from different tissues including the heart, lung, kidney, liver, small intestine, large intestine, and brain from wild-type and transgenic mice using RNA STAT-60 reagents (Tel-Test, Friendswood, TX). Poly(A)-rich RNA was subsequently prepared using oligo-dT cellulose columns. For RT-PCR of transgenic mRNA, first strand cDNA was synthesized with oligo(dT)$_{12-18}$ primers using Superscript II reverse transcriptase as recommended by the manufacturer (Gibco BRL, Gaithersburg, MD). Oligonucleotide primers (upstream, 5′-GCAATGGTGCGCCTGCTGGAAGATGG-3′; downstream, 5′-GGAGCTGGTGCAAGTGCAGGAGCC-3′), which flank the intron in the transgene were used to PCR amplify the transgene. Two cycle conditions were used. The first 19 cycles used the conditions of 94°C for 1 min, 68–0.5°C/cycle for 2 min. The subsequent 15 cycles used the conditions of 94°C for 1 min, 58°C for 2 min, and 72°C for 2 min. The resulting ~350-bp product (as opposed to the ~900-bp genomic DNA-derived product) was isolated electrophoretically in 1.4% agarose and visualized by ethidium bromide staining.

LacZ Staining

Tie2-Cre transgenic mice were bred with *CAG-CAT-Z* transgenic mice (Araki *et al.,* 1995) or *R26R* transgenic mice (Soriano, 1999) for histological analyses. The *CAG-CAT-Z* transgene has a *choline acetyltransferase* (*CAT*) cassette which is flanked by two *loxP* sites, followed by a *lacZ* cassette. The transgene is under control of the ubiquitous *CAG* promoter (Niwa *et al.,* 1991); however, *lacZ* expression is only activated by Cre-mediated recombination. The *R26R* transgenic mice is made by homologous recombination of a gene trap cassette into a 5′ intron of the *ROSA26* locus, which is expressed ubiquitously and uniformly at all developmental and postnatal stages (Friedrich and Soriano, 1991). The gene trap cassette includes a splice acceptor, neomycin-resistant cassette which is flanked by two *loxP* sites, and a *lacZ* cassette (Soriano, 1999). Similar to the *CAG-CAT-Z* transgenic mice, *lacZ* expression is only activated by Cre-mediated recombination in *R26R* transgenic mice. As a control, embryos of *Tie2-lacZ* transgenic mice were also used for lacZ staining (Schlaeger *et al.,* 1997). Whole-mount lacZ staining of embryos was performed as follows: First, embryos were fixed with 4% paraformaldehyde/phosphate buffered saline (PBS) on ice for 1 h, then rinsed with buffer containing 0.2 M sodium phosphate (pH 7.3), 2 mM magnesium chloride, 0.02% NP-40, and 0.01% sodium deoxycholate (Rinse Buffer). Rinsed embryos were stained in the same buffer containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mg/ml X-gal (5-bromo-4-chloro-3-indoyl β-D-galactopyranoside). Whole-mount stained embryos were embedded in paraffin, sectioned at 7 μm, and mounted on the slides. LacZ-stained sections were lightly counterstained with eosin, mounted in Permount (Fisher Scientific), and photographed under an Olympus BX-50 microscope.

RESULTS

Eight independent lines of *Tie2-Cre* transgenic mice were generated and screened for expression by RT-PCR. RT-PCR confirmed *Cre* transgene expression in tissues of all of eight lines (data not shown). We analyzed each of these lines histologically by lacZ staining of *Tie2-Cre* transgenic mice bred with either *CAG-CAT-Z* transgenic mice or *R26R* transgenic mice. Two independent lines, which carry ~2 (line 155-1) and ~20 (line 161-8) copies of the transgene (as estimated by dot blot hybridization with radiolabeled probes), show strong β-galactosidase activity in virtually all blood vessels of E11.5 *Tie2-Cre;CAG-CAT-Z* double transgenic mouse embryos (Fig. 1). This staining pattern is highly similar to the E11.5 *Tie2-lacZ* embryos (Schlaeger *et al.,* 1997) and no background staining is observed in *CAG-CAT-Z* embryos. This indicates that the *Tie2* promoter/enhancer regions we used in this study faithfully drive *Cre* transgene expression in a pan-endothelial-specific fashion. Sections of *Tie2-Cre;CAG-CAT-Z* embryos confirm lacZ staining in the endocardium and endothelial cells in the aorta, umbilical arteries, and veins, as well as small blood vessels (Fig. 2).

