

# Development of heart valves requires *Gata4* expression in endothelial-derived cells

José Rivera-Feliciano<sup>1</sup>, Kyu-Ho Lee<sup>2,\*</sup>, Sek Won Kong<sup>2</sup>, Satish Rajagopal<sup>2</sup>, Qing Ma<sup>2</sup>, Zhangli Springer<sup>2</sup>, Seigo Izumo<sup>3</sup>, Clifford J. Tabin<sup>1</sup> and William T. Pu<sup>2,†</sup>

Cardiac malformations due to aberrant development of the atrioventricular (AV) valves are among the most common forms of congenital heart disease. At localized swellings of extracellular matrix known as the endocardial cushions, the endothelial lining of the heart undergoes an epithelial to mesenchymal transition (EMT) to form the mesenchymal progenitors of the AV valves. Further growth and differentiation of these mesenchymal precursors results in the formation of portions of the atrial and ventricular septae, and the generation of thin, pliable valves. *Gata4*, which encodes a zinc finger transcription factor, is expressed in the endothelium and mesenchyme of the AV valves. Using a *Tie2-Cre* transgene, we selectively inactivated *Gata4* within endothelial-derived cells. Mutant endothelium failed to undergo EMT, resulting in hypocellular cushions. Mutant cushions had decreased levels of *ErbB3*, an EGF-family receptor essential for EMT in the atrioventricular cushions. In *Gata4* mutant embryos, *ErbB3* downregulation was associated with impaired activation of Erk, which is also required for EMT. Expression of a *Gata4* mutant protein defective in interaction with Friend of Gata (FOG) cofactors rescued the EMT defect, but resulted in a decreased proliferation of mesenchyme and hypoplastic cushions that failed to septate the ventricular inlet. We demonstrate two novel functions of *Gata4* in development of the AV valves. First, *Gata4* functions as an upstream regulator of an *ErbB3-Erk* pathway necessary for EMT, and second, *Gata4* acts to promote cushion mesenchyme growth and remodeling.

**KEY WORDS:** Heart development, Endocardial cushion, *Gata4*, Heart valves, Cardiac development, Cardiac morphogenesis, Epithelial-mesenchymal transition, EMT, Atrioventricular valves

## INTRODUCTION

Cardiac malformations attributable to aberrant development of the atrioventricular (AV) valvuloseptal complex are among the most common forms of congenital heart disease (Pierpont et al., 2000). Development of the AV valvuloseptal complex can be considered to occur in several steps (reviewed by Armstrong and Bischoff, 2004). First, endocardial cells at the AV canal (AVC) undergo an epithelial to mesenchymal transition (EMT), forming mesenchymal cells that invade swellings of extracellular matrix to form the endocardial cushions (EC). Next, the cellularized superior and inferior EC grow and fuse, forming the AV valvuloseptal complex that divides the ventricular inflow into a right and a left AV valve. Further elongation and remodeling of the cushions results in formation of the mature valve leaflets.

Transformation of AV endocardium to cushion mesenchyme requires the input of multiple signaling molecules secreted from the adjacent myocardium, including Tgf $\beta$  and Egf family members (Camenisch et al., 2000; Nakajima et al., 2000a; Sugi et al., 2004; Rivera-Feliciano and Tabin, 2006). Ablation of *ErbB3*, which encodes an Egf family receptor, results in failure of endocardial cells of the AV cushions to undergo EMT to form cushion mesenchyme (Camenisch et al., 2000). One mechanism by which *ErbB3* promotes

EMT is to activate *Ras* (Camenisch et al., 2000). Decreased *Ras* signaling results in defective EMT (Camenisch et al., 2000; Lakkis and Epstein, 1998), and, conversely, increased *Ras* signaling results in increased EMT and hypercellular, enlarged EC (Gitler et al., 2003; Lakkis and Epstein, 1998).

The transcription factor *Gata4* is essential for heart formation (Kuo et al., 1997; Molkentin et al., 1997; Watt et al., 2004; Zeisberg et al., 2005). In humans, *GATA4* heterozygous mutations have been associated with defects in the muscular septum separating atria or ventricles, and variably associated with valvular pulmonary stenosis (Garg et al., 2003; Hirayama-Yamada et al., 2005; Okubo et al., 2004). In addition to its expression in the myocardium, *Gata4* is robustly expressed in the endocardium and the EC (Charron and Nemer, 1999; Heikinheimo et al., 1994). This expression pattern, and the presence of EC defects in mouse embryos homozygous for two different hypomorphic *Gata4* alleles (Crispino et al., 2001; Pu et al., 2004), suggested that *Gata4* might be an important regulator of EC development.

To further investigate the role of *Gata4* in EC development, we specifically inactivated *Gata4* in endothelium and endothelium-derived cushion mesenchyme. We show that *Gata4* expression in endothelium-derived cells is required at two stages of AV valve formation, illustrating novel cell-autonomous roles for *Gata4* in the endocardium. First, *Gata4* is required to promote EMT of endocardial cells to generate AV cushion mesenchyme. Second, *Gata4* activity in endocardial-derived cells is required later during AV valve maturation for growth and remodeling of the AV cushions to septate the ventricular inlet.

## MATERIALS AND METHODS

### Mice

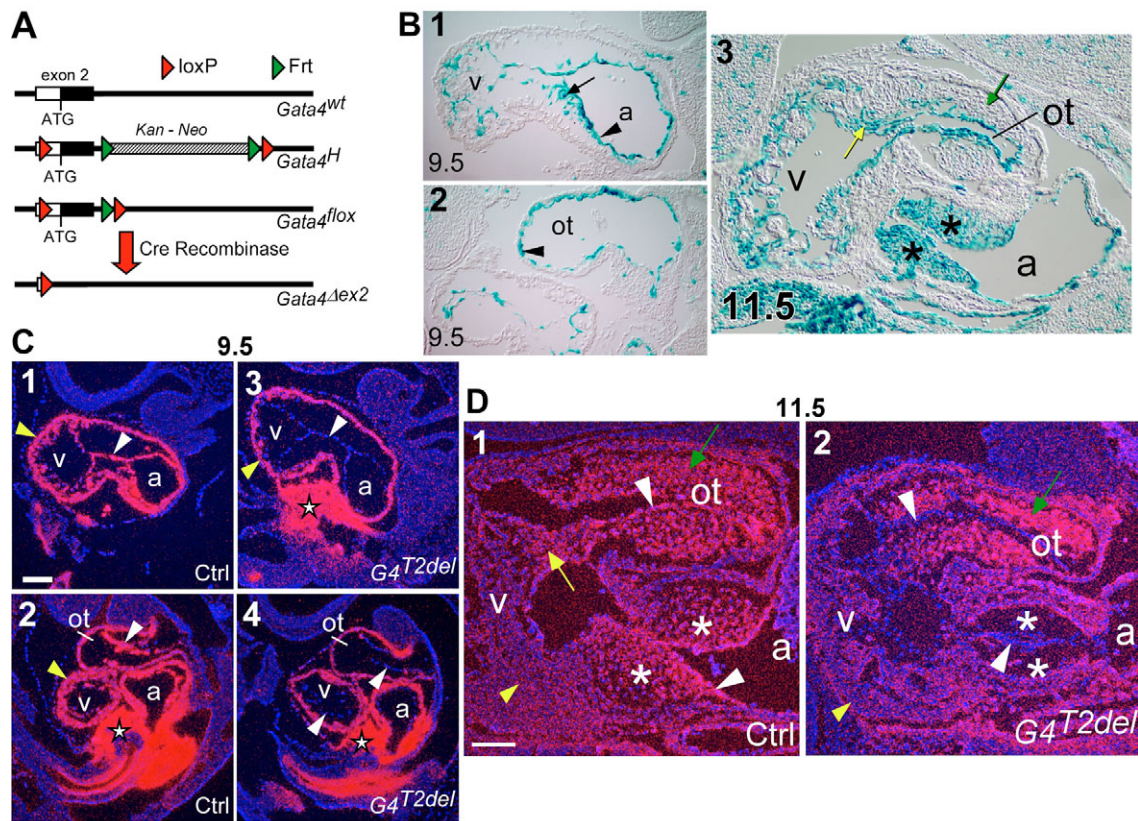
*Gata4<sup>H</sup>* and *Gata4<sup>fllox</sup>* alleles (Fig. 1A) have been described previously (Pu et al., 2004; Zeisberg et al., 2005). *Gata4<sup>H</sup>* expresses reduced amounts of protein compared with the wild-type allele (Pu et al., 2004). *Gata4<sup>w/Ki</sup>* and

<sup>1</sup>Department of Genetics, Harvard Medical School, Boston, MA 02115, USA.

<sup>2</sup>Department of Cardiology, Children's Hospital and Department of Pediatrics, Harvard Medical School, Boston, MA 02115, USA. <sup>3</sup>Cardiovascular Division, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA 02215, USA.

\*Present address: Departments of Pediatric Cardiology and Cell Biology and Anatomy, Medical University of South Carolina, BSB-601, Suite 250508, 173 Ashley Avenue, Charleston, SC 29445, USA

<sup>†</sup>Author for correspondence (e-mail: wpu@enders.tch.harvard.edu)



**Fig. 1. Endocardial-restricted inactivation of *Gata4*.** (A) Structure of wild-type (wt), hypomorphic (H), floxed, and deleted ( $\Delta$ ex2) *Gata4* alleles. (B) Fate mapping of *T2Cre*-recombined endothelial cells. (B1, B2) In *T2Cre*+; *R26RstoplacZ* embryos at E9.5,  $\beta$ -galactosidase reporter expression (blue) was seen in the endocardium (arrow) and EC mesenchyme (arrowhead) of the AVC, and in the OT endothelium. (B3) By E11.5, the  $\beta$ -galactosidase-positive cells populated the AV cushions (asterisks). Endothelial-derived OT cushion mesenchyme (yellow arrow) was largely confined to the most proximal portion of the OT cushion. Most of the outflow cushion was not recombined by *T2Cre* (green arrow). (C) Endocardial-restricted inactivation of *Gata4* by *T2Cre* at E9.5. Control (C1, C2) and *Gata4<sup>T2del</sup>* (C3, C4) embryos, hybridized to a *Gata4* exon 2-specific probe (red pseudocolor). Blue, DAPI counterstain. The *Gata4* exon 2 in situ hybridization signal was present in the endocardium of control embryos but not of mutant embryos (white arrowheads). *Gata4* expression in the myocardium (yellow arrowheads) and proepicardium (star) was unaffected. (D) Inactivation of *Gata4* in endothelium and endothelium-derived cells at E11.5. In control embryos (D1), *Gata4* exon 2 in situ hybridization signal was present in the endocardium (white arrowhead), myocardium (yellow arrowhead), and AV and OT cushion mesenchyme. In mutant embryos (D2), *Gata4* was not detected in endothelium (white arrowhead) or AVC mesenchyme. Strong signal was still present in mid and distal OT cushion mesenchyme (green arrow) and myocardium (yellow arrowhead). a, atria; v, ventricle; ot, OT. Scale bars in C and D: 100  $\mu$ m.

