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Generation of Axin1 Conditional Mutant Mice

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Summary

Axin1 is a critical negative regulator of the canonical Wnt-signaling pathway. It is a concentration-limiting factor in the β-catenin degradation complex. *Axin1* null mutant mouse embryos died at embryonic day 9.5, precluding direct genetic analysis of the roles of Axin1 in many developmental and physiological processes using these mutant mice. In this study, we have generated mice carrying two directly repeated *loxP* sites flanking the exon 2 region of the *Axin1* gene. We show that floxed-allele-carrying mice ($Axin1^{fx/fx}$) mice appear normal and fertile. Upon crossing the $Axin1^{fx/fx}$ mice to the *CMV-Cre* transgenic mice, the *loxP*-flanked exon 2 region that encodes the N-terminus and the conserved regulation of G-protein signaling domain was efficiently deleted by Cre-mediated excision in vivo. Moreover, we show that mouse embryos homozygous for the Cre/loxP-mediated deletion of exon 2 of the *Axin1* gene display embryonic lethality and developmental defects similar to those reported for $Axin1^{-/-}$ mice. Thus, this $Axin1^{fx/fx}$ mouse model will be valuable for systematic tissue-specific dissection of the roles of Axin1 in embryonic and postnatal development and diseases.

Keywords

Axin1; conditional inactivation; Cre-LoxP

INTRODUCTION

Axin1 is a multidomain scaffolding protein that interacts with multiple proteins and serves as a key negative regulator of canonical Wnt signaling by the β-catenin destruction complex (Clevers, 2006; MacDonald *et al.*, 2009). Axin1 has been reported to be the rate limiting factor for the β-catenin destruction complex assembly (Lee *et al.*, 2003; Salic *et al.*, 2000). In addition, Axin1 is also involved in regulation of TGF-β, SAPK/JNK, and P53 signaling pathways (Liu *et al.*, 2006; Rui *et al.*, 2004; Zhang *et al.*, 1999). Recent data demonstrated that Axin1 is a central coordinator of Myc degradation (Arnold *et al.*, 2009).

Complete inactivation of Axin1 function leads to early embryonic lethality at E9.5 with forebrain truncation, neural tube defects, and embryonic axis duplications (Chia and Costantini, 2005; Chia *et al.*, 2009; Perry *et al.*, 1995; Zeng *et al.*, 1997). This does not allow identification of potential function in later developmental processes in which Axin1 is also likely to play critical roles (e.g., bone development and remodeling). To investigate Axin1 function at later stages of embryogenesis and in postnatal mice, a conditional allele for

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Axin1 inactivation where Axin1 function is disrupted in tissue- and stage-specific manner would be valuable.

The *Axin1* gene consists of 10 exons spanning ~56 kb (Zeng *et al.*, 1997). In the Axin1 null allele (Perry *et al.*, 1995), exon 2 and parts of the two flanking introns are deleted and exons 1 and 3 are separated by a ~600-kb transgene insertion (Zeng *et al.*, 1997). We designed a targeting construct to disrupt exon 2, which encodes the first two AUG codons and highly conserved regulation of G-protein signaling domain (Zeng *et al.*, 1997). Two loxP sites were inserted in the same orientation upstream and downstream of exon 2. A Neo-TK (thymidine kinase) cassette with two flanking Frt sites was inserted downstream of second loxP site. The AclI-linearized targeting vector was electroporated into SV129 embryonic stem (ES) cells. One hundred and twenty G418-resistant ES cell colonies were selected and then screened by Southern blot analysis for homologous recombination using 5′ external probe (Fig. 1A). Two colonies demonstrated the 6.1-kb wild-type and 4.6-kb targeted bands by Southern blotting with the 5′ probe (Fig. 1B). Two Axin1-targeted ES clones were injected into C57BL/6J blastocysts for the production of chimeric animals. Three male chimeras produced only one transmitted the floxed allele to the germline as demonstrated by PCR genotyping and Southern blot analysis. The F1 heterozygous (fn/+; fn denotes floxP-neo) mice were mated, and homozygous (fn/fn) progenies were identified by PCR and Southern blot analysis (Fig. 1B). Both $Axin1^{fn/+}$ and $Axin1^{fn/fn}$ mice were viable, fertile, and did not display any obvious phenotypic abnormality, indicating that the insertion of loxP and Frt-Neo-Frt cassette does not alter *Axin1* gene expression.