Interestingly, we detect strong lacZ staining in the mesenchymal cells of the AV canal and part of the outflow tract in *Tie2-Cre;CAG-CAT-Z* embryos (Fig. 2). A small number of circulating cells are also positive for lacZ (Fig. 6F). The endocardial cushion of *Tie2-lacZ* embryos shows no lacZ staining (Fig. 3). To investigate lacZ staining in mesenchymal cells of the developing heart of *Tie2-Cre;CAG-CAT-Z* embryos further, we examined earlier time points. In E7.5 embryos, lacZ staining is observed in a subset of cells in the extra-embryonic mesodermal component of visceral yolk sac (Figs. 4A–4C). In E8.5 embryos (when the cardiac jelly is still acellular), only a fraction of endothelial cells in the aorta and common atrial chamber show lacZ staining (Fig. 4D); not all of the endocardial cells are lacZ-positive. In the blood islands of E8.5 yolk sac, we detect lacZ staining in the majority of endothelial cells; however, we do not see lacZ staining in hematopoietic cells (Fig. 4H). In E9.5 embryos, lacZ staining is observed in almost all endothelial cells and endocardial cells. This is the first stage that mesenchymal cells in the AV regions are noticeable. In addition to endocardial cells, mesenchymal cells in the AV regions also show strong lacZ staining (Fig. 5B). The number of mesenchymal cells in the OT region is much smaller than in the AV canal in this embryonic stage. A subset of these mesenchymal cells are lacZ positive (Fig. 5C). We also examined the omphalomesenteric artery as well as the vitelline artery, where native Tie2 receptor-expressing hematopoietic cells are located (Takakura *et al.,* 1998). However, we did not detect lacZ staining in the hematopoietic cells in these regions (data not shown). In E10.5 embryos, the number of mesenchymal cells in the OT region increases significantly; however, lacZ staining is only observed in the proximal or conal cushions (Fig. 5D). LacZ staining is not observed in mesenchymal cells in the distal outflow tract and branchial arch arteries, although endothelial cells in these regions are lacZ positive (Fig. 5E). In contrast to the OT region, almost all of mesenchymal cells in the AV canal show strong lacZ staining (Fig. 5F). In the OT regions of E11.5 embryos, lacZ-positive mesenchymal cells are observed in the developing leaflets of pulmonary valve and aortic valve (Figs. 6A and 6C). Mesenchymal cells in the outflow septum at the level of the conus show lacZ staining throughout the depth of the cushion. Interestingly, however, mesenchymal cells in the distal outflow tract are negative for lacZ (Figs. 6B, 6E). We observed very weak lacZ staining primarily in the endothelial cells in *Tie2-Cre; CAG-CAT-Z* transgenic embryos older than E12.5 (data not shown).

DISCUSSION

Endothelial Lineage Analyses Using Tie2-Cre; CAG-CAT-Z Transgenic Mice

In *Tie2-lacZ* mice, the lacZ staining pattern demonstrates transcriptional activity of the *Tie2* promoter/enhancer at a given time point (Schlaeger *et al.,* 1997, 1995). In contrast, the lacZ staining pattern of *Tie2-Cre;CAG-*
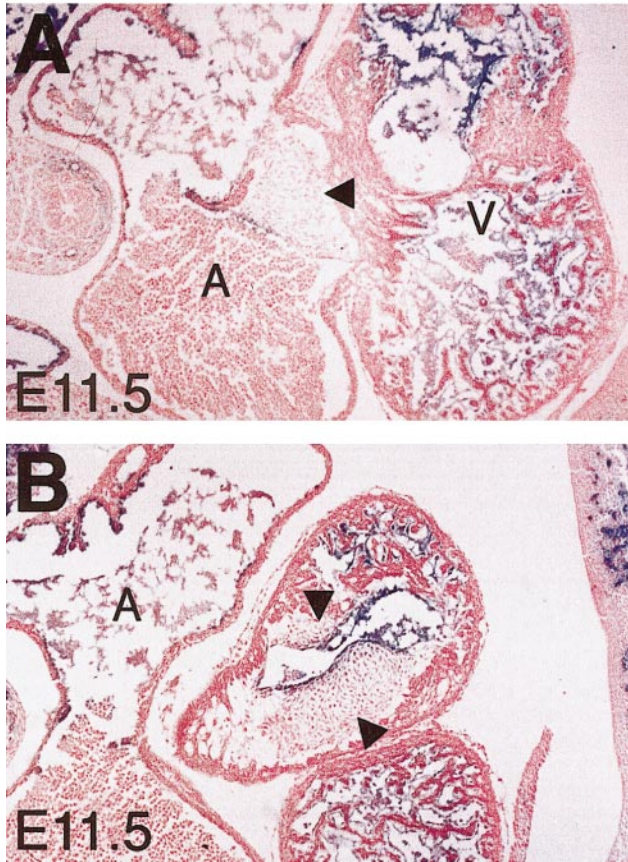
Tie2-LacZ

FIG. 3. Transverse sections of X-Gal stained *Tie2-lacZ* embryos. In an E11.5 *Tie2-lacZ* embryo, expression of *lacZ* is observed specifically in endocardium, but not in endocardial cushion (arrowheads). (A) Transverse section at the level of AV canal. (B) Transverse section at the level of OT region. A, atrium; V, ventricle.

