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Generation of conditional alleles for *Foxc1* and *Foxc2* in mice

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Abstract

The Forkhead box transcription factors *Foxc1* and *Foxc2* are crucial for development of the eye, cardiovascular network, and other physiological systems, but their cell type-specific and post-developmental functions are unknown, in part because conventional (i.e., whole-organism) homozygous-null mutations of either factor result in perinatal death. Here, we describe the generation of mice with conditional-null *Foxc1^{fllox}* and *Foxc2^{fllox}* mutations that are induced via Cre-mediated recombination. Mice homozygous for the unrecombined alleles are viable and fertile, indicating that the conditional alleles retain their wild-type function. The embryos of *Foxc1^{fllox}* or *Foxc2^{fllox}* mice crossed with *Cre*-deleter mice that are homozygous for the recombined allele (i.e., *Foxc1^{Δ/Δ}* or *Foxc2^{Δ/Δ}* embryos) lack expression of the corresponding gene and show the same developmental defects observed in conventional homozygous mutant embryos. We expect these conditional mutations to enable characterization of the cell-type specific functions of *Foxc1* and *Foxc2* in development, disease, and adult animals.

Keywords

conditional knockout; gene targeting; Cre recombination; *Foxc1*; *Foxc2*

Introduction

FoxC1 and *FoxC2* are closely related members of the Fox (Forkhead box) family of transcription factors (Benayoun *et al.*, 2011; Hannenhalli and Kaestner, 2009). Mutations of human *FOXC1* are associated with Axenfeld-Rieger anomaly (ARA), a dominantly inherited syndrome characterized by dysgenesis of the anterior chamber in the eye and congenital glaucoma [Online Mendelian Inheritance in Man (OMIM) no. 601090] (Mears *et al.*, 1998; Nishimura *et al.*, 1998), and human *FOXC2* mutations lead to the autosomal dominant syndrome Lymphedema-distichiasis (LD), which is characterized by the obstruction of lymphatic drainage in the limbs and the growth of an extra set of eyelashes (OMIM no. 602402) (Fang *et al.*, 2000; Petrova *et al.*, 2004; Tammela *et al.*, 2005). There is also evidence that some humans heterozygous for mutations in *FOXC1* or *FOXC2* have

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Gene symbols for human Foxc are presented with all uppercase letters (e.g., *FOXC1*), the first letter is capitalized for mouse genes (e.g., *Foxc1*), and the first and subclass letters are capitalized for chordates (e.g., *FoxC1*) (Kaestner *et al.*, 2000).

congenital heart defects (Fang *et al.*, 2000; Maclean *et al.*, 2005; Winnier *et al.*, 1999), whereas FoxC2 is implicated in tumor angiogenesis and metastasis (Kume, 2012; Mani *et al.*, 2007; Sano *et al.*, 2010).

In mice, *Foxc1* and *Foxc2* expression largely overlap in many embryonic tissues derived from the non-axial (i.e., paraxial, intermediate, and lateral plate) mesoderm (Hiemisch *et al.*, 1998; Iida *et al.*, 1997; Kume *et al.*, 2000b; Kume *et al.*, 1998; Kume *et al.*, 2001; Sasaki and Hogan, 1993; Seo *et al.*, 2006; Seo and Kume, 2006; Winnier *et al.*, 1997; Winnier *et al.*, 1999). *Foxc1* and *Foxc2* are also expressed in neural-crest derivatives, including cells in the pharyngeal arches, the endocardial cushions of the cardiac outflow tract, the meninges and the periocular mesenchyme (Gage *et al.*, 2005; Gitler *et al.*, 2004; Kume *et al.*, 1998; Seo *et al.*, 2012; Siegenthaler *et al.*, 2009; Winnier *et al.*, 1999). Mice that are homozygous for either a spontaneous mutation in *Foxc1* (congenital hydrocephalus, *Foxc1^{ch}*) or an engineered null mutation (*Foxc1^{lacZ}*) die prenatally or perinatally with identical phenotypic abnormalities (Gruneberg, 1943; Hong *et al.*, 1999; Kume *et al.*, 1998), including hemorrhagic hydrocephalus and multiple skeletal, ocular, genitourinary and cardiovascular defects (Kume, 2009). Similar skeletal, genitourinary, and cardiovascular defects, as well as pre- and perinatal death, occur in *Foxc2*-null mutant mice (Iida *et al.*, 1997; Kume *et al.*, 2000b; Kume *et al.*, 2001; Takemoto *et al.*, 2006; Winnier *et al.*, 1997; Winnier *et al.*, 1999).