The Frt-flanked neo selection marker was deleted in vivo by breeding the $Axin1^{fn/+}$ or $Axin1^{fn/fn}$ mice with the FLPeR mice, which constitutively express the FLPe recombinase in most tissue types, including cells of the developing germ line (Farley *et al.*, 2000). The $Axin1^{fx/+}$ (fx denotes floxP with neo cassette deleted) mice, which have two loxP sites flanking Exon 2 of the *Axin1* gene and one Frt site in the *Axin1* allele, were confirmed by PCR and Southern blot analysis using 5′ and neo probes (Fig. 1C). $Axin1^{fx/+}$ mice were then inter-crossed to generate the homozygous $Axin1^{fx/fx}$ conditional mice (Fig. 1D). The $Axin1^{fx/fx}$ mice were viable and fertile and did not present any recognizable phenotype.

To demonstrate that the loxP flanked exon 2 of the *Axin1* gene could be deleted in vivo and is essential for Axin1 function, the $Axin1^{fx/fx}$ mice were mated with *TgCMV-Cre* transgenic mice (Schwenk *et al.*, 1995), in which Cre recombinase is expressed under the control of the human cytomegalovirus (CMV) promoter that is active in most cells and tissues. The exon 2-deleted mouse was confirmed by PCR and Southern blot analysis using exon 2 probes (Fig. 2A). Both 6.1 kb and 2.1 kb bands representing the wild-type allele were detected in $Axin1^{fx/+}$, $Axin1^{+/del}$ (del denotes exon 2 deleted), and wild-type mice, but absent in $Axin1^{fx/fx}$ mice. About 1.3 kb and 1.6 kb bands representing in targeted allele were observed in $Axin1^{fx/+}$ and $Axin1^{fx/fx}$ mice but were absent in $Axin1^{+/del}$ and wild-type mice. The genotypes of the mice were confirmed by hybridizing the same membrane to the 5′ external probe (Fig. 2A). $Axin1^{+/del}$ mice heterozygous for the *Axin1* gene are viable, fertile, and did not display any obvious visible abnormalities. To analyze the effect of a homozygous deletion of exon 2 of the *Axin1* gene, $Axin1^{+/del}$ mice were intercrossed. The $Axin1^{del/del}$ homozygous mouse was confirmed by PCR analysis. Next, we performed Western blot analysis using cell lysates extracted from whole embryos (Fig. 2B). A strong Axin1 band was observed in the $Axin1^{+/del}$ extracts, but no signal was observed in $Axin1^{del/del}$ extracts. These data demonstrated that, as expected, the Axin1 protein is not produced in $Axin1^{del/del}$ mutant embryos.

Loss of Axin1 results in early embryonic lethality. The $Axin1^{-/-}$ homozygous embryos died at E9.5, displaying a wide spectrum of abnormalities including incomplete closure or

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malformation of head folds, crooked neural tube, cardia bifida, and duplication of embryonic axis (Perry *et al.*, 1995; Zeng *et al.*, 1997). The $Axin1^{del/del}$ mice exhibit recessive embryonic defects very similar to those caused by the null allele $Axin1^{Tg1}$ (Chia *et al.*, 2009; Perry *et al.*, 1995; Zeng *et al.*, 1997). Twenty-seven embryos from intercrossed among $Axin1^{+/del}$ mice were examined at E10.5 and seven $Axin1^{del/del}$ homozygotes were found, consistent with the expected Mendelian ratio. Eighteen embryos from intercrossed among $Axin1^{+/del}$ mice were also examined at E9.5 and four $Axin1^{del/del}$ homozygotes were found as the expected frequency. All embryos of the homozygotes were severely abnormal. They were significantly smaller than their wild-type or heterozygous littermates (Fig. 2C) and displayed underdeveloped head folds (Fig. 2C2) and open head folds (Fig. 2C4). The embryos of heterozygotes were indistinguishable from that of wild-type littermates (data not shown). Therefore, the deleted allele should represent a null allele of *Axin1*.

In summary, we have successfully generated a conditional null *Axin1* allele and showed that deletion of exon 2 of the *Axin1* gene leads to recessive lethal phenotype very similar to those of $Axin1^{-/-}$ mice. These mice provide a powerful tool to determine the physiological role of Axin1 during late embryonic development and postnatal life and a mouse model to investigate organ and tissue-specific function of Axin1.