CAT-Z double transgenic mice provides a lineage history of transcriptional activity because once *Cre*-mediated recombination occurs and activates *lacZ* expression, the transgene cannot revert back to the original allele, and the recombined *lacZ* transgene is inherited through the cellular lineage. Since the *Tie2* promoter/enhancer drives expression of the transgene in a pan-endothelial-specific manner, *Tie2-Cre;CAG-CAT-Z* mice are useful for endothelial cell lineage analyses.

In addition to the strong, pan-endothelial lacZ staining in *Tie2-Cre;CAG-CAT-Z* mouse embryos, mesenchymal cells in the AV canal of these embryos show strong lacZ staining. This observation is consistent with observations of endocardial–mesenchymal transformation in heart explants of the chick and mouse (Camenisch *et al.,* 2000; Nakajima *et al.,* 1997; Runyan and Markwald, 1983; Yamamura *et al.,*

1997) and provide further support for these previous reports in an unperturbed *in vivo* setting. Formation of the endocardial cushion is less well understood in the mouse heart. The lacZ staining in the AV cushion of the heart in *Tie2-Cre;CAG-CAT-Z* embryos suggests that mesenchymal cells in the AV cushion are of endocardial cell lineage. Lo *et al.* constructed a *Connexin 43* (*Cx43*)-*lacZ* transgenic mouse line using the promoter region of *Cx43,* a member of the gap junction proteins (Lo *et al.,* 1997). Judging from the similarity of the lacZ expression in *Cx43-lacZ* transgenic mouse with the migration of cardiac neural crest cells in chick-quail chimeras, the *Cx43-lacZ* transgenic mouse is an *in vivo* mouse model for cardiac neural crest patterning (Waldo *et al.,* 1999). Interestingly, *Cx43-lacZ* transgenic mice do not show lacZ staining in the AV cushion. The absence of lacZ expression in the AV cushion of *Cx43-lacZ* transgenic mice and strong lacZ expression in the AV cushion in *Tie2-Cre;CAG-CAT-Z* transgenic mice further supports the endocardial origin of the mesenchymal cells in AV canal.

In contrast to the AV canal, only a limited number of mesenchymal cells in the OT regions, such as the outflow septum at the level of the conus and the developing valve leaflets, show lacZ staining. Experiments using chick-quail chimeras demonstrated that the mesenchymal cells in the OT regions are derived from both cardiac neural crest cells and nonneural crest cells (Waldo *et al.,* 1998, 1999). The lacZ staining in the OT regions of the *Tie2-Cre;CAG-CAT-Z* embryos is consistent with these observations in the avian system. Cardiac neural crest cells migrate into the outflow tract and form the prongs of the aorticopulmonary septum around E9.5 in mice. In *Cx43-lacZ* embryos, mesenchymal cells surrounding the lumen of the aortic sac and distal outflow tract are lacZ positive at E9.5 [Fig. 2 in (Waldo *et al.,* 1999)]. In *Tie2-Cre;CAG-CAT-Z* embryos, lacZ-positive mesenchymal cells are detected in the proximal outflow tract. However, mesenchymal cells in the distal outflow tract are lacZ-negative. In the avian system, the proximal outflow cushions fuse by a completely different mechanism from the distal outflow cushions with little contribution from extracardiac cells. The proximal outflow tract is not formed from the aorticopulmonary septation complex, but by closure of the myocardializing conal cushions (van den Hoff *et al.,* 1999). The clear demarkation of proximal (lacZ positive) from distal outflow cushions (lacZ negative) in *Tie2-Cre;CAG-CAT-Z* embryos (Figs. 6B and 6E) also supports the fact that the proximal outflow tract develops differently from the distal outflow tract. Interestingly, this demarkation coincides with the region where BMP-7 (proximal) expression is replaced by BMP-4 (distal) (Yamada *et al.,* 1999). The distal cardiac outflow tract is divided into aortic and pulmonary channels by the aorticopulmonary septum around E11.5. In *Cx43-lacZ* embryos, *lacZ* expression is observed in the common wall shared by aortic and pulmonary channels [Fig. 3 in (Waldo *et al.,*