Heterozygous *Foxc1*- (*Foxc1*^{+/-}) and *Foxc2*- (*Foxc2*^{+/-}) mice survive to adulthood, but most of the compound heterozygous offspring from crosses of *Foxc1*^{+/-} and *Foxc2*^{+/-} mice (i.e., *Foxc1*; *Foxc2* heterozygotes) die pre- or perinatally with cardiovascular and genitourinary abnormalities similar to those associated with each individual homozygous-null mutation (Kume *et al.*, 2000b; Kume *et al.*, 2001; Seo *et al.*, 2006; Seo and Kume, 2006; Smith *et al.*, 2000; Wilm *et al.*, 2004; Winnier *et al.*, 1999). Furthermore, our group has shown that the severity of the mutant phenotype, including the abnormalities in blood- and lymphatic-vessel formation, is dependent on the cumulative “dose” of functional *Foxc1* and *Foxc2* genes; abnormalities are more extensive in *Foxc1*^{-/-}; *Foxc2*^{-/-} mice than in *Foxc1*^{+/-}; *Foxc2*^{-/-} mice or *Foxc1*^{-/-}; *Foxc2*^{+/-} mice (Seo *et al.*, 2006; Seo and Kume, 2006). Thus, *Foxc1* and *Foxc2* functionally overlap and cooperatively regulate many aspects of embryonic development in a gene-dose dependent manner (Kume, 2009, 2010). However, these experiments were performed with conventional (i.e., global) knockout mice, so the cell type-specific functions of *Foxc1* and *Foxc2* expression remain to be elucidated. Mice carrying a conditional *Foxc* knockout mutation, crossed with (inducible) Cre mice, may also live longer than mice with conventional *Foxc* mutations and, consequently, could be analyzed in postnatal development and disease. For these reasons, we generated lines of mice with conditionally mutant alleles of these genes. These mouse lines will be useful to define the particular roles of Foxc1 and Foxc2 in specific cell lineages during embryonic development as well as in various pathological conditions in adults.

Results and Discussion

The entire coding region of *Foxc1* is located within a single exon and contains no introns. To generate the conditional *Foxc1*-null (*Foxc1^{flx}*) allele in mice, a genomic DNA fragment containing the coding exon of *Foxc1* was isolated from the RPCI-22 129S6/SvEvTac mouse BAC library; loxP sequences were inserted into the 5' upstream region and the 3' untranslated region (UTR) to flank the coding region, and an frt-flanked *neo^r* expression cassette was inserted immediately upstream of the 5' loxP sequence to enable positive selection (Fig. 1a). The resulting target vector was transfected into TL1 mouse embryonic stem (ES) cells by electroporation; then, the ES cell colonies were selected with G418 and gancyclovir, screened via southern blot hybridization with the 5' probe (Fig. 1b), and verified via rehybridization with the 3' probe (data not shown). Chimeric mice were

generated by microinjecting *Foxc1^{flx-neo/+}* ES cells (Fig. 1b) into C57BL/6 blastocysts, and germline transmission was verified via obtaining F1 *Foxc1^{flx-neo/+}* offspring from crossing chimeric and Black Swiss mice. The F1 *Foxc1^{flx-neo/+}* mice were crossed with FLPe mice (Rodriguez *et al.*, 2000) to remove the *neo^r* cassette in the germ line; then, the *Foxc1^{flx/+}* offspring were interbred, and F2 mice were genotyped via PCR after weaning (Fig. 1c). The homozygous *Foxc1^{flx/flx}* mice appeared normal, were fertile, and were born in the expected Mendelian ratio.