METHODS

Generation of Mice Carrying the Floxed and Knockout Axin Alleles

A bacterial artificial chromosome (BAC) clone containing the *Axin1* genomic region was obtained by screening the RPCI-22 129/SvEvTac mouse BAC library (BACPAC Resources, Children's Hospital of Oakland, Oakland, CA). A 14.1-kb NotI-XhoI fragment containing exons 1 and 2 was subcloned into pBluescript plasmid vector for the construction of the targeting vector. A replacement targeting vector was constructed with a diphtheria toxin (DTA) expression cassette, a 2.6-kb NotI/NheI fragment as 5′ homologous arm, a loxP site, a 2.6-kb NheI/PmeI fragment containing exon 2 of the *Axin1* gene, a second loxP site (in the same orientation as the first one), an Frt-flanked *Neo*/thymidine kinase (*Neo-TK*) positive selection cassette, a 7.4-kb PmeI/SpeI fragment as the 3′ homology arm and another *PGK-DTA* expression cassette as negative selection marker.

The targeting vector was linearized with AclI and electroporated into SV129 mouse ES cells, which were selected with 175 mg/ml of G418. G418-resistant ES clones were screened by Southern blot analysis of BamHI/BglII-digested ES cells genomic DNA with a 5′ external probe. The targeting frequencies were 2 of 120. The two independent targeted ES clones were microinjected into C57BL/6J host blastocysts. Three chimeras were bred to C57BL/6 mice, and F1 agouti off-spring mice were genotyped by PCR and Southern blot hybridization to validate the germline transmission. The Frt-flanked neo gene was deleted in vivo by breeding the $Axin1^{fn/+}$ or $Axin1^{fn/fn}$ mice with the FLPeR mice (Farley *et al.*, 2000). Exon 2 of the *Axin1* gene was deleted by crossing the $Axin1^{fx/fx}$ mice with *TgCMV-Cre* transgenic mice (Schwenk *et al.*, 1995). The C57BL/6, FLPeR (Farley *et al.*, 2000), and *TgCMV-Cre* (Schwenk *et al.*, 1995) mice were obtained from The Jackson Laboratory.

Genotyping

Genomic DNA was isolated from mouse tail and analyzed by Southern blotting according to standard protocols. Mice carrying wild-type and $Axin1^{fx}$ alleles were genotyped in a single PCR reaction using a forward primer 3F (TGAACTCTTGATCAGGTCTTG) and reverse primer 2R (TATGATCTTTGGTCCTTTCTG), which amplify the wild-type (372-bp) and $Axin1^{fx}$ (463-bp) alleles. PCR reactions for genotyping were carried out under the following

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conditions: 94°C for 5 min, 35 cycles at 94°C, and 59°C each for 30 s, followed by extension at 72°C for 1 min and final extension at 72°C for 5 min.

Western Blot

Embryos were dissected at E9.5 and homogenized in a homogenizer with RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate) with protease inhibitor cocktail (Sigma). Protein concentrations were measured using Coomassie Plus Protein Assay kit (Pierce, Rockford, IL). About 30 micrograms protein samples were separated in a denaturing 10% SDS–PAGE gel (Invitrogen) and transferred to nitrocellulose membrane. The membranes were washed, blocked (5% milk in PBS-T), and incubated in the appropriate antibodies overnight at 4°C. Antibodies for Western blot analysis: rabbit anti-Axin1 (Zymed Laboratories, South San Francisco, CA) at a dilution of 1:500 and mouse anti-β-actin (Sigma) at a dilution of 1:5000. Secondary antibodies were horseradish peroxidase conjugated. Visualization was done with Pico or Femto chemiluminescent agents (Pierce).

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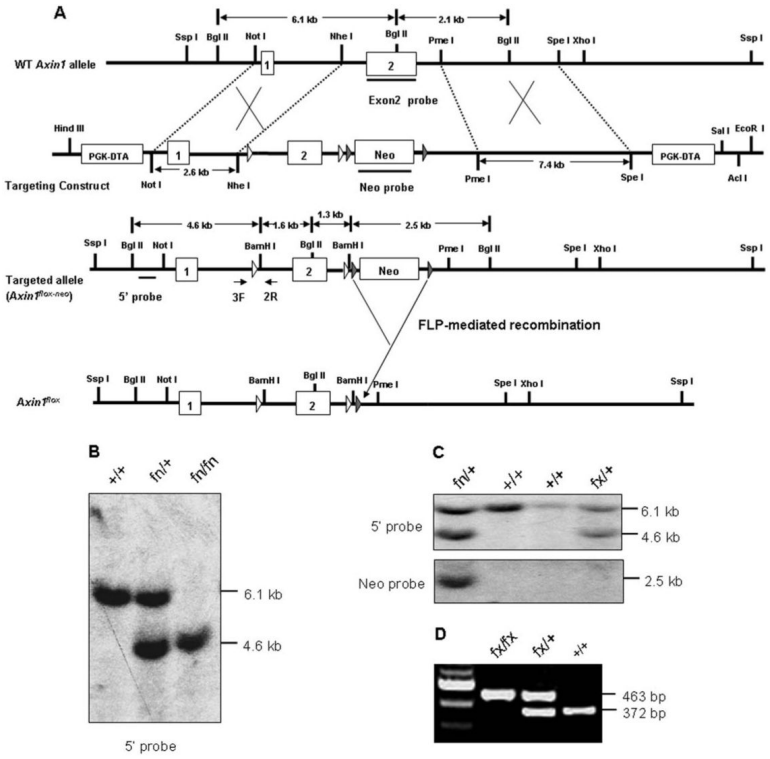