Tie2-Cre;CAG-CAT-Z
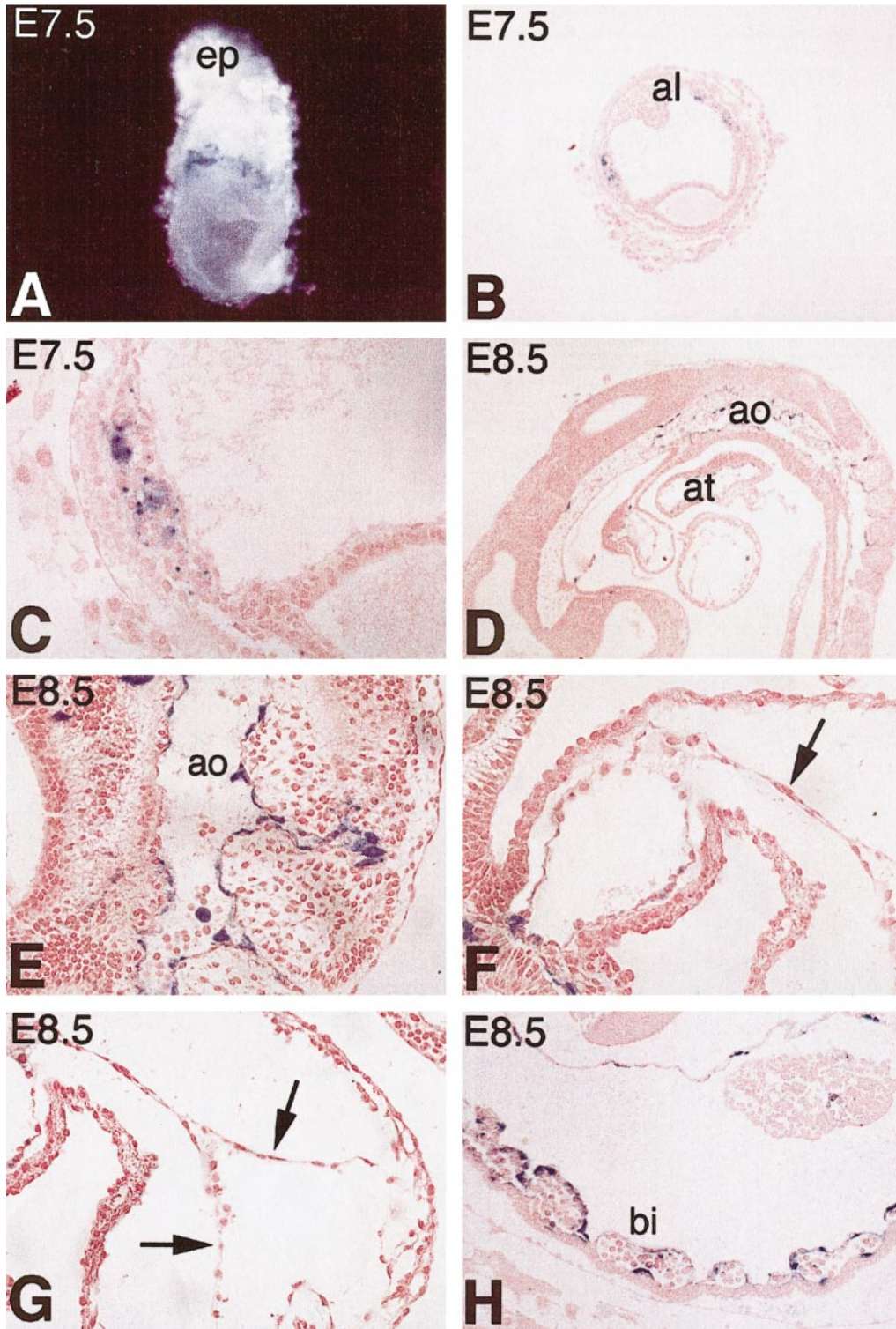
1999)]. Conversely, in *Tie2-Cre;CAG-CAT-Z* E11.5 embryos, lacZ staining is observed in the endothelial cells of aortic and pulmonary channels but not in the mesenchymal cells which compose the aorticopulmonary septum.

Using *Wnt1-Cre;R26R* double transgenic mice, Jiang *et al.* reported lineage analysis of cardiac neural crest cells (Jiang *et al.,* 2000). The *Wnt1* gene is expressed specifically in the neural plate, in the dorsal neural tube, and in the early migratory neural crest population at all axial levels. When the crest cell lineage migrates away from the neural tube, the *Wnt1* expression is abolished and is not expressed at any other time or in any other place during later stages of development (Echelard *et al.,* 1994). LacZ staining of the *Wnt1-Cre;R26R* transgenic mice showed results very similar to that in *Cx43-lacZ* transgenic mice. In embryos older than E12.5, the distribution of lacZ-positive cells of the *Wnt1-Cre;R26R* transgenic mice is closer to the results from avian chimeras than the *Cx43-lacZ* transgenic mice. This difference can be explained by a decreased ongoing activity of the *Cx43* promoter after E12.5.