*R26RstoplacZ* mice (Crispino et al., 2001; Mao et al., 1999) were obtained from Stuart Orkin (Harvard Medical School). Transgenic mice expressing *Cre* from *Tie2* (also known as *Tek*) regulatory elements (*T2Cre*) were obtained from M. Yanagisawa (Kisanuki et al., 2001). *Wnt1-Cre* mice (Danielian et al., 1998) were obtained from Jackson Laboratories. All mice were maintained in a mixed C57BL6/129 genetic background. *Gata4<sup>wil/flox</sup>*, *T2Cre*+ mice were crossed with *Gata4<sup>flox/flox</sup>* mice to yield *Gata4<sup>flox/flox</sup>*, *T2Cre*+ (*Gata4<sup>T2del</sup>*) mice. All animal care and procedures were performed under protocols approved by the Institutional Animal Care and Use Committee.

#### Histology

Embryos were fixed in 4% paraformaldehyde overnight at 4°C, and then paraffin-wax embedded and sectioned at 10  $\mu$ m. Alcian Blue staining was performed by using 0.15 mg/ml Alcian Blue in 5% acetic acid. BrdU labeling and TUNEL staining were performed as described previously (Zeisberg et al., 2005).

#### Tissue culture

For short-term embryo culture, E9.5 embryos were incubated in M199 supplemented with 1% FBS for 30 minutes at 37°C in 5% CO<sub>2</sub>. Heregulin (Sigma; 100 ng/ml) or U0126 (Calbiochem; 10  $\mu$ M) were added as indicated.

AV explant culture was carried out as described (Rivera-Feliciano and Tabin, 2006). Where indicated, U0126 (Calbiochem), vehicle (DMSO) or a growth factor cocktail [Tgf $\beta$ 2 (Cell Biosciences), 50 ng/ml; Bmp2 (R&D Systems), 200 ng/ml; hyaluronic acid (Sigma), 500 ng/ml; heregulin (Sigma), 50 ng/ml] was added to the media at the start of the explant culture.

Human umbilical vein endothelial cells (HUVEC; passage <6; Cambrex) were cultured in complete endothelial growth media (Cambrex). BT20 human breast cancer cells (ATCC) were transfected using Fugene6 (Roche).

#### Gene expression

Whole-mount staining for detection of  $\beta$ -galactosidase activity was performed as described (Lobe et al., 1999). Immunostaining was performed using the following antibodies: Nfatc1 (1:200, Santa Cruz), Desmin (1:4, Biomed), Chd5 (1:20, Santa Cruz), phospho-Erk1/2 (1:200, Cell Signaling),  $\alpha$ -SMA-Cy3-conjugated (Clone 1A4, Sigma 1:200) and biotin-conjugated CD31 (Pecam1, 1:100) monoclonal antibody (Clone MEC 13.3, BD Pharmingen). Erbb3 western blotting was performed with antibody C-17 (Santa Cruz, 1:200) and normalized to Gapdh (Research Diagnostics, 1:10,000).

For RNA analysis, four mutant and four control RNA samples were prepared (Pico-Pure RNA Isolation Kit, Arcturus), each consisting of 10 microdissected AVCs. Probe was prepared from 50 ng total RNA using an isothermal amplification protocol (NuGen), and hybridized to Affymetrix Mouse 430 2.0 microarrays. Two control samples were excluded because of

**Table 1. Primer and probe sequences**

Gene	Forward	Reverse	Note
<b>Real-time PCR assays</b>			
<i>Ankrd1</i>	CAGTTGTAGAGAAATTCCTGTCAGACA	GTGGAGTGCCGTCCGTTTAT	Sybr Green
<i>ErbB3</i>	TAGACGTCAATGGTGCAGATCTG	TAGACGTCAATGGTGCAGATCTG	Sybr Green, mouse
<i>ErbB3</i>	GTGGCACTCAGGGAGCATTA	TCTGGGACTGGGAAAAGG	Sybr Green, human
<i>Gapdh</i>	Proprietary taqman assay from Applied Biosystems		Taqman
<i>Gata2</i>	GGCAGTGGCCCTGTGATC	CGAAGCTTGGGACACTTTTTATG	Sybr Green
<i>Igf1</i>	ACAGGCTATGGCTCCAGCAT	GCTCCGGAAGCAACACTCAT	Sybr Green
<i>Nrp2</i>	ACGTGGCATTGGGCATT	GAGCATCCCCAGCATGTTG	Sybr Green
<i>Pdga</i>	GGTCCACCACCCGAGTGT	CCTGGACCTCTTTCAATTTTGG	Sybr Green
<i>Plx1</i>	AGATGACCACTGCCACTGTATTC	CTTTGAACCTGTGTTCCCTCGAT	Sybr Green
<i>Sgk3</i>	TGCTGGGCGCTGTTC	AGCAACATCTCGGCAGTAAAAAG	Sybr Green
<i>Thbs1</i>	CCTCAAGAAAATGCAGTTTTCAAA	AAACCAAGGAACTCTTCATGGT	Sybr Green
<i>Tnc</i>	TCCCAAGAGAAATTTACAGCTACAG	GAGGTATCCAGTGACCGATGCT	Sybr Green
<i>Vcam1</i>	CGTCGCGAGGTTGTTTAGAGTA	CAGTCCAAGCAACACTCTCTGATT	Sybr Green
<b>In situ hybridization probes</b>			
<i>Bmp2</i>	AACTAGAAGCCGTGGAGGAACTCCA	TTGTGGAGGGCTGCGGGTGCCTTAG	PCR template
<i>Cdh5</i>	ACAACCATGACAACACCCGCAACA	ATCCAGGTTGCAATGAGGTTGGGT	PCR template
<i>ErbB3</i>	BE947142		EST template
<i>Gata4</i> exon 2	195-981 of accession AB075549		PCR template
<i>HRT1</i>	A1316788		EST template
<i>Nfatc1</i>	TGGTGGTTGAGATACCACCTTTCC	TAGAAGCGTCACCATGAGGACAAC	PCR template
<i>Nkx2-5</i>			(Tanaka et al., 1999)
<i>Notch1</i>	AW047868		EST template
<i>Nppa</i>			(Tanaka et al., 1999)
<i>Pecam</i>	CCAACAGAGCCAGCAGTATGAGGACCAG	CACCCCCGAAACACAAGGAAGATAGG	PCR template
<i>Plxnc1</i>	BE981975		EST template
<i>Snail</i>	CAAACCACTCGGATGTGAAGAGA	GTGAAACAGGTGTACCAGGACAA	PCR template
<i>Tbx2</i>			(Bruneau et al., 2001)
<i>Tgfb2</i>			(Pelton et al., 1989)
<i>Thbs1</i>	AW122997		EST template
<b>Primers for amplifying <i>ErbB3</i> promoter and intronic enhancer homology arms</b>			
Prom 5' arm	GCGGTACCCGAGAAAGCCTGTTGACTTGAGTTCCATC	GGCTCGAGACTAGTCACCATATCCGGCTCCTCTGCTAC	
Prom 3' arm	GGACTAGTCTAATCGCTGGCTGTTTCAGGTGG	GACCATGGTACCTGCTGAGAGTTGCCATC	
Enh 5' arm	GCGGTACCCGCTCCCTTACCCTCCACTGTAATC	GGCTCGAGACTAGTCAGAACCAAACTCCTTGCCAGG	
Enh 3' arm	GGACTAGTCTCGGATAGAATTGCTTATTGTTG	GGCTCGAGAACATTTACCCTCCTCTCAACGG	
<b>Site-directed mutagenesis</b>			
Prom top	ATCTCCTCTTAACCGTCTCCGGGGGAAAT		
Prom bot	ATTTCCCCGGGAGACGGTTAAGAGGAGAT		
Enh m1 top	CTTAGCTCTCCCTAGATGAGGAGAAGGGTTG		
Enh m1 bot	CAACCTTCTCCTCATCTAGGGGAGGAGCTAAG		
Enh m2 top	GGAGGCCACCAGTTCCTTAGAATCCAGCTTTAAAGACAAAG		
Enh m2 bot	CTTTGTCTTTAAAGCTGGATTCTAAGGAACTGGTGGCCCTC		

excessive noise. We excluded probe sets that may cross-hybridize to unrelated targets (probe name ending with '\_x\_at') or that received 'Absent' calls across all samples. The 27,082 remaining probe sets were ranked by the 'relative difference' d-score (Tusher et al., 2001), using the Significance Analysis of Microarray (SAM) software package (<http://www-stat.stanford.edu/~tibs/SAM/>).

For quantitative RT-PCR, RNA samples were converted to cDNA and amplified by Ovation isothermal amplification. The cDNA was then used for quantitative PCR on an ABI7300 thermal cycler, with Sybr Green or Taqman detection. Primer sequences are provided in Table 1.

In situ hybridization was performed on 10 µm paraffin sections using digoxigenin-labeled or S<sup>35</sup>-labeled RNA probes as described (Brent et al., 2003; Tanaka et al., 1999). Signal from S<sup>35</sup>-labeled probes was detected in dark field and pseudocolored red using PhotoShop. Sections were counterstained with DAPI. For in situ hybridization probes refer to Table 1. Results shown are representative of at least two embryos.

#### Plasmids

The *Gata4* expression construct has been described previously (Lee et al., 1998). The *Gata4*<sup>Δex2</sup> expression construct was generated by RT-PCR amplification of the Cre-recombined *Gata4* transcript. *Gata4*<sup>ΔDBD-*enr*</sup>

was constructed by PCR cloning the *Gata4* DNA-binding domain upstream of the engrailed repressor domain. The murine *ErbB3* promoter and intron 1 enhancer was cloned from a bacterial artificial chromosome by Red/ET recombineering (GeneBridges) into pGL3-BASIC (Promega) or pGL3-promoter (Promega), respectively. Luciferase assays were normalized for transfection efficiency using pRL-null (Promega).