FIG. 1.

Generation of mice carrying *Axin1*^{fn} (flox-neo) and *Axin1*$^{fx/fx}$ alleles. **A:** Targeting construct for inactivation of the *Axin1* gene. The wild-type *Axin1* locus, targeting construct, targeted allele, and flox allele are shown schematically. The boxes numbered 1–2 represent the exons 1 and 2. Open triangles represent the loxP sequence. The filled triangles represent the frt sites. The PKG-promoter-driven DT-A and Neo-TK cassette are indicated by boxes. The bars represent the probes for Southern blot analysis. The flox allele was generated by crossing targeted allele with FLPeR mice. A 2.6-kb NotI/NheI fragment represents 5′ homologous arm. A 7.4-kb PmeI/SpeI fragment represents the 3′ homology arm. The arrows indicate the priming position for primers 3F and 2R, which were used to detect both wild-type and floxed alleles. **B:** Southern blot analysis of BamHI-BglII genomic DNA prepared from tail biopsies of wild-type (+/+), heterozygous (fn/+, fn denotes floxP-neo), and homozygous (fn/fn) *Axin1* flox-neo mice using 5′ external probe. Presence of wild-type and targeted alleles are indicated by 6.1-kb and 4.6-kb fragments, respectively. **C:** Southern blot analysis of genomic DNA using 5′ probe and Neo probe confirmed that Neo-TK cassette was removed by the Flp recombinase. The 2.5-kb band represents the Neo-TK cassette. **D:** A representative agarose gel image of PCR genotyping using primers 3F and 2R. A 372-bp band represents wild-type alleles, and the 463-bp band represents the floxed allele.

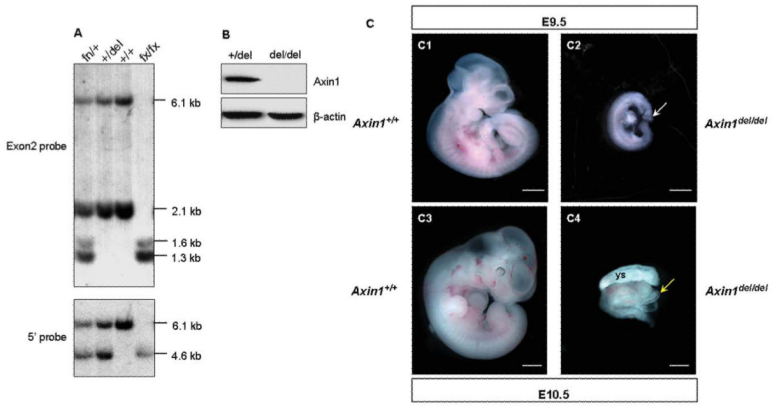

FIG. 2.

Axin1$^{del/del}$ embryos died during early embryonic development. **A:** Southern blot analysis using 5′ probe and exon 2 probe confirmed the deletion of exon 2 of the *Axin1* gene in *Axin1*$^{+/del}$ mice. The genomic DNA was digested with BamHI and BglII. The 5′ probe detected 6.1-kb and 4.6-kb fragments for wild-type allele and flox allele, respectively. The exon 2 probe detected 6.1-kb and 2.1-kb fragments for wild-type allele and 1.3-kb and 1.6-kb fragments for flox allele. **B:** Western blot analysis on Axin1 in *Axin1*$^{+/del}$ and *Axin1*$^{del/del}$ embryos. Total protein extract from E9.5 embryos were blotted for Axin1 and β-actin (the loading control). **C:** *Axin1*$^{del/del}$ mice display an embryonic lethal phenotype. E9.5 and E10.5 *Axin1*$^{del/del}$ embryos are much smaller than their wild-type littermates (C1–C4). *Axin1*$^{del/del}$ embryos show underdeveloped head folds (C2, white arrow) and open head folds (C4, yellow arrow). Ys, yolk sac. Bars: 0.5 mm.

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