Furthermore, using *Pax3-Cre;R26R* transgenic mice, Epstein *et al.* also reported lineage analysis of cardiac neural crest cells (Epstein *et al.,* 2000). The *Pax3* gene encodes a transcription factor expressed in the dorsal neural tube which gives rise to neural crest cells (Goulding *et al.,* 1991). LacZ staining of *Pax3-Cre;R26R* transgenic mice was similar to that of *Wnt1-Cre;R26R* transgenic mice and *Cx43-lacZ* transgenic mice. Overall, these findings are precisely complementary to our present data.

Our results suggest that endocardial cells contribute to the non-crest-derived mesenchyme in the OT regions. For example, using *Cx43-Cre* transgenic mice and *CAG-CAT-eGFP* transgenic mice, in which green fluorescent protein acts as a Cre-dependent reporter instead of lacZ (Kawamoto *et al.,* 2000), we may be able to simultaneously label cardiac neural crest-derived and endothelial cell-derived structures in the OT region. In the present study, we cannot formally rule out the possibility of ectopic expression of the *Tie2-Cre* transgene in cell lineages unrelated to endothelial cells. However, we feel that this is unlikely, because we cannot detect lacZ staining in the endocardial cushion at any stage of *Tie2-lacZ* transgenic mice, whose *lacZ* expression is controlled by the identical promoter/enhancer region that we use for *Tie2-Cre* transgenic mice. The absence of lacZ staining in the endocardial cushion in *Tie2-lacZ* transgenic mice indicates that transgene expression driven by this promoter/enhancer shuts off upon endocardial–mesenchymal transformation.

A study using gold-labeled quail endothelial cells of dorsal aorta showed that endothelial cells migrate into the subendothelial mesenchyme and express smooth muscle actins (DeRuiter *et al.,* 1997). To test the hypothesis that endothelial–mesenchymal transformation occurs in the aorta of the developing mouse, we examined the dorsal aorta of *Tie2-Cre;CAG-CAT-Z* mouse embryos and *Tie2-Cre;R26R* mouse embryos at various stages. No detectable lacZ staining was observed in subendothelial mesenchymal regions (data not shown), suggesting that endothelial–mesenchymal transformation does not occur in this region during mouse development. However, the absence of lacZ staining in the mesenchyme of the dorsal aorta of *Tie2-Cre; CAG-CAT-Z* and *Tie2-Cre;R26R* embryos does not completely rule out the possibility of endothelial–mesenchymal transformation in the aorta in the mouse because we cannot exclude the possibility that the *CAG* promoter or *ROSA26* allele is simply not active in the subendothelial mesenchyme of the aorta.

Circulating endothelial precursors or angioblast?

In addition to endothelial cells and endocardial cushion mesenchymal cells, we observed circulating lacZ-positive cells in *Tie2-Cre;CAG-CAT-Z* embryos. The nature of these cells is not yet clear. In *Tie2-lacZ* embryos, hematopoietic cells do not show lacZ staining (Schlaeger *et al.,* 1995). Hematopoiesis begins in the yolk sac at E7.5 and subsequently shifts to the fetal liver and then to the spleen and bone marrow (Johnson and Moore, 1975). Before hepatic hematopoiesis, intraembryonic hematopoietic sites are the para-aortic splanchnopleural mesoderm (P-Sp) region and the aorta-gonad-mesonephros (Medvinsky and Dierzak, 1996). The omphalomesenteric artery is formed from splanchnic mesodermal cells around E8.0 and migrates to the yolk sac, where the vitelline artery is formed by E9.0. The native *Tie2* receptor is expressed not only in the endothelial cells of these arteries but also in the hematopoietic cells which aggregate and adhere to the endothelial cells of these arteries (Takakura *et al.,* 1998). However, in