## RESULTS

### Endothelial-restricted *Gata4* inactivation

To determine the function of *Gata4* within the endocardium and its derivatives, we inactivated a floxed *Gata4* allele (*Gata4*<sup>fllox</sup>; Fig. 1A) (Pu et al., 2004; Zeisberg et al., 2005) by expressing *Cre* recombinase from a *Tie2* promoter (*T2Cre*) (Kisanuki et al., 2001). *Cre*-mediated recombination of *Gata4*<sup>fllox</sup> resulted in excision of a portion of exon 2, including the start codon and 46% of the *Gata4* coding region. The recombined allele (*Gata4*<sup>Δex2</sup>) expresses a truncated protein containing both zinc fingers and the C-terminal activation domain, but lacking the N-terminal transactivation domains (see Fig. S1 in the supplementary material). The truncated



protein failed to activate multiple cardiac and intestinal *Gata4*-dependent promoters in vitro (Fig. S1 in the supplementary material; T. Bosse and S. Krasinski, personal communication), consistent with previous results (Morrisey et al., 1997). These data, along with the observation that embryos homozygous for this mutation in their germline (*Gata4*<sup>Δex2/Δex2</sup>) resemble previously reported mice carrying *Gata4* null alleles (Kuo et al., 1997; Molkenin et al., 1997) (data not shown), suggest that the *Gata4*<sup>Δex2</sup> allele behaves as a loss-of-function mutation. However, we cannot exclude the possibility that *Gata4*<sup>Δex2</sup> retains partial function.

In control experiments, we characterized the spatiotemporal pattern of recombination catalyzed by *T2Cre* using the reporter *R26RstoplacZ*, which expresses *lacZ* only after activation by *Cre* (Mao et al., 1999). In *T2Cre*<sup>+</sup>; *R26RstoplacZ* embryos, *T2Cre* activated reporter expression in the majority of endocardial cells by E9.5 (Fig. 1B1,B2). *lacZ* expression was not observed in epicardial or myocardial cells. Between E9.5 and E11.5, endocardial cells at the AVC transform into mesenchymal cells and populate the AV cushions. Consistent with this lineage history, mesenchymal cells of the AV cushions were recombined by *Cre* recombinase, and consequently expressed the *lacZ* reporter at E9.5 and E11.5 (Fig. 1B1, arrow; 1B3, asterisks).

The endocardium and EC mesenchyme of the developing heart express high levels of *Gata4* (Fig. 1C,D) (Heikinheimo et al., 1994). To specifically inactivate *Gata4* in endocardial-derived cells, we generated embryos with the genotype *Gata4*<sup>fllox/fllox</sup>; *T2Cre*<sup>+</sup> (*Gata4*<sup>T2del</sup>). We examined expression of *Gata4* in these embryos by in situ hybridization using an exon 2-specific probe. In control embryos, *Gata4* exon 2 transcripts were present in endocardial, epicardial and myocardial cells (Fig. 1C1,C2). In *Gata4*<sup>T2del</sup> embryos, expression from the *Gata4* exon 2 probe was unchanged in the epicardium and myocardium (star and yellow arrowheads, Fig. 1C3,C4), but expression in the endocardium was absent (white arrowheads, Fig. 1C3,C4). At E11.5, robust expression was detected in AVC mesenchyme in control embryos, but this tissue was largely deficient in *Gata4*<sup>T2del</sup> embryos (asterisks, Fig. 1D; see below). The lack of detectable *Gata4* transcripts in mutant endocardium was not due to non-specific transcript degradation, or a general failure of endothelium to subspecialize into endocardium, as mutant endocardium continued to express normal levels of *Pecam*, an endothelial marker, and *Nfatc1*, a marker of endothelium subspecialized to line the heart (data not shown).

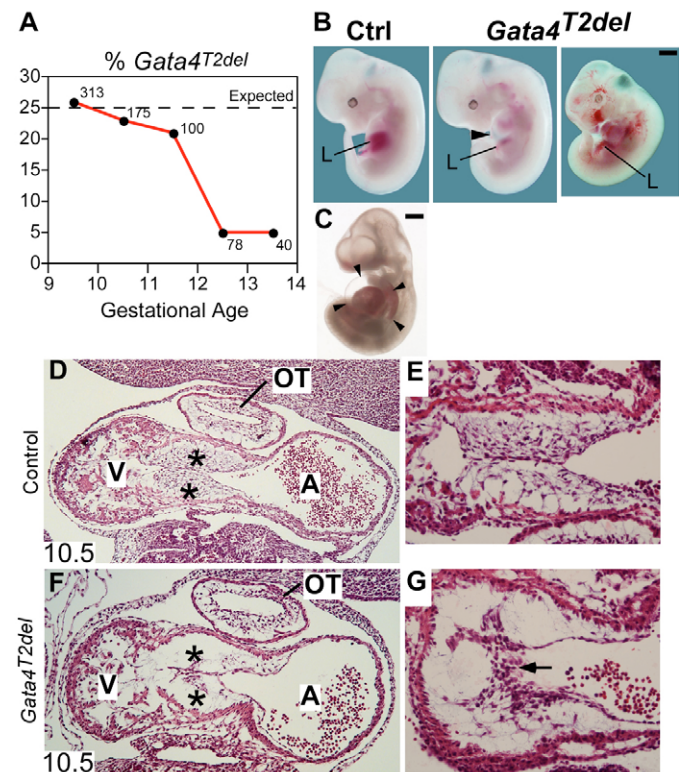
EC also contribute to formation of the outflow (OT) tract and the OT valves. However, only the most proximal portion of OT cushion mesenchyme derives from endothelial progenitors that were recombined by the *T2Cre* transgene (yellow arrow, Fig. 1B). The bulk of OT cushion mesenchyme is derived from neural crest, as demonstrated by fate mapping using the neural crest restricted *Wnt1Cre* transgene (Gitler et al., 2003; Verzi et al., 2005) (see Fig. S2 in the supplementary material). *Gata4* is expressed in OT endothelium and both endocardial- and neural crest-derived OT mesenchyme (green arrow, Fig. 1D). Consistent with the lack of *T2Cre*-mediated recombination in the mid and distal OT mesenchyme (green arrow, Fig. 1B3), *Gata4* expression in these regions was not affected in *Gata4*<sup>T2del</sup> embryos (green arrow, Fig. 1D).

### Phenotypic characterization of *Gata4*<sup>T2del</sup> embryos

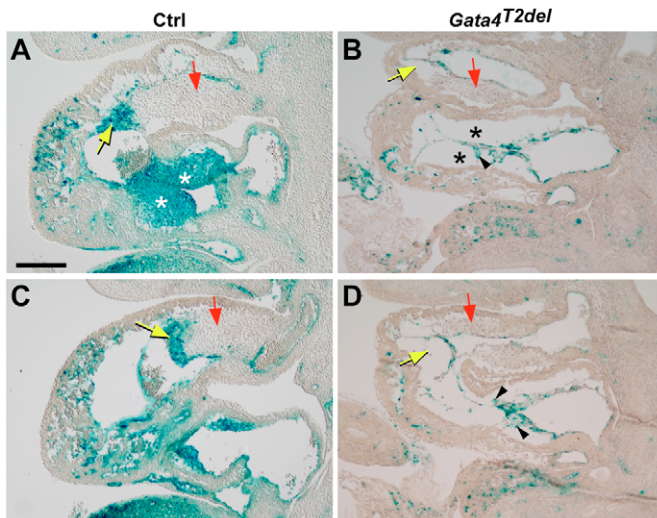
Out of 34 litters genotyped, no *Gata4*<sup>T2del</sup> mice survived to weaning. By E12.5, the prevalence of *Gata4*<sup>T2del</sup> embryos was 80% less than expected based on Mendelian ratios (Fig. 2A). At E12.5, surviving *Gata4*<sup>T2del</sup> embryos had pericardial effusion and peripheral

hemorrhage, which are hallmarks of embryos with heart failure (Fig. 2B,C). The liver was hypoplastic (Fig. 2B; see also Fig. S3 in the supplementary material). Histological examination of the heart showed that these mutant embryos displayed a paucity of mesenchymal cells within the AV cushions (Fig. 2D-G). Additionally, the mutant AV endocardium was multiple cell layers thick at certain foci (arrow, Fig. 2G), whereas the AV endocardium of controls remained as a single cell layer epithelial sheet (Fig. 2E). Although the myocardium appeared normal in the majority of mutant embryos, in 30% of embryos (six out of 17 examined) the compact myocardium was abnormally thin (Fig. S4 in the supplementary material).

The hypocellular AV cushion phenotype was 100% penetrant and was not due to increased apoptosis, as measured by TUNEL staining (data not shown). A small and variable number of mesenchymal cells were observed in mutant AV cushions. To determine the origin of these cells, we fate mapped *T2Cre*-expressing cells using the *R26RstoplacZ* reporter in control and *Gata4*<sup>T2del</sup> mutant embryos

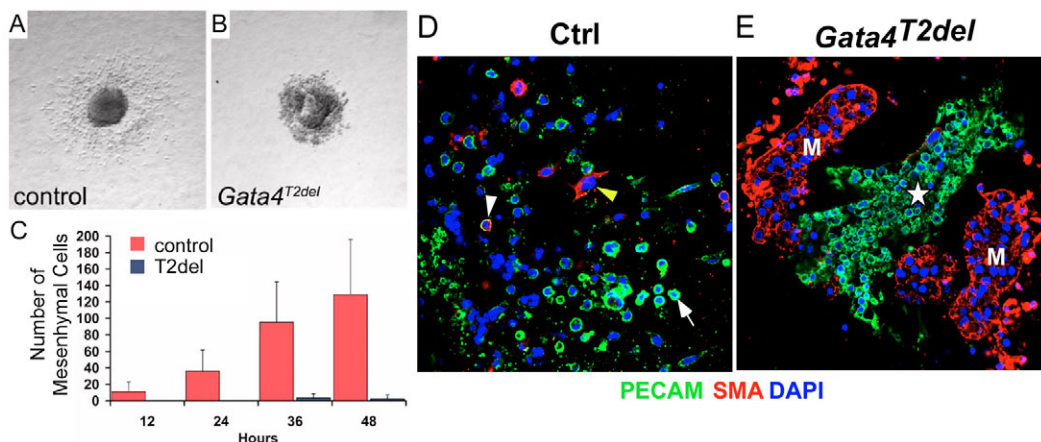


**Fig. 2. Phenotype of embryos with endothelial-restricted inactivation of *Gata4* (*Gata4*<sup>T2del</sup>).** (A) *Gata4*<sup>T2del</sup> embryos die by E12.5-E13.5. The incidence of *Gata4*<sup>T2del</sup> embryos is plotted against gestational age; expected incidence was 25%. Numbers indicate total number of embryos genotyped. (B) Gross appearance of E12.5 control and *Gata4*<sup>T2del</sup> littermates. Mutants showed liver hypoplasia (L) and variable growth retardation, peripheral hemorrhage and pericardial effusion (arrowhead). (C) E12.5 *Gata4*<sup>T2del</sup> embryo with severe pericardial effusion (arrowhead). Scale bars: 50  $\mu$ m. (D-G) Formation of AV cushion mesenchyme requires *Gata4* activity in endothelial-derived cells. By E10.5, the AV cushions of control embryos were populated by numerous mesenchymal cells (asterisk, D), whereas in *Gata4*<sup>T2del</sup> embryos the AV cushions were severely hypocellular (asterisk, F). The endocardium overlying the AV cushions became several cell layers thick in mutant embryos (arrow, G), whereas it was one cell layer thick in control embryos (E).