To verify that expression of the *Foxc1*-null allele could be induced via Cre-mediated recombination, *Foxc1^{flx/+}* mice were crossed with *Elia-Cre* mice (Lakso *et al.*, 1996), which carry a *Cre* transgene that induces the expression of Cre recombinase in the germ cells. The *Foxc1^{+/-}* offspring of this cross (Fig. 1d) were interbred, and *Foxc1^{Δ/Δ}* embryos were examined to confirm that *Foxc1* expression was completely abolished (Fig. 2a). This observation is consistent with previously published results from our group, in which tissue-specific deletion of *Foxc1* in vascular endothelial cells was achieved by crossing *Foxc1^{flx}* mice with endothelial *Tie2-Cre* mice (Hayashi and Kume, 2008; Seo *et al.*, 2012). The *Foxc1^{Δ/Δ}* mutant embryos also displayed the hydrocephalic and abnormal ocular phenotypes (Fig. 2b-iii, iv) observed in conventional *Foxc1*-null mutant embryos (Kidson *et al.*, 1999; Kume *et al.*, 1998).

Conditional *Foxc2*-null mice were generated in a similar manner. Like *Foxc1*, *Foxc2* is coded within a single exon and contains no introns. A targeting vector containing the conditional *Foxc2^{flx}* allele and a *neo^r* expression cassette (Fig. 3a) was generated and transfected into mouse ES cells; then, ES cell colonies were selected and screened via Southern blot hybridization (Fig. 3b), and *Foxc2^{flx-neo/+}* ES cells were injected into blastocysts to generate chimeric mice with germ-line *Foxc2^{flx-neo}* expression. The chimeric mice were crossed with Black Swiss mice to obtain *Foxc2^{flx-neo/+}* F1 mice, which were subsequently bred with FLPe mice to generate *Foxc2^{flx/+}* mice, and then the *Foxc2^{flx/+}* mice were interbred (Fig. 3c). Homozygous *Foxc2^{flx/flx}* mice were born at the expected Mendelian ratio and appeared normal. *Foxc2^{flx/+}* mice were bred with *Elia-Cre* mice to produce *Foxc2^{+/-}* mice (Fig. 3d); then, the *Foxc2^{+/-}* mice were interbred, and the lack of *Foxc2* expression in *Foxc2^{Δ/Δ}* embryos was confirmed (Fig. 4a). The *Foxc2^{Δ/Δ}* embryos also had cardiac defects, such as ventricular septal defects and thinner myocardium (Fig. 4b-iii, iv), similar to those reported in conventional *Foxc2^{-/-}* mutant embryos (Iida *et al.*, 1997; Winnier *et al.*, 1999).

To determine where *Foxc1* and *Foxc2* are expressed in the adult, we isolated CD31+ endothelial cells from the kidney, heart, and lung of adult wild-type mice and performed real-time RT-PCR analysis (Fig. 5). Both *Foxc1* and *Foxc2* were highly expressed in CD31+ vascular endothelial cells in all three organs, particularly the heart. To a lesser extent, they were also expressed in CD31- cell populations, which could include fibroblasts and other types of nonvascular cells. Based on these results, the newly generated conditional *Foxc^{flx}* mice can be used to analyze the postnatal role of *Foxc1* and *Foxc2* in vascular endothelial cells by crossing these mice with tamoxifen-inducible Cre mouse lines (Gothert *et al.*, 2004; Wang *et al.*, 2010).