FIG. 4. X-Gal stained *Tie2-Cre;CAG-CAT-Z* double transgenic E7.5 (A–C) and E8.5 (D–H) embryos. (A) Whole-mount stained E7.5 embryo of *Tie2-Cre;CAG-CAT-Z* transgenic mouse. (B) Transverse sections of E7.5 embryo of *Tie2-Cre;CAG-CAT-Z* transgenic mouse. (C) A higher magnification of Fig. 4B. Expression of *lacZ* is observed in a subset of cells in the extra-embryonic mesodermal component of visceral yolk sac. (D–G) Parasagittal sections of an E8.5 *Tie2-Cre;CAG-CAT-Z* transgenic mouse embryo. (D) Only a small fraction of endothelial cells in the aorta and common atrial chamber show lacZ staining in the E8.5 embryo. (E) In dorsal aorta, lacZ-positive endothelial cells are observed. (F, G) In the OT region, endocardial cells do not show expression of *lacZ* (arrows). At this stage, the cardiac jelly is acellular. (H) In the blood islands of the E8.5 yolk sac, expression of *lacZ* is observed in the majority of endothelial cells, but not in the hematopoietic cells. ep, ectoplacental cone; al, allantois; ao, dorsal aorta; at, common atrial chamber; bi, blood island.
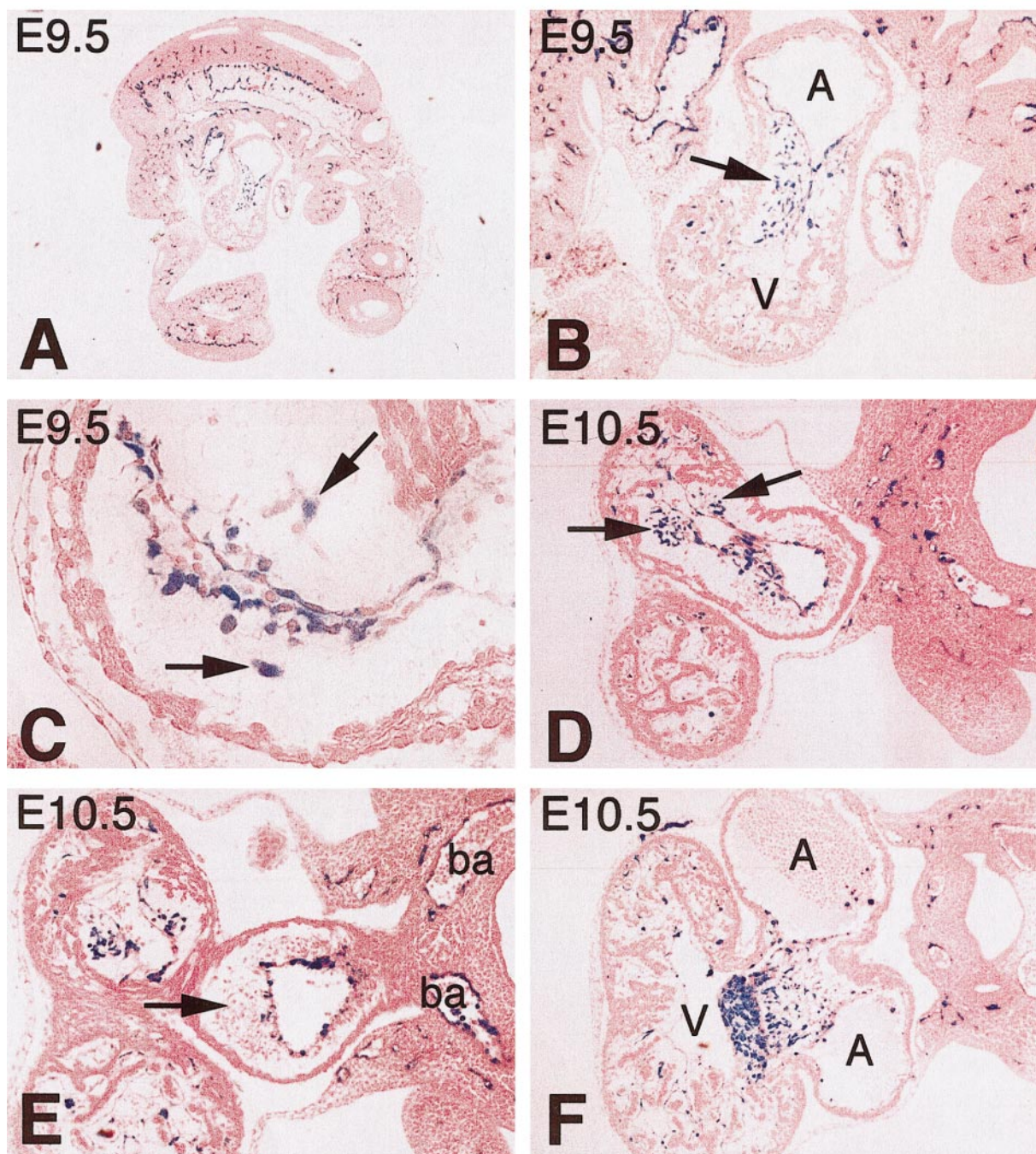
Tie2-Cre;CAG-CAT-Z