**Fig. 3. Fate map of  $T2Cre$ -recombined cells in  $Gata4^{T2del}$  hearts.** (A–D)  $T2Cre$ -mediated activation of the  $R26RstoplacZ$  reporter (blue) was mapped in E11.5 control (A,C) or  $Gata4^{T2del}$  (B,D) embryo hearts. Whole-mount embryos were X-gal stained, then sectioned in the sagittal plane. Sections through the AV (A,B) and OT (C,D) cushions are shown. In control hearts,  $T2Cre$ -recombined cells of endothelial origin gave rise to the AV cushion mesenchyme (asterisk), as well as the proximal tip of the OT cushion (yellow arrow). The mid- and distal OT cushions were not recombined by  $T2Cre$  (red arrows). In  $Gata4^{T2del}$  embryos, the corresponding regions were markedly hypocellular. Residual cells in the AV cushions were descended from  $T2Cre$ -recombined endothelium (arrowhead). Scale bar: 100  $\mu$ m.

(Fig. 3). We found that these cells were *lacZ* positive (arrowhead, Fig. 3B), indicating that they are derived from endothelium and not from an alternative tissue compartment, such as epicardium. In situ hybridization demonstrated that the residual mesenchymal cells did not express *Gata4* (data not shown).  $T2Cre$ -recombined mesenchyme at the proximal tip of the OT cushions (yellow arrows, Fig. 3A,C) was missing in  $Gata4^{T2del}$  mutants (yellow arrows, Fig. 3B,D).



**Fig. 4. Endocardial  $Gata4$  activity is required for mesenchyme formation in AV cushion explants.** (A,B)  $Gata4^{T2del}$  AV explants produced markedly fewer mesenchymal cells than control explants. (C) Quantitation of the number of cells with mesenchymal morphology from control and  $Gata4^{T2del}$  explants ( $n=3$ ). (D,E) Failure of endocardial activation in  $Gata4^{T2del}$  AV explant cultures. In control embryos, Pecam-expressing endothelial cells lost cell-cell contacts and adopted a rounded shape (arrow). Some rounded cells expressed SMA (white arrowhead) and acquired a mesenchymal morphology (yellow arrowhead). In mutant explants, endothelial cells remained in a sheet (star) and did not adopt a rounded shape. No mesenchymal cells or cells positive for both Pecam and SMA were detected ( $n=4$ ). Myocardium (M) of control and mutant explants stained positive for SMA.

### ***Gata4* is necessary for AV cushion EMT**

The process of EMT can be recapitulated in vitro by culturing explants of the AVC in a three-dimensional collagen gel (Runyan and Markwald, 1983). During EMT, endothelial cells first undergo an activation step during which they lose their cell-cell contacts. The cells then adopt an elongated morphology, upregulate mesenchymal markers such as SMA, and downregulate endothelial markers such as Pecam. These activated cells subsequently invade and migrate through the extracellular matrix to complete the transformation process.

When cultured in a collagen gel, control AVC explants produced a halo of invasive, migrating cells with mesenchymal morphology (Fig. 4A). By contrast, mutant explants failed to generate mesenchymal cells (Fig. 4B,C). Immunostaining with antibodies for SMA and Pecam delineated three classes of endothelial cells at different stages of activation in all explants examined: (1) rounded cells expressing Pecam but not SMA (arrow, Fig. 4D); (2) rare, round transitional cells expressing SMA (white arrowhead, Fig. 4D); and (3) elongated, SMA-expressing cells (yellow arrowhead, Fig. 4D). By contrast, explants from  $Gata4^{T2del}$  embryos failed to produce mesenchymal, SMA-expressing cells (Fig. 4E). Mutant endothelium appeared multi-layered in a manner reminiscent of the morphology of sectioned  $Gata4^{T2del}$  hearts (star, Fig. 4E; arrow, Fig. 2G). None of the aforementioned signs of endothelial cell activation were detected in explants examined by immunostaining for Pecam and SMA. These data suggest that endothelial recombination of *Gata4* impairs the activation of endothelial cells and blocks their transformation to invasive mesenchyme.

Having established an essential role for *Gata4* in the transformation of AVC endocardial cells to mesenchymal cells, we sought to elucidate the mechanism by which *Gata4* acts in this process. Because adjacent myocardium is known to influence endocardial EMT, we investigated whether the endocardial inactivation of *Gata4* blocked EMT in a non-cell-autonomous manner. Myocardial differentiation and specification of atrioventricular canal myocardium remained intact, as indicated by the patterns of expression of sarcomeric myosin, the myocardial



**Table 2. Genes differentially expressed between control and *Gata4*<sup>T2del</sup> AVCs**

Rank	Probe set ID	Gene symbol	Gene name	UniGene ID	Fold change
<b>Downregulated genes</b>					
1	1418788_at	Tek	Endothelial-specific receptor tyrosine kinase	Mm.14313	-1.6
2	1423213_at	Plxnc1 <sup>a,c</sup>	Plexin C1	Mm.256712	-1.8
3	1434606_at	ErbB3 <sup>a,c</sup>	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	Mm.29023	-2.1
4	1421811_at	Thbs1 <sup>a,c</sup>	Thrombospondin 1	Mm.4159	-2.2
5	1426294_at	Hapln1	Hyaluronan and proteoglycan link protein 1	Mm.266790	-1.8
6	1456144_at	Nav3	Neuron navigator 3	Mm.225050	-1.8
7	1416342_at	Tnca	Tenascin C	Mm.980	-2.0
8	1444403_at	Cbfa2t2h	Core-binding factor, runt domain, alpha subunit 2, translocated to, 2 homolog (human) (Cbfa2t2h), mRNA	Mm.29914	-1.5
9	1449303_at	Sesn3	Sestrin 3	Mm.227443	-1.7
10	1422286_a_at	Tgjf	TG interacting factor	Mm.101034	-2.2
11	1421908_a_at	Tcf12	Transcription factor 12	Mm.171615	-1.6
12	1448293_at	Ebf1	Early B-cell factor 1	Mm.215971	-1.5
13	1426295_at	Hapln1	Hyaluronan and proteoglycan link protein 1	Mm.266790	-1.9
14	1442019_at	-	16 days neonate cerebellum cDNA, RIKEN full-length enriched library, clone:9630033M11 product: unclassifiable, full insert sequence	Mm.152121	-2.7
15	1435727_s_at	D15Erdt366e	DNA segment, Chr 15, ERATO Doi 366, expressed	Mm.33207	-1.5
16	1452366_at	4732435N03Rik	RIKEN cDNA 4732435N03 gene	Mm.334569	-2.6
17	1432029_a_at	Smap1	Stromal membrane-associated protein 1	Mm.329963	-1.9
18	1434957_at	Cdon	Cell adhesion molecule-related/downregulated by oncogenes	Mm.80509	-1.8
19	1434510_at	Papss2	3'-phosphoadenosine 5'-phosphosulfate synthase 2	Mm.203916	-3.9
20	1437401_at	Igf1b	Insulin-like growth factor 1	Mm.268521	-2.0
21	1434413_at	-	Transcribed locus	Mm.380595	-2.1
22	1451740_at	Paip1	Polyadenylate binding protein-interacting protein 1	Mm.132584	-2.0
23	1439641_at	LOC553090	Hypothetical LOC553090	-	-2.4
24	1418120_at	Rbm8a	RNA binding motif protein 8a	Mm.261972	-1.5
25	1426862_at	9130023F12Rik	RIKEN cDNA 9130023F12 gene	Mm.356580	-1.7
26	1417625_s_at	Cmkor1	Chemokine orphan receptor 1	Mm.6522	-2.3
27	1421917_at	Pdgfra	Platelet derived growth factor receptor, alpha polypeptide	Mm.221403	-1.7

specific transcription factor *Nkx2-5*, the chamber myocardium marker *Nppa*, and the AVC myocardial marker *Tbx2* (Fig. 5; data not shown). Myocardial expression of *Bmp2* and *Tgfβ2*, known paracrine activators of EMT (Nakajima et al., 2000b; Rivera-Feliciano and Tabin, 2006; Sugi et al., 2004), was unchanged in *Gata4*<sup>T2del</sup> hearts (data not shown). Furthermore, culture of control AVC explants directly next to *Gata4*<sup>T2del</sup> explants did not rescue the EMT defect in mutant explants. Similarly, culture of explants in the presence of a mixture of growth factors known to promote EMT (*Tgfβ2*, *Bmp2*, heregulin and hyaluronic acid) did not rescue the EMT defect in mutant explants, but did robustly stimulate the formation of mesenchymal cells in control explants. These data suggest that the phenotype of *Gata4*<sup>T2del</sup> explants was not due to an absence of diffusible factor(s) produced by the myocardium that promote EMT.

We also considered the possibility that *Gata4* could regulate EMT through modulation of the extracellular matrix, which is necessary for cushion mesenchyme formation (Camenisch et al., 2000). However, the extracellular matrix of the AV cushions was still present in *Gata4*<sup>T2del</sup> hearts, as assessed by binding of the stain Alcian Blue to acidic glycosaminoglycans present in the cushion extracellular matrix (Fig. 5D). Moreover, addition hyaluronic acid directly to explant cultures did not rescue the EMT defect of mutant explants (data not shown).

### ***Gata4* regulates expression of *ErbB3***

We next turned our attention to cell-autonomous mechanisms that could account for the loss of EMT in *Gata4*<sup>T2del</sup> embryos. Downregulation of *Snail* as a result of *Notch* signaling leads to

downregulation of *VE-cadherin* (*Cdh5*), and is necessary for endocardial EMT (Timmerman et al., 2004). In *Gata4*<sup>T2del</sup> hearts, we did not find altered expression of components of this pathway (*Notch1*, *HRT1* and *Snail*) by in situ hybridization (Fig. 5C; data not shown) or of *Cdh5* by immunohistochemistry (Fig. 5E).