Collectively, the results reported here demonstrate that we have successfully generated conditional *Foxc1*-null and *Foxc2*-null mutant mouse lines, that the *Foxc1^{flx}* and *Foxc2^{flx}* alleles retain the function of their corresponding wild-type genes, and that the inactive *Foxc1*-null and *Foxc2*-null alleles can be induced via Cre-mediated recombination. When used with currently available techniques for restricting Cre expression to individual cell lineages or various stages of development, we expect these conditional *Foxc* mutant lines to enable characterization of the tissue-type and cell-type-specific functions of *Foxc1* and

Foxc2 in many developmental processes and disease, as well as the function of Foxc1 and Foxc2 in adult animals. Therefore, our conditional mutant mouse lines will be a powerful tool in elucidating the mechanisms by which Foxc1 and Foxc2 function in various embryonic and pathological processes.

Materials and Methods

Mice

Ell1a-Cre (Stock 003724) (Lakso *et al.*, 1996) and *Flpe* deleter (Stock 005703) (Rodriguez *et al.*, 2000) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were maintained under pathogen-free conditions as approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All animal procedures were reviewed and approved by the Northwestern University Institutional Animal Care and Use Committee (IACUC) and comply with all applicable laws and guidelines.

Foxc1 and Foxc2 targeting vectors

Clones containing genomic DNA of the *Foxc1* and *Foxc2* locus were obtained from a 129S6/SvEvTac BAC library (RPCI-22) (BACPAC Resources, Children's Hospital Oakland, CA), and loxP sites were inserted into the 5' upstream and 3' downstream regions. For the *Foxc1* vector, a neomycin resistance cassette (*neo^r*) was placed 5' to the first loxP site, and a *Herpes Simplex Virus*-thymidine kinase cassette (*tk*) was placed at the end of the 5' homology region; for the *Foxc2* vector, the selection cassettes were placed in the 3' downstream region (*neo^r*) and at the end of the 3' homology region (*tk*). The *neo^r* cassettes were flanked by two FRT sites to facilitate deletion by Flp recombinase. The resulting vectors were verified by sequencing.

Targeted ES cells and generation of chimeric mice

Linearized targeting vectors (100 µg) for *Foxc1^{flx-neo}* or *Foxc2^{flx-neo}* were electroporated into TL1 ES cells (129S6), and then successfully transfected cells were selected via resistance to G418 and gancyclovir. Double-resistant ES cell colonies were screened by Southern blot analysis as described previously (Kume *et al.*, 2000a; Kume *et al.*, 1998); one clone containing the *Foxc1^{flx-neo}*-targeted allele was identified out of 350 screened clones, and two clones containing the *Foxc2^{flx-neo}*-targeted allele were identified out of 251 screened clones. To confirm the correct targeting of the homologous regions, the *Foxc1* and *Foxc1^{flx-neo}* alleles were detected by using a 5' probe and a 3' probe after BglII digestion; the *Foxc1* allele was detected as 8.5-kb (5') and 12.5-kb (3') bands, and the *Foxc1^{flx-neo}* allele was detected as 5.2-kb (5') and 16-kb (3') bands. The *Foxc2* and *Foxc2^{flx-neo}* alleles were detected by using a probe after XbaI digestion and after MfeI/NdeI digestion; the *Foxc2* allele was detected as 12.6-kb (5') and 7.6-kb (3') bands, and the *Foxc2^{flx-neo}* allele was detected as 7.3-kb (5') and 14.8-kb (3') bands.

Targeted ES cells for *Foxc1^{flx-neo}* or *Foxc2^{flx-neo}* were injected into host (C57BL/6) blastocysts and produced germline chimeras. Chimeric male mice were mated with Black Swiss (Taconic) females and the offspring were genotyped by PCR with the primers described below; then, the *Foxc1^{flx-neo/+}* or *Foxc2^{flx-neo/+}* F1 mice (on a mixed, 129 × Black Swiss, genetic background) were bred with *Flpe* deleter mice to generate conditional *Foxc1^{flx/+}* or *Foxc2^{flx/+}* mice.