FIG. 5. X-Gal stained *Tie2-Cre;CAG-CAT-Z* double transgenic E9.5 (A–C) and E10.5 (D–F) embryos. (A–C) Parasagittal sections of E9.5 *Tie2-Cre;CAG-CAT-Z* transgenic embryos. (A) Almost all of the endothelial cells show lacZ staining. (B) In the AV canal, both mesenchymal cells (arrow) and endocardial cells show lacZ staining. (C) In the OT region, the number of mesenchymal cells is very small compared to the AV canal. A subset of the mesenchymal cells in the OT region shows lacZ staining (arrows). (D–F) Transverse sections of E10.5 *Tie2-Cre;CAG-CAT-Z* transgenic embryos. (D) In the OT region, the number of mesenchymal cells are increased compared to E9.5 embryos. LacZ staining is observed in the proximal or conal cushions (arrows), but not in the other areas of mesenchymal cells. (E) Endothelial cells of the distal outflow tract and branchial arch arteries are lacZ-positive. Note that no lacZ staining is observed in the mesenchymal cells of distal outflow tract (arrow). (F) Almost all of the mesenchymal cells in the AV canal show strong lacZ staining. A, atrium; V, ventricle; ba, branchial arch artery.
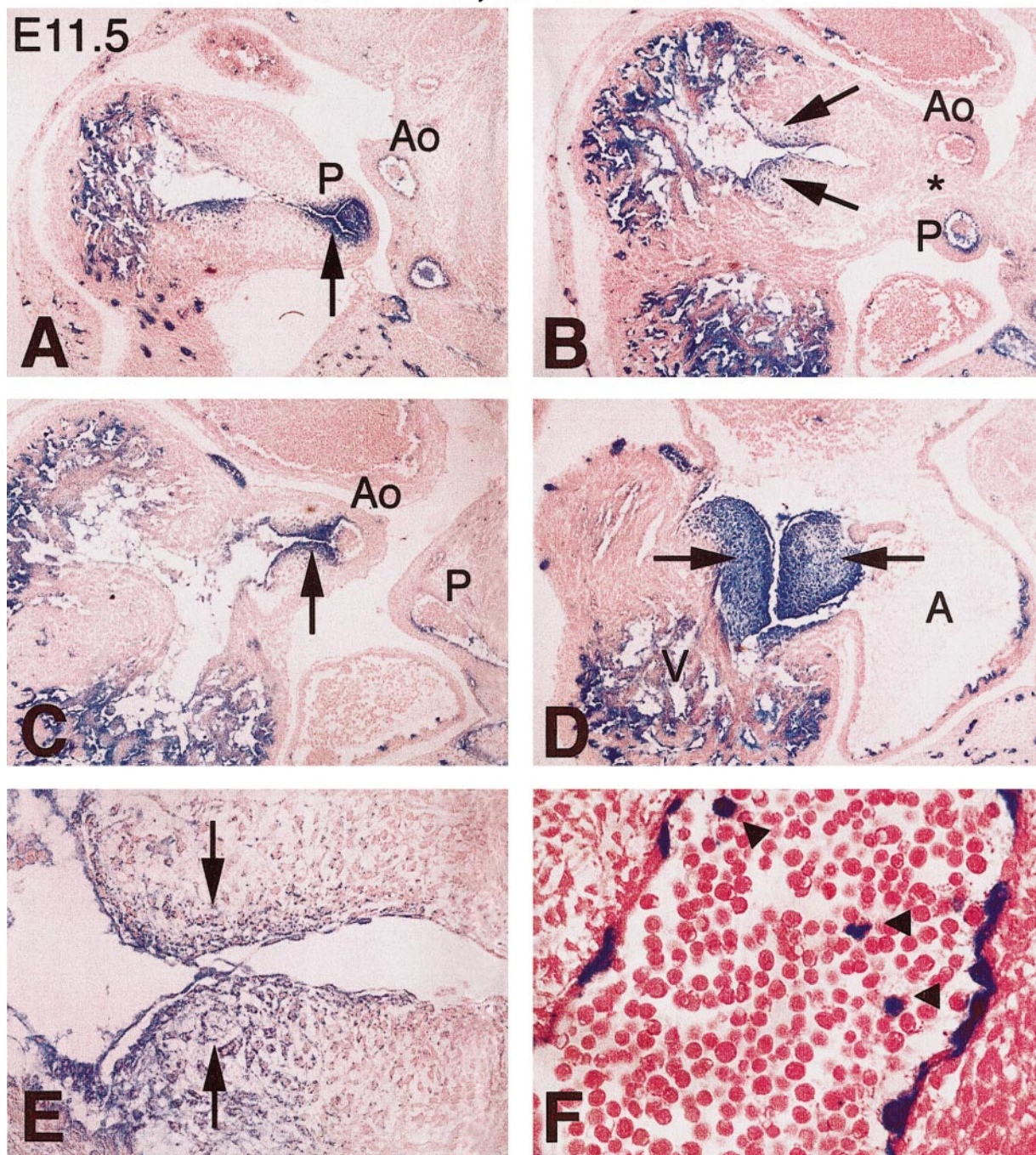
Tie2-Cre;CAG-CAT-Z