To identify genes whose altered expression might contribute to the *Gata4*<sup>T2del</sup> phenotype, we performed genome-wide expression analysis using microarrays. We isolated RNA from the AV region of mutant and control E9.5 hearts (between 30 and 33 somites), and used the RNA to probe Affymetrix microarrays. We used the SAM algorithm (Tusher et al., 2001) to identify genes with significantly altered expression (Table 2). For a subset of these, we used qRT-PCR to validate differences in gene expression between mutant and control tissue (Fig. 6A). Out of 11 genes tested, qRT-PCR confirmed differential gene expression in four: *ErbB3*, thrombospondin 1 (*Thbs1*), plexin C1 (*Plxnc1*), and tenascin C (*Tnc*; Fig. 6A). We also confirmed differential expression of *ErbB3*, *Thbs1* and *Plxnc1* by in situ hybridization (Fig. 6B).

The downregulation of *ErbB3* in *Gata4*<sup>T2del</sup> AV tissue was of particular interest. EGF family ligands signal through *ErbB3* to activate *Ras* and promote EMT, and in the absence of *ErbB3* AV endothelium fails to form cushion mesenchyme (Camenisch et al., 2002). We confirmed downregulation of *ErbB3* transcripts by qRT-PCR (Fig. 6A) and in situ hybridization (Fig. 6B), and downregulation of *ErbB3* protein by western blotting (Fig. 6C).

To obtain further evidence that *Gata4* regulates *ErbB3* expression, we investigated whether ectopic expression of *Gata4* in endothelial cells that normally lack this transcription factor is sufficient to

Table 2. Continued

Rank	Probe set ID	Gene symbol	Gene name	UniGene ID	Fold change
<b>Upregulated genes</b>					
1	1449187_at	Pdgfa <sup>a</sup>	Platelet derived growth factor, alpha	Mm.2675	1.6
2	1439713_at	Itga1	Integrin alpha 1	–	1.5
3	1423100_at	Fos	FBJ osteosarcoma oncogene	Mm.246513	1.9
4	1426114_at	Hnnpab	Heterogeneous nuclear ribonucleoprotein A/B	Mm.280842	1.7
5	1421126_at	Ryr2	Ryanodine receptor 2, cardiac	Mm.239871	1.7
6	1433657_at	A130092J06Rik	RIKEN cDNA A130092J06 gene	Mm.250441	1.5
7	1415949_at	Cpe	Carboxypeptidase E	Mm.31395	1.5
8	1419647_a_at	Ier3	Immediate early response 3	Mm.25613	1.6
9	1456768_a_at	–	–	–	1.8
10	1430781_at	Ak7	Adenylate kinase 7	Mm.36006	1.6
11	1425196_a_at	Hint2	Histidine triad nucleotide binding protein 2	Mm.25285	1.5
12	1420991_at	Ankrd1 <sup>b</sup>	Ankyrin repeat domain 1 (cardiac muscle)	Mm.10279	1.8
13	1458917_at	–	–	–	1.9
14	1439518_at	–	–	–	1.8
15	1449897_a_at	Mtcp1	Mature T-cell proliferation 1	Mm.16366	1.5
16	1460519_a_at	Mettl5	Methyltransferase like 5	Mm.268657	1.7
17	1447845_s_at	Vnn1	Vanin 1	Mm.27154	1.7
18	1445938_at	5930427L02Rik	RIKEN cDNA 5930427L02 gene	Mm.379523	1.7
19	1418486_at	Vnn1	Vanin 1	Mm.27154	1.5
20	1422529_s_at	Casq2	Calsequestrin 2	Mm.15343	1.6
21	1429317_at	Qrs1	Glutaminyl-tRNA synthase (glutamine-hydrolyzing)-like 1	Mm.5904	1.6
22	1417313_at	Lsm7	LSM7 homolog, U6 small nuclear RNA associated ( <i>S. cerevisiae</i> )	Mm.379101	3.8
23	1451370_at	–	Adult male kidney cDNA, RIKEN full-length enriched library, clone:0610042I08 product:RNA component of mitochondrial RNAase P, 1, full insert sequence	Mm.289109	1.7
24	1430787_at	2310050B05Rik	RIKEN cDNA 2310050B05 gene	–	1.6
25	1455251_at	Itga1	Integrin alpha 1	–	1.6
26	1431248_at	5031426D15Rik	RIKEN cDNA 5031426D15 gene	–	1.6
27	1426902_at	Coq6	Coenzyme Q6 homolog (yeast)	Mm.280062	1.6
28	1437904_at	MGI:2387367	Developmentally regulated RNA binding protein 1	Mm.33310	2.1
29	1428960_at	4933434I06Rik	RIKEN cDNA 4933434I06 gene	Mm.27658	1.5
30	1417150_at	Slc6a4	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	Mm.300318	2.2
31	1455227_at	Aadacl1	Arylacetamide deacetylase-like 1	Mm.24576	1.6
32	1434865_a_at	Exoc7	Exocyst complex component 7	Mm.22530	1.7
33	1435436_at	–	Transcribed locus	Mm.234875	2.2
34	1417713_at	Eif2s2	Eukaryotic translation initiation factor 2, subunit 2 (beta)	Mm.383218	1.7
35	1452840_at	1500009L16Rik	RIKEN cDNA 1500009L16 gene	Mm.271188	2.4
36	1440397_at	–	–	–	2.0
37	1430023_at	5133400G04Rik	RIKEN cDNA 5133400G04 gene	Mm.151498	1.6
38	1427263_at	Xist	Inactive X specific transcripts	–	1.9
39	1449146_at	Notch4	Notch gene homolog 4 ( <i>Drosophila</i> )	Mm.173813	2.8
40	1436723_at	Fshprh1	FSH primary response 1	Mm.34903	1.6

Genes differentially expressed between between control ( $n=2$ ) and mutant ( $n=4$ ) were identified by SAM ( $\Delta>0.4$  and fold-change  $>1.5$ ) and ranked by the relative difference score.

<sup>a</sup>Differential expression validated by qRT-PCR ( $P<0.05$ ).

<sup>b</sup>Differential expression failed validation by qRT-PCR ( $P>0.05$ ).

<sup>c</sup>Differential expression validated by in situ hybridization.

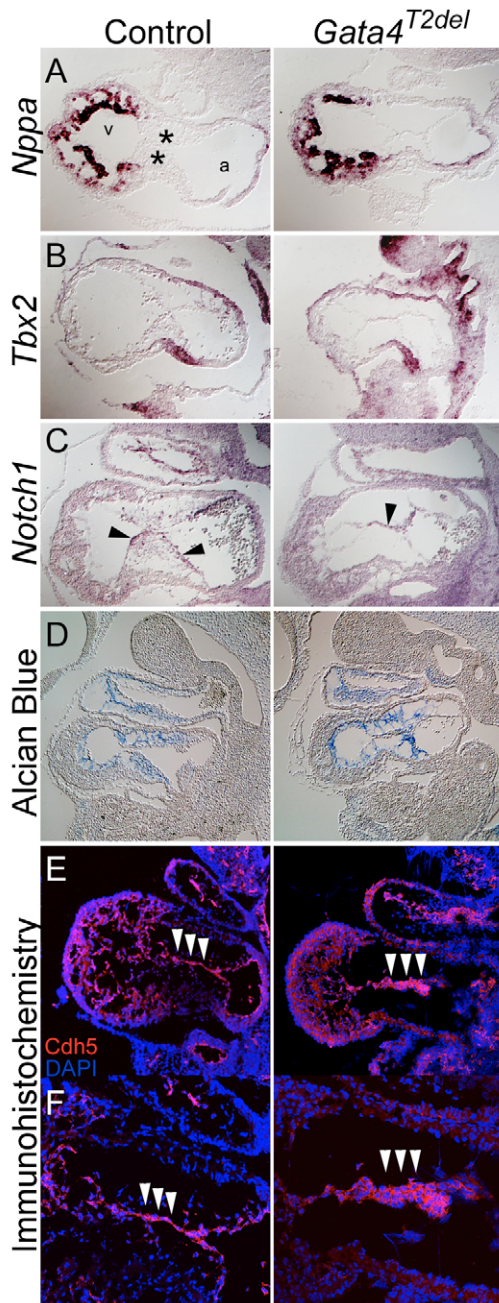
activate *ErbB3* expression. Adenoviral expression of *Gata4* in human umbilical vein endothelial cells (HUVEC) caused a 5-fold upregulation of *ErbB3* expression compared with cells treated with a GFP-expressing adenovirus (Fig. 6D). The degree of upregulation is likely to be even higher in *Gata4*-expressing cells, as adenovirus transduced only 15–20% of cells. Conversely, we investigated whether *Gata* factors are necessary for *ErbB3* expression in at least some cellular contexts. For this experiment, we used BT20 breast carcinoma cells, which natively express both *Gata4* and *ErbB3* proteins (Bouchard et al., 2005) (data not shown). Expression of a dominant-negative *Gata4*DBD-engrailed fusion protein strongly decreased *ErbB3* transcript levels (Fig. 6E).

To determine whether *Gata4* regulates *ErbB3* expression at the level of transcription, we used rVista 2.0 (Loots and Ovcharenko, 2004) to find *ErbB3* non-coding sequences

conserved between mouse and human. Conserved non-coding sequences upstream of the putative transcriptional start site and within the first intron were used to drive expression of luciferase reporters. We found that co-transfected *Gata4* stimulated transcription from both the promoter and the intronic enhancer (Fig. 6F). By contrast, the truncated protein produced by the *Gata4*<sup>Δex2</sup> allele failed to stimulate transcription from these *ErbB3* regulatory elements (Fig. 6F). These regulatory elements contain three predicted GATA motifs conserved between mouse and human. In mobility shift experiments, the two consensus sites in the enhancer strongly bound *Gata4*, whereas the site in the promoter (nonconsensus GATG site) did not. Mutation of these sites did not significantly alter transcriptional stimulation by *Gata4* (see Fig. S5 in the supplementary material).