Genotyping

Embryonic tissues or tail tips were lysed by proteinase K (Roche), diluted in TE (10:1) buffer for 2 hours at 55 °C; then, the proteinase was inactivated at 94 °C for 15 min. The conditional-mutant mice were genotyped by performing PCR with primers for *Foxc1^{flx}* (c1-

F1, 5'-ATTTTTTTTCCCCCTACAGCG-3'; c1-R1, 5'-ATCTGTTAGTATCTCCGGGTA-3') or *Foxc2^{flx}* (c2-F1, 5'-CTCCTTTGCGTTTCCAGTGA-3'; c2-R1, 5'-ATTGGTCCTTCGTCTTCGCT-3'), and the Cre-mediated deletion of *Foxc1* (*Foxc1^Δ*) or *Foxc2* (*Foxc2^Δ*) was confirmed by performing PCR with primers for *Foxc1^Δ* (c1-F2, 5'-ACTGCTGACTCTGGTACTTC-3'; c1-R1, 5'-ATCTGTTAGTATCTCCGGGTA-3') or *Foxc2^Δ* (c2-F1, 5'-CTCCTTTGCGTTTCCAGTGA-3'; c2-R2, 5'-CAACAGGGGCAACAAGTCC-3').

Embryo analysis

Embryos were obtained at several different stages of development, with embryonic day 0.5 (E0.5) defined as the time when the vaginal plug was detected. Gross evaluations of embryos and histological analyses were performed as described previously (Kume *et al.*, 2000a, 2000b; Kume *et al.*, 1998; Kume *et al.*, 2001; Seo *et al.*, 2006); images of whole embryos were obtained with a DFC320 camera (Leica).

Endothelial cell isolation

WT male mice were anesthetized and perfused with PBS. The heart, lungs, and kidneys were dissected in cold DMEM/20% FBS/1% penicillin-streptomycin, minced well with a razor blade, and then enzymatically digested in 1 mg/mL type I collagenase at 37° for 40 min. The tissues were then mechanically dissociated by trituration and filtered (70 μm). The cell suspension was incubated with CD31-Dynabeads, prepared with rat anti-mouse CD31 (BD Pharmingen, clone MEC 13.3) and sheep anti-rat IgG Dynabeads (Invitrogen), for 1 hr at 4°. After extensive washing with 0.1% FBS/2 mM EDTA/PBS, the bead-bound endothelial cells and the negative fraction were resuspended in 1 mL of Trizol (Invitrogen).

RNA isolation and real-time RT-PCR

For analysis of embryonic gene expression, total RNA was isolated from the tail, which includes the presomitic mesoderm and somites where *Foxc1* and *Foxc2* are expressed, with the Absolutely RNA Nanoprep Kit (Stratagene), and cDNA was synthesized with iScript reverse transcriptase (Bio-Rad) as directed by the manufacturer's instructions. Real-time RT-PCR was performed with a Bio-Rad iCycler, Absolute SYBR Fluorescein Mix, and primer sets for the respective genes. Peptidylprolyl isomerase A (*Ppia*) was used as an internal standard for mRNA expression. The primers used in this study were as follows: *Ppia*-sense, 5'-CAAATGCTGGACCAAACACA-3'; *Ppia*-anti, 5'-TGCCATCCAGCCATTCAGTC-3'; *Foxc1*-sense: 5'-TTCTTGCGTTCAGAGACTCG-3'; *Foxc1*-anti: 5'-TCTTACAGGTGAGAGGCAAGG-3'; *Foxc2*-sense, 5'-GCAACCCAACAGCAAACCTTTC-3'; *Foxc2*-anti, 5'-GACGGCGTAGCTCGATAGG-3'.

For analysis of gene expression in endothelial cells isolated from adult mice, total RNA was isolated using TRIzol Reagent (Invitrogen). cDNA was synthesized with Taqman Reverse Transcription kit (Applied Biosystems) and amplification was performed on a Taqman 7500 (Applied Biosystems). The relative expression of each gene was calculated by the ddCt comparative threshold method and normalized to endogenous 18S rRNA expression. The 18S primers and probe used in this study were as follows: 18S sense, 5'-ACGAGACTCTGGCATGCTAACTAGT-3', 18S anti, 5'-CGCCACTTGTCCCTCTAAGAA-3', 18S probe, 5'-ACGCGACCCCCGAGCGGT-3'.