FIG. 6. X-Gal stained *Tie2-Cre;CAG-CAT-Z* transgenic E11.5 embryos. (A–E) Transverse sections of E11.5 *Tie2-Cre;CAG-CAT-Z* transgenic embryos. (A, C) In the developing leaflets of pulmonary valve and aortic valves, lacZ-positive mesenchymal cells are observed (arrows). (B) In the outflow septum at the level of the conus, mesenchymal cells show lacZ staining (arrows). Note that no lacZ staining is observed in mesenchymal cells of the aorticopulmonary septation complex (star). (D) Strong lacZ staining is observed in the mesenchymal cells of AV canal (arrows). (E) A higher magnification of panel B. Mesenchymal cells in the proximal outflow tract show lacZ staining throughout the depth of the cushion. (F) Parasagittal section of *Tie2-Cre;CAG-CAT-Z* transgenic E11.5 embryo. In the dorsal aorta, a small number of circulating cells are lacZ-positive (arrowheads). Ao, aortic channel of the outflow tract; P, pulmonary channel of the outflow tract; A, atrium; V, ventricle.

Tie2-Cre;CAG-CAT-Z embryos, we did not detect lacZ-positive hematopoietic cells in the blood islands of the yolk sac, in the omphalomesenteric artery, or in the vitelline artery. Interestingly, Takahashi *et al.* showed that bone marrow-derived endothelial cells can be recruited to form endothelial cells in blood vessels (Takahashi *et al.,* 1999). They subjected wild-type mice to lethal irradiation followed by bone marrow transplantation. Donor bone marrow was derived from *Tie2-lacZ* transgenic mice. Their results indicate that endothelial progenitor cells can be recruited from the bone marrow to the areas where neovascularization takes place. Furthermore, using human peripheral blood samples, Lin *et al.* reported the endothelial outgrowth potential and kinetics of bone marrow-derived hematopoietic cells (Lin *et al.,* 2000). These results suggest that circulating endothelial progenitor cells exist and that they are derived from bone marrow. The circulating, lacZ-positive cells in *Tie2-Cre;CAG-CAT-Z* transgenic embryos may therefore be circulating endothelial progenitor cells. We hypothesize that the *Tie2* promoter/enhancer becomes active after some hematopoietic cells differentiate into circulating endothelial progenitor cells; this may explain why hematopoietic cells in the blood island, omphalomesenteric artery, and vitelline artery do not show lacZ staining.

Endothelial Specific-Gene Targeting

Tie2-Cre transgenic mice can provide valuable information regarding the roles of molecules which may be involved with the endocardial–mesenchymal transformation *in vivo* (Camenisch *et al.,* 2000). In *Tie2-Cre;CAG-CAT-Z* transgenic embryos, not all of the AV cushion cells are lacZ positive but the number of lacZ positive cells seems to increase during the development (Figs. 5B, 5F, and 6D). This can be because more lacZ-positive endocardial cells participate in endocardial–mesenchymal transformation, or because the lacZ positive cushion cells proliferate. At this moment, we cannot differentiate these possibilities. *Tie2-Cre* transgenic mice also enable endothelial cell (endothelial cell-lineage)-specific gene targeting by the *Cre/loxP* system. Lethal developmental abnormalities that result from systemic disruption of the genes expressed during embryonic development frequently impede the (patho)physiologic analyses of the roles of these gene products in adult mice. Tissue-specific gene targeting may overcome these problems and thus allow (patho)physiologic analyses of the genes in adult mice. For example, conventional targeting of *endothelin-1* (*Et-1*), of which the main expression site is endothelial cells in adult mammals, results in developmental abnormalities and neonatal death (Kurihara *et al.,* 1994). We are using *Tie2-Cre* transgenic mice to create mice with an endothelial cell-specific disruption of the *ET-1* in order to reveal the physiologic role of endothelial-endothelin-1 in adult animals (Kisanuki *et al.,* unpublished data).

In conclusion, we generated *Tie2-Cre* transgenic mice,

which facilitate endothelial cell-lineage analyses and endothelial cell-specific gene targeting. LacZ staining of *Tie2-Cre;CAG-CAT-Z* embryos were consistent with endocardial–mesenchymal transformation in the AV canal and part of the OT regions. Importantly, these observations were made in the setting of the intact, unmanipulated embryos, not in the organ culture or transplantation chimeras. *Tie2-Cre* transgenic mice will be a powerful genetic model to improve the understanding of the roles of the genes expressed in endothelial cells during embryonic development as well as (patho)physiologic conditions such as atherosclerosis, cancer, and wound healing.

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