### **Gata4 regulates an *ErbB3*-*Erk* pathway required for EMT**

Next, we wished to determine whether *Gata4*<sup>T2del</sup> AV endothelium was functionally deficient in the transduction of *Egf* signals. We treated E9.5 *Gata4*<sup>T2del</sup> and control embryos with the *ErbB3* ligand



**Fig. 5. Expression of myocardial and endocardial markers in *Gata4*<sup>T2del</sup> mutant hearts.** (A,B) In situ hybridization for *Nppa* (A) and *Tbx2* (B) in E9.5 embryos, showing intact expression in *Gata4*<sup>T2del</sup> hearts. Asterisk indicates the EC. (C) In situ hybridization for *Notch1* in E10.5 embryos, showing unperturbed expression in AV endocardium (arrowheads). (D) Alcian Blue staining of acidic glycosaminoglycans, including hyaluronic acid, in E10.5 control and mutant cushions. (E,F) *Cdh5* immunostaining of E9.5 embryo sections. Note the bunched up phenotype of the mutant AV endocardium compared with control AV endocardium (arrowheads). Panels in F are higher magnifications of the sections shown in E.

heregulin, and measured the phosphorylation of *Erk*, which is activated downstream of *Ras*. In heregulin-treated control embryos, activated *Erk* was readily observed in AV cushion endothelium (arrowheads, Fig. 7A, middle panel). By contrast, in heregulin-treated *Gata4*<sup>T2del</sup> embryos, *Erk* activation was strongly reduced in AV cushion endothelium (arrowheads, Fig. 7A, bottom panel). *Erk* activation in myocardium did not differ between genotypes (arrows, Fig. 7A), indicating that the downregulation in cushion endothelium was specific and unlikely to be due to technical factors.

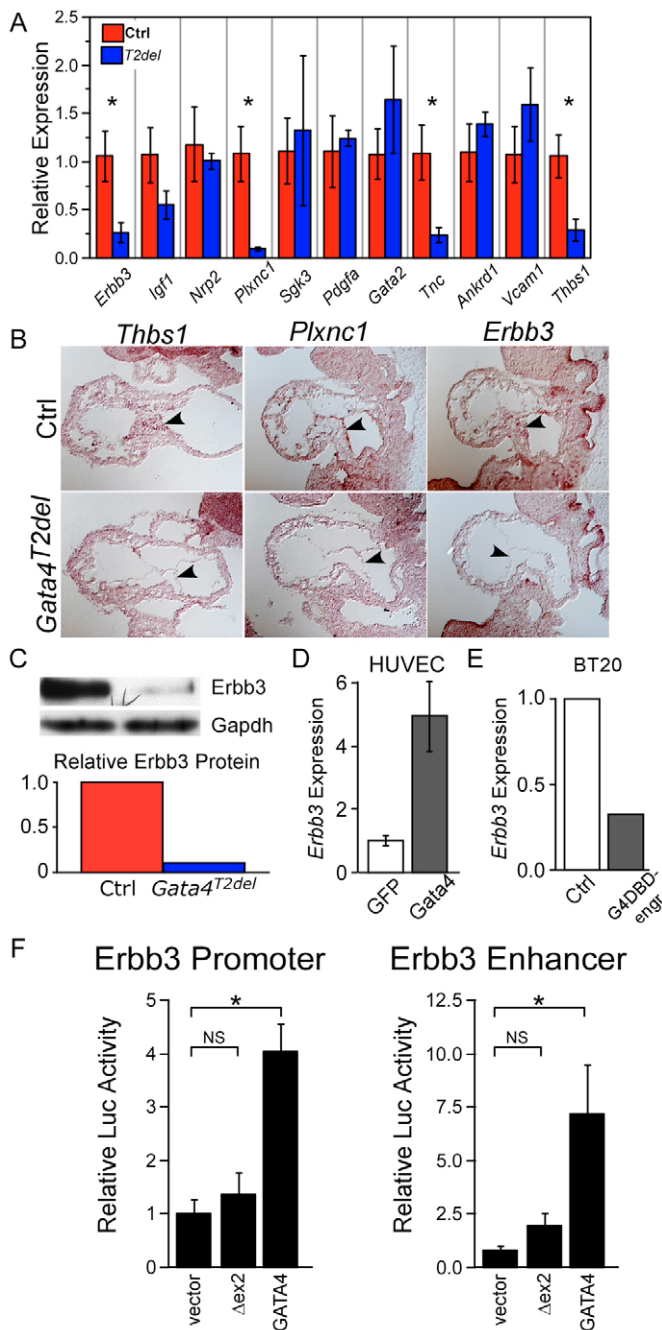
We then investigated whether defective *Erk* activation downstream of *ErbB3* might account for the marked reduction in EMT seen in the *Gata4*<sup>T2del</sup> AV cushions. We treated control and mutant explants with U0126, a selective inhibitor of *Erk* activation. We found that U0126 strongly reduced the extent of EMT in wild-type explants (Fig. 7B). We reasoned that if *Gata4* and *Erk* are mutually required for EMT, then partial antagonism of each would inhibit EMT. To test this hypothesis, we generated AVC explants homozygous for *Gata4*<sup>H</sup>, a *Gata4* allele that expresses reduced levels of *Gata4* protein (Pu et al., 2004). We treated *Gata4*<sup>H/H</sup> and littermate control explants with a 50% inhibitory concentration of U0126 (400 nM). In the presence of vehicle, *Gata4*<sup>H/H</sup> and control explants generated comparable numbers of mesenchymal cells. However, the number of mesenchymal cells generated by *Gata4*<sup>H/H</sup> explants treated with 400 nM U0126 was significantly reduced when compared with the number generated by similarly treated control explants (Fig. 7C). This result is consistent with a model in which *Gata4* and *Erk* act in the same genetic pathway to promote EMT.

### **Gata4 is required for growth and remodeling of the AV cushions**

Constitutive, partial loss of *Gata4* function, as a result of either decreased protein expression (*Gata4*<sup>H/H</sup>) or a point mutation abrogating *Fog1* and *Fog2* (*Zfp1* and *Zfp2*, respectively – Mouse Genome Informatics) interaction (*Gata4*<sup>Ki/Ki</sup>), resulted in embryonic lethality between E12.5–E16.5 (Crispino et al., 2001; Pu et al., 2004). These embryos had common atrioventricular canal defects, indicating a severe abnormality of EC maturation such that the superior and inferior cushions failed to fuse and divide the ventricular inflow tract into separate inlets for the right and left ventricle. This abnormality could, in principle, have been caused by impaired *Gata4* function in the endocardium, or by defective *Gata4* function in the myocardium with secondary abnormalities in the EC due to abnormal paracrine signaling. To determine whether there is a cell-autonomous requirement for *Gata4* in later atrioventricular valve maturation, we generated embryos with the genotype *Gata4*<sup>fllox/Ki</sup>; *T2Cre*<sup>+</sup> (abbreviated *Gata4*<sup>T2del/Ki</sup>). In these embryos, *Gata4*<sup>fllox</sup> complements *Gata4*<sup>Ki</sup> except in the endothelium and its derivatives, where *T2Cre*-mediated recombination inactivates it.

*Gata4*<sup>Ki/fllox</sup>; *T2Cre*<sup>-</sup> control mice were present at weaning at the expected Mendelian frequency and had no obvious heart defects. By contrast, *Gata4*<sup>T2del/Ki</sup> embryos were present in the expected Mendelian ratio at E16.5, but were not present at weaning (out of eight litters genotyped), indicating lethality in late gestation or in the perinatal period. Mutant embryos examined at E16.5 had severe peripheral hemorrhage and edema, consistent with heart failure (data not shown). At E12.5, the heart (Fig. 8A–D) and the liver appeared normal (Fig. 8A,B). However, in later stage embryos, the AV valve leaflets were hypoplastic, and failure of fusion of the superior and inferior AV cushions resulted in a common atrioventricular canal defect (asterisk, Fig. 8E,F). Cell death was not increased in mutant cushions, as measured by TUNEL staining at E12.5 and E15.5 (data not shown). However, cell proliferation, as measured by BrdU





**Fig. 6. *Gata4* regulates *Erbb3* expression.** (A) qRT-PCR of control and mutant AVC samples. Gene expression was normalized to *Gapdh*. \* $P < 0.05$ ,  $n = 3$  per group. (B) In situ hybridization demonstrating downregulation of *Thbs1*, *Plxnc1* and *Erbb3* in *Gata4*<sup>T2del</sup> EC. Arrowheads indicate AV cushions. (C) Downregulation of *Erbb3* protein in AVC whole cell lysates by western blotting. Each sample contained AVCs pooled from 10 hearts. Expression was quantitated by densitometry and normalized to *Gapdh*. (D) Adenoviral expression of *Gata4* upregulated *Erbb3* in HUVEC cells, compared with GFP expression from a control virus. *Erbb3* expression was measured by qRT-PCR and normalized to *Gapdh*. The graph shows the average of three independent experiments. (E) BT20 cells were co-transfected with an EGFP expression vector and either *Gata4*DBD-engrailed or an empty expression vector. After sorting for transfected (GFP-expressing) cells, *Erbb3* expression was measured by qRT-PCR and normalized to *Gapdh*. Results are representative of two independent experiments. (F) Constructs containing *Erbb3* promoter (−1356 to +214) and intron 1 enhancer (+104 to +3076) sequences driving luciferase were co-transfected with *Gata4* or *Gata4* <sup>$\Delta$ ex2</sup> expression constructs into BT20 cells. Luciferase activity was normalized to pRL-null. Results are the average of five (enhancer) or eight (promoter) independent experiments (\* $P < 0.05$ ).

The AV cushions are largely formed by mesenchyme derived from endothelial cells (see Fig. 1B, Fig. 3). Inactivation of *Gata4* within endothelial-derived cells results in a marked decrease in the number of mesenchymal cells in the AV cushions (Fig. 2). By contrast, the OT cushions are formed by cells derived from at least two sources. OT endothelium undergoes EMT to form the mesenchyme of the proximal OT cushions, whereas neural crest contributes to the mid- and distal OT cushions (Fig. 1B; Fig. 3; see also Fig. S2 in the supplementary material). *Gata4* is expressed in both the neural crest-derived and endothelial-derived portions of the OT cushions. However, its expression in the neural crest-derived portion is not required for normal OT development, as *Gata4* inactivation by *Wnt1Cre* was compatible with normal survival and normal OT morphogenesis (W.T.P., unpublished). *Gata4* expression was required to form the proximal, endothelial-derived portion of the OT cushion (Fig. 3). Because *Gata4*<sup>T2del</sup> embryos do not survive to a stage at which mature OT valves are evident, we were unable to determine what effect the loss of this portion of OT cushion has on OT valve development. Congenital abnormalities of the pulmonary valve are associated with human *GATA4* mutation (Garg et al., 2003; Hirayama-Yamada et al., 2005; Okubo et al., 2004), suggesting that *Gata4* activity within the proximal, endothelial-derived portion of the OT cushions may be important for development of the OT valves.