Statistical analyses

Data are shown as mean and SD. Statistical significance was determined by Student's *t* test (two-tailed). The analyses were conducted with GraphPad Prism.

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Fig. 1. Generation of the conditional *Foxc1^{flx}* allele in mice. (a) The *Foxc1* gene (top) contains a single protein-coding exon (black box) flanked by 5' and 3' UTRs (open boxes).

Arrowheads identify the locations of the primers used for PCR genotyping. The 5' and 3' probes are indicated by bars. B, BamHI; Bg, BglII; K, KpnI; Xb, XbaI. **(b)** Southern blot analysis of ES cell clones with the 5' probe after BglI digestion. **(c)** PCR genotyping of a representative offspring from interbreeding between two *Foxc1^{flx/+}* mice; the wild-type and *Foxc1^{flx}* alleles produce 230-bp and 330-bp PCR products, respectively. **(d)** PCR genotyping of three offspring from breeding between *Foxc1^{flx/+}* mice and *Elia-Cre* mice. The *Foxc1^Δ* allele produces a 500-bp PCR product, and the asterisk indicates partial deletion of the *Foxc1^{flx}* allele.

Fig. 2. Analysis of conditional *Foxc1*^{Δ/Δ} embryos at E15.5. **(a)** Quantitative RT-PCR analysis of *Foxc1* expression in wild-type (WT) and conditional *Foxc1*^{Δ/Δ} embryos. **(b)** Images of (i, ii) WT and (iii, iv) conditional *Foxc1*^{Δ/Δ} embryos. Arrows identify (iii) the enlarged cerebral hemisphere (hydrocephalus) and (iv) abnormally thickened cornea observed in conditional *Foxc1*^{Δ/Δ} embryos. The conditional *Foxc1*^{Δ/Δ} embryo also (iv) lacked an anterior chamber in the eye. ac, anterior chamber. Scale bar = 100 μm.

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Fig. 3.

Generation of the conditional *Foxc2^{flx}* allele in mice. **(a)** The *Foxc2* gene (top) contains a single protein-coding exon (black box) flanked by 5' and 3' UTRs (open boxes). Arrowheads identify the locations of primers used for PCR genotyping. The probe is indicated by a bar. H, HindIII, M, MfeI, Nd, NdeI, Nh, NheI; Xb, XbaI. **(b)** Southern blot analysis of ES cell clones with the probe after XbaI digestion. **(c)** PCR genotyping of a representative offspring from interbreeding between two *Foxc2^{flx/+}* mice. The wild-type and *Foxc2^{flx}* alleles give 300 bp and 400 bp PCR products, respectively. **(d)** PCR genotyping of three offspring from breeding between *Foxc2^{flx/+}* mice and *Elia-Cre* mice. The *Foxc2^Δ* allele gives a 505-bp PCR product, and the asterisk indicates partial deletion of the *Foxc2^{flx}* allele.

Fig. 4. Analysis of conditional *Foxc2*^{Δ/Δ} embryos at E13.5. **(a)** Quantitative RT-PCR analysis of *Foxc2* expression in wild-type (WT) and conditional *Foxc2*^{Δ/Δ} embryos. **(b)** Images of (i, ii) WT and (iii, iv) conditional *Foxc2*^{Δ/Δ} embryos. (ii, iv) show the high power image of the regions indicated in i and iii, respectively. A ventricular septal defect (arrow in iii) and abnormally thin myocardium (double arrows in iv) were observed in conditional *Foxc2*^{Δ/Δ} mutant embryos. Scale bars = 500 μm in B and 100 μm in C.

Fig. 5.

Foxc1 and Foxc2 gene expression in endothelial cells. Real-time RT-PCR was performed to determine the levels of Foxc1 and Foxc2 in CD31+ endothelial cells isolated from mouse kidney, heart, and lung. Gene expression is relative to 18S rRNA. Values are mean $\pm$ SD of three independent experiments.