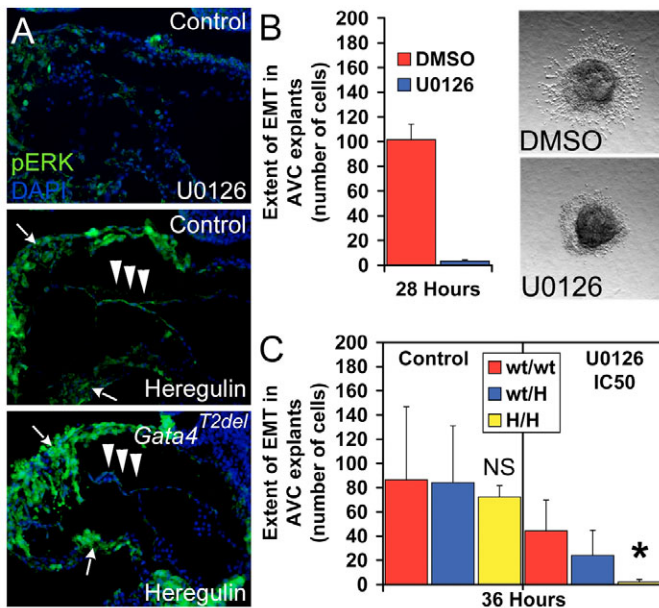
### ***Gata4* function in formation of AV mesenchyme**

Inactivation of *Gata4* within endothelial-derived cells blocked endocardial EMT, resulting in a paucity of mesenchymal cells within the AV cushions. This was associated with strong downregulation of *Erbb3* (Fig. 6). Using heterologous expression systems, we show that *Gata4* modulates *Erbb3* transcript levels and transcriptional activity of *Erbb3* regulatory elements (Fig. 6F). Although these regulatory elements contain evolutionarily conserved GATA binding sites, these binding sites were not required for transcriptional stimulation by *Gata4* in vitro (see Fig. S5 in the supplementary material). These data suggest that *Gata4* may regulate *Erbb3* indirectly. Alternatively, these findings might

uptake, was decreased in mutant cushion mesenchyme at E12.5 and E13.5 (Fig. 8G, data not shown). These data suggest that *Gata4-Fog* interaction within endocardium-derived cells is required for normal proliferation of the AV cushions.

### **DISCUSSION**

We used a *T2Cre* transgene to recombine a conditional *Gata4* allele (*Gata4*<sup>lox</sup>) within endothelial-derived cells and demonstrated that *Gata4* is required at two stages of AVC morphogenesis. First, *Gata4* is necessary for endothelial EMT to form AV cushion mesenchyme. Second, *Gata4* is necessary for the growth and remodeling of the AV cushions, after they have been populated by mesenchymal cells. *Gata4* interaction with *Fog* cofactors within endothelial-derived cells is dispensable for the former, but required for the latter.



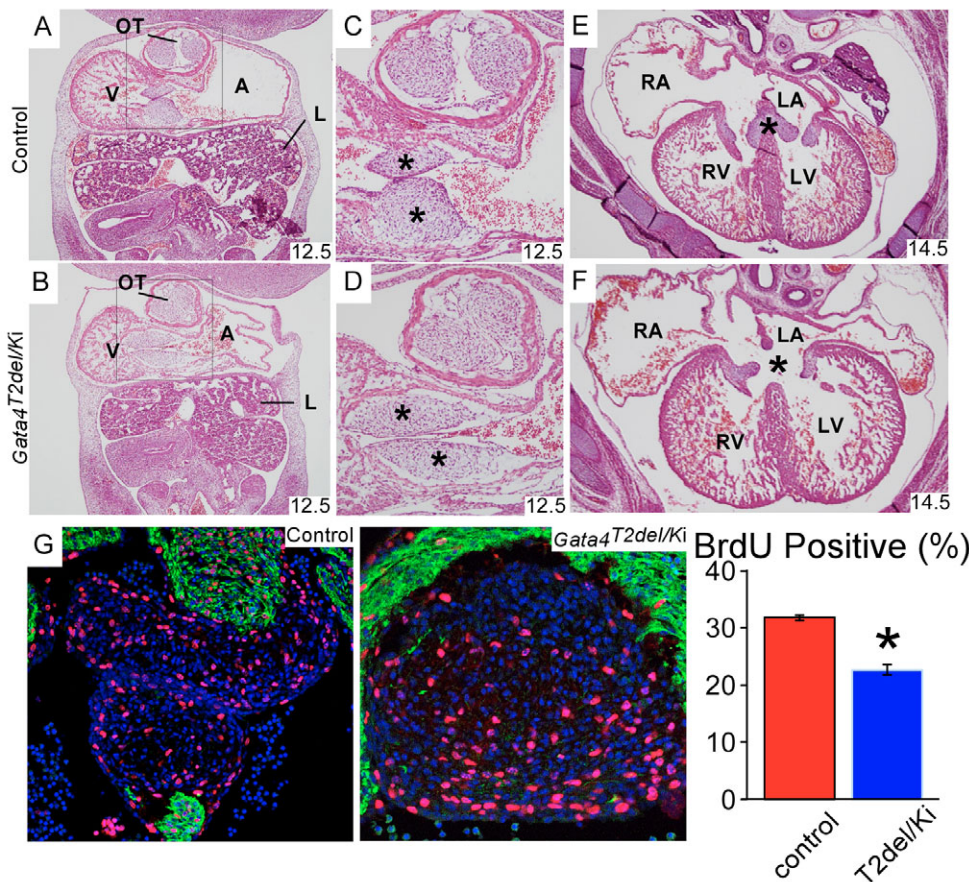
**Fig. 7. *Gata4* regulates an ErbB3-Erk pathway required for EMT.**

(A) E9.5 control or *Gata4*<sup>T2del</sup> embryos were cultured in M199 containing 100 ng/ml heregulin. A phosphospecific Erk antibody (pErk; green staining) showed defective Erk activation in the endocardium of mutant embryos (arrowheads, bottom panel) compared with control embryos (arrowheads, middle panel). Myocardial pErk levels were unchanged between genotypes. To demonstrate antibody specificity, a control embryo was incubated with U0126, a selective inhibitor of Erk activation (top panel). (B) U0126 (10  $\mu$ M) blocked the formation of mesenchymal cells in wild-type AV explants. (C) Synthetic phenotype resulting from the combination of reduced *Gata4* expression and partial Erk inhibition. Explants from embryos homozygous for a hypomorphic *Gata4* allele (H/H) underwent EMT at the same rate as controls in the presence of vehicle (NS, no significant difference). However, in the presence of U0126 at a 50% inhibitory dose, EMT was strongly inhibited in hypomorphic explants ( $*P < 0.05$  compared with control-treated H/H or U0126-treated wt/wt;  $n = 4$ ). The extent of EMT was measured by the number of cells with mesenchymal morphology that invaded the collagen gel.

represent limitations of the in vitro assay system. Additional in vivo studies of *ErbB3* regulatory elements will be necessary to elucidate the mechanism by which *Gata4* regulates *ErbB3* in the endocardial cushions.

Downregulation of *ErbB3* in *Gata4*<sup>T2del</sup> AVCs was associated with impaired Erk activation in response to the *ErbB3* ligand heregulin (Fig. 7A). Erk activation was necessary for endocardial EMT (Fig. 7B). Partial inhibition of both *Gata4* and Erk impaired the formation

of cushion mesenchyme (Fig. 7C). This synthetic phenotype suggests a strong interaction between *Gata4*- and *Erk*-dependent pathways. Collectively, these data are consistent with a model in which *Gata4* functions upstream of an *ErbB3*-*Ras*-*Erk* pathway that is necessary for the formation of cushion mesenchyme. Intriguingly, *Gata4* itself is activated by Erk phosphorylation (Liang et al., 2001), suggesting the possibility of a positive-feedback loop that promotes endocardial EMT.



**Fig. 8. AV valve remodeling requires *Gata4* expression in endocardium and its derivatives.**

*Gata4*<sup>T2del/Ki</sup> (*Gata4*<sup>Kilflox</sup>; *Tie2*<sup>Cre</sup>) embryos are deficient for *Gata4*-Fog interaction in the endocardium and its derivatives. (A-D) AV cushions were normally populated by mesenchymal cells at E12.5 (asterisks, C,D), indicating that the *Gata4*-Fog interaction is not essential for EMT. The liver (L) was normal in size and morphology. Boxed regions of A,B are enlarged in C,D. (E,F) A common atrioventricular canal (asterisk) was present in all *Gata4*<sup>T2del/Ki</sup> embryos at E14.5, whereas in control embryos the AVC was septated by the fusion of the superior and inferior cushions. (G) Decreased proliferation of cushion mesenchyme in *Gata4*<sup>T2del/Ki</sup> embryos. Proliferation was measured as the percentage of nuclei that stained positive for BrdU (red). Nuclei were stained with DAPI. Myocytes were labeled with desmin (green). Three sections from three different E12.5 embryos were examined per group by a blinded observer ( $*P < 0.05$ ).



Although *Gata4* is required for AV EMT, *Gata4* interaction with Fog is dispensable for this step of AV valve development. Embryos deficient in *Fog1* or *Fog2*, or in *Gata4-Fog* interactions (*Gata4<sup>Ki/Ki</sup>*), did not show any defect in the generation of AV valve mesenchyme by EMT (Crispino et al., 2001; Katz et al., 2003; Tevosian et al., 2000), indicating that *Gata4* regulation of AV cushion EMT does not require Fog interaction. Consistent with this conclusion, *Gata4<sup>T2del/Ki</sup>* EC were normally populated with mesenchymal cells (Fig. 8A-D).

### Gata4 function in AV valve growth and remodeling

After AV cushion mesenchyme is formed by EMT, it rapidly proliferates to fill the expanding AV cushions with mesenchymal cells. The growing superior and inferior AV cushions meet and fuse, dividing the ventricular inlet into a right and left channel. *Gata4* is necessary for this process, as a decrease in *Gata4* levels or germline abrogation of the *Gata4-Fog* interaction resulted in an unseptated ventricular inlet (Crispino et al., 2001; Pu et al., 2004). Here, we show that *Gata4-Fog* interaction within endothelial-derived cells is required for septation of the ventricular inlet (Fig. 8).

### Conclusion

This study demonstrates an essential role for expression of *Gata4* in the endothelium and its derivatives. During the initial steps of AV valve formation, *Gata4* is necessary for the expression of *ErbB3*, which acts through a *Ras-Erk* pathway to promote EMT. During subsequent steps of AV valve maturation, *Gata4*, in cooperation with a Fog cofactor, promotes the growth and fusion of the AV cushions, resulting in the division of the ventricular inlet into two separate channels guarded by two AV valves. These data suggest that *Gata4* mutations might contribute to EC defects in humans; indeed, a *Gata4* mutation has been reported to occur in association with AV septal defects (Garg et al., 2003).

The authors thank J. L. Galloway for comments on the manuscript, and members of the Tabin, Cepko, Dymecki, Izumo and Pu labs for their advice and support. This work was supported by grants from the NIH (C.J.T., R01 HD0045499; W.T.P., K08 HL004387-04 and 1 PO1 HL074734) and by a charitable donation from Edward Marran and Karen Carpenter. J.R.-F. was supported by a predoctoral National Research Service Award.

### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/133/18/3607/DC1>

### References

- Armstrong, E. J. and Bischoff, J. (2004). Heart valve development: endothelial cell signaling and differentiation. *Circ. Res.* **95**, 459-470.
- Bouchard, M. F., Taniguchi, H. and Viger, R. S. (2005). Protein kinase A-dependent synergism between GATA factors and the nuclear receptor, liver receptor homolog-1, regulates human aromatase (CYP19) PII promoter activity in breast cancer cells. *Endocrinology* **146**, 4905-4916.
- Brent, A. E., Schweitzer, R. and Tabin, C. J. (2003). A somitic compartment of tendon progenitors. *Cell* **113**, 235-248.
- 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.
- Camenisch, T. D., Spicer, A. P., Brehm-Gibson, T., Biesterfeldt, J., Augustine, M. L., Calabro, A., Jr, Kubalak, S., Klewer, S. E. and McDonald, J. A. (2000). Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme. *J. Clin. Invest.* **106**, 349-360.
- Camenisch, T. D., Schroeder, J. A., Bradley, J., Klewer, S. E. and McDonald, J. A. (2002). Heart-valve mesenchyme formation is dependent on hyaluronan-augmented activation of ErbB2-ErbB3 receptors. *Nat. Med.* **8**, 850-855.
- Charron, F. and Nemer, M. (1999). GATA transcription factors and cardiac development. *Semin. Cell Dev. Biol.* **10**, 85-91.
- Crispino, J. D., Lodish, M. B., Thurberg, B. L., Litovsky, S. H., Collins, T., Molkentin, J. D. and Orkin, S. H. (2001). Proper coronary vascular development and heart morphogenesis depend on interaction of GATA-4 with FOG cofactors. *Genes Dev.* **15**, 839-844.
- Danielian, P. S., Muccino, D., Rowitch, D. H., Michael, S. K. and McMahon, A. P. (1998). Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. *Curr. Biol.* **8**, 1323-1326.
- Garg, V., Kathiriyai, I. S., Barnes, R., Schluterman, M. K., King, I. N., Butler, C. A., Rothrock, C. R., Eapen, R. S., Hirayama-Yamada, K., Joo, K. et al. (2003). GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5. *Nature* **424**, 443-447.
- Gitler, A. D., Zhu, Y., Ismat, F. A., Lu, M. M., Yamauchi, Y., Parada, L. F. and Epstein, J. A. (2003). Nf1 has an essential role in endothelial cells. *Nat. Genet.* **33**, 75-79.
- Heikinheimo, M., Scandrett, J. M. and Wilson, D. B. (1994). Localization of transcription factor GATA-4 to regions of the mouse embryo involved in cardiac development. *Dev. Biol.* **164**, 361-373.
- Hirayama-Yamada, K., Kamisago, M., Akimoto, K., Aotsuka, H., Nakamura, Y., Tomita, H., Furutani, M., Imamura, S., Takao, A., Nakazawa, M. et al. (2005). Phenotypes with GATA4 or NKX2.5 mutations in familial atrial septal defect. *Am. J. Med. Genet. A* **135**, 47-52.
- Katz, S. G., Williams, A., Yang, J., Fujiwara, Y., Tsang, A. P., Epstein, J. A. and Orkin, S. H. (2003). Endothelial lineage-mediated loss of the GATA cofactor Friend of GATA 1 impairs cardiac development. *Proc. Natl. Acad. Sci. USA* **100**, 14030-14035.
- Kisanuki, Y. Y., Hammer, R. E., Miyazaki, J., Williams, S. C., Richardson, J. A. and Yanagisawa, M. (2001). Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. *Dev. Biol.* **230**, 230-242.
- Kuo, C. T., Morrissey, E. E., Anandappa, R., Sigrist, K., Lu, M. M., Parmacek, M. S., Soudais, C. and Leiden, J. M. (1997). GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes Dev.* **11**, 1048-1060.
- Lakkis, M. M. and Epstein, J. A. (1998). Neurofibromin modulation of ras activity is required for normal endocardial-mesenchymal transformation in the developing heart. *Development* **125**, 4359-4367.
- Lee, K. H., Evans, S., Ruan, T. Y. and Lassar, A. B. (2004). SMAD-mediated modulation of YY1 activity regulates the BMP response and cardiac-specific expression of a GATA4/5/6-dependent chick Nkx2.5 enhancer. *Development* **131**, 4709-4723.
- Lee, Y., Shioi, T., Kasahara, H., Jobe, S. M., Wiese, R. J., Markham, B. E. and Izumo, S. (1998). The cardiac tissue-restricted homeobox protein Csx/Nkx2.5 physically associates with the zinc finger protein GATA4 and cooperatively activates atrial natriuretic factor gene expression. *Mol. Cell. Biol.* **18**, 3120-3129.
- Liang, Q., Wiese, R. J., Bueno, O. F., Dai, Y. S., Markham, B. E. and Molkentin, J. D. (2001). The transcription factor gata4 is activated by extracellular signal-regulated kinase 1- and 2-mediated phosphorylation of serine 105 in cardiomyocytes. *Mol. Cell. Biol.* **21**, 7460-7469.
- Lobe, C. G., Koop, K. E., Kreppner, W., Lomeli, H., Gertsenstein, M. and Nagy, A. (1999). Z/AP, a double reporter for cre-mediated recombination. *Dev. Biol.* **208**, 281-292.
- Loots, G. G. and Ovcharenko, I. (2004). rVISTA 2.0: evolutionary analysis of transcription factor binding sites. *Nucleic Acids Res.* **32**, W217-W221.
- Mao, X., Fujiwara, Y. and Orkin, S. H. (1999). Improved reporter strain for monitoring Cre recombinase-mediated DNA excisions in mice. *Proc. Natl. Acad. Sci. USA* **96**, 5037-5042.
- Molkentin, J. D., Lin, Q., Duncan, S. A. and Olson, E. N. (1997). Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes Dev.* **11**, 1061-1072.
- Morrissey, E. E., Ip, H. S., Tang, Z. and Parmacek, M. S. (1997). GATA-4 activates transcription via two novel domains that are conserved within the GATA-4/5/6 subfamily. *J. Biol. Chem.* **272**, 8515-8524.
- Nakajima, H., Nakajima, H. O., Salcher, O., Dittie, A. S., Dembowsky, K., Jing, S. and Field, L. J. (2000a). Atrial but not ventricular fibrosis in mice expressing a mutant transforming growth factor-beta(1) transgene in the heart. *Circ. Res.* **86**, 571-579.
- Nakajima, Y., Yamagishi, T., Hokari, S. and Nakamura, H. (2000b). Mechanisms involved in valvuloseptal endocardial cushion formation in early cardiogenesis: roles of transforming growth factor (TGF)-beta and bone morphogenetic protein (BMP). *Anat. Rec.* **258**, 119-127.
- Okubo, A., Miyoshi, O., Baba, K., Takagi, M., Tsukamoto, K., Kinoshita, A., Yoshiura, K., Kishino, T., Ohta, T., Niikawa, N. et al. (2004). A novel GATA4 mutation completely segregated with atrial septal defect in a large Japanese family. *J. Med. Genet.* **41**, e97.
- Pelton, R. W., Nomura, S., Moses, H. L. and Hogan, B. L. (1989). Expression of transforming growth factor beta 2 RNA during murine embryogenesis. *Development* **106**, 759-767.
- Pierpont, M. E., Markwald, R. R. and Lin, A. E. (2000). Genetic aspects of atrioventricular septal defects. *Am. J. Med. Genet.* **97**, 289-296.
- Pu, W. T., Ishiwata, T., Juraszek, A. L., Ma, Q. and Izumo, S. (2004). GATA4 is a dosage-sensitive regulator of cardiac morphogenesis. *Dev. Biol.* **275**, 235-244.

- Rivera-Feliciano, J. and Tabin, C. J. (2006). Bmp2 instructs cardiac progenitors to form the heart-valve-inducing field. *Dev. Biol.* **295**, 580-588.
- Runyan, R. B. and Markwald, R. R. (1983). Invasion of mesenchyme into three-dimensional collagen gels: a regional and temporal analysis of interaction in embryonic heart tissue. *Dev. Biol.* **95**, 108-114.
- Sugi, Y., Yamamura, H., Okagawa, H. and Markwald, R. R. (2004). Bone morphogenetic protein-2 can mediate myocardial regulation of atrioventricular cushion mesenchymal cell formation in mice. *Dev. Biol.* **269**, 505-518.
- Tanaka, M., Chen, Z., Bartunkova, S., Yamasaki, N. and Izumo, S. (1999). The cardiac homeobox gene *Csx/Nkx2.5* lies genetically upstream of multiple genes essential for heart development. *Development* **126**, 1269-1280.
- Tevosian, S. G., Deconinck, A. E., Tanaka, M., Schinke, M., Litovsky, S. H., Izumo, S., Fujiwara, Y. and Orkin, S. H. (2000). FOG-2, a cofactor for GATA transcription factors, is essential for heart morphogenesis and development of coronary vessels from epicardium. *Cell* **101**, 729-739.
- Timmerman, L. A., Grego-Bessa, J., Raya, A., Bertran, E., Perez-Pomares, J. M., Diez, J., Aranda, S., Palomo, S., McCormick, F., Izpisua-Belmonte, J. C. et al. (2004). Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. *Genes Dev.* **18**, 99-115.
- Tusher, V. G., Tibshirani, R. and Chu, G. (2001). Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Natl. Acad. Sci. USA* **98**, 5116-5121.
- Verzi, M. P., McCulley, D. J., De Val, S., Dodou, E. and Black, B. L. (2005). The right ventricle, outflow tract, and ventricular septum comprise a restricted expression domain within the secondary/anterior heart field. *Dev. Biol.* **287**, 134-145.
- Watt, A. J., Battle, M. A., Li, J. and Duncan, S. A. (2004). GATA4 is essential for formation of the proepicardium and regulates cardiogenesis. *Proc. Natl. Acad. Sci. USA* **101**, 12573-12578.
- Zeisberg, E. M., Ma, Q., Juraszek, A. L., Moses, K., Schwartz, R. J., Izumo, S. and Pu, W. T. (2005). Morphogenesis of the right ventricle requires myocardial expression of Gata4. *J. Clin. Invest.* **115**, 1522-1531.