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Generation of mice with a conditional null allele of the Jagged2 gene

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Summary

The Notch signaling pathway is an evolutionarily-conserved intercellular signaling mechanism, and mutations in its components disrupt embryonic development in many organisms and cause inherited diseases in humans. The Jagged2 (*Jag2*) gene, which encodes a ligand for Notch pathway receptors, is required for craniofacial, limb and T cell development. Mice homozygous for a *Jag2* null allele die at birth from cleft palate, precluding study of *Jag2* function in postnatal and adult mice. We have generated a *Jag2* conditional null allele by flanking the first two exons of the *Jag2* gene with loxP sites. Cre-mediated deletion of the $Jag2^{flox}$ allele generates the $Jag2^{del2}$ allele, which behaves genetically as a *Jag2* null allele. This *Jag2* conditional null allele will enable investigation of *Jag2* function in a variety of tissue-specific contexts.

Keywords

Notch signaling; conditional null allele; Cre-loxP; gene targeting

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The Notch signaling pathway is an evolutionarily conserved intercellular signaling mechanism. Mutations in Notch pathway components disrupt embryonic development in diverse multicellular organisms and cause cancers and inherited disease syndromes in humans (Bray, 2006; Fiuza and Arias, 2007). In mammals, genes of the Notch family (*Notch1* through *Notch4*) encode Type 1 transmembrane protein receptors that interact with Type 1 transmembrane ligands encoded by genes of the Delta-like (*Dll1*, *Dll3* and *Dll4*) and Jagged (*Jag1* and *Jag2*) families. We have previously described the construction and analysis of a targeted null allele of the Jagged2 (*Jag2*) gene, termed $Jag2^{del1}$ (originally named $Jag2^{\Delta DSL}$) (Jiang *et al.*, 1998). $Jag2^{del1}/Jag2^{del1}$ homozygous mutant mice die at birth from completely penetrant cleft palate. The $Jag2^{del1}/Jag2^{del1}$ mice also exhibit soft tissue syndactyly, the fusion of the digits on the fore- and hindlimbs (Jiang *et al.*, 1998). Cleft palate and soft tissue syndactyly are also exhibited at reduced penetrance by mice homozygous for the syndactylism mutation, a spontaneous *Jag2* missense mutant allele ($Jag2^{sm}$) that behaves genetically as a *Jag2* hypomorphic allele (Casey *et al.*, 2006; Sidow *et al.*, 1997). To circumvent the neonatal lethality exhibited by $Jag2^{del1}/Jag2^{del1}$ null mutant mice and permit the study of *Jag2* function in postnatal and adult mice, we describe here construction of an allele for conditional inactivation of *Jag2* gene function using the Cre-loxP system.

The *Jag2* gene spans approximately 21 kb on mouse Chromosome 12, and consists of 26 exons. To generate the $Jag2^{floxneo}$ targeting vector (Fig. 1a), a PGK*neo* selection cassette was introduced into intron 2. The PGK*neo* cassette was flanked by FRT sites for removal by

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Flpe recombinase (Farley *et al.*, 2000), and by a loxP site distal to the PGK*neo* cassette in intron 2. A second loxP site was introduced approximately 1.1 kb upstream of the *Jag2* translational start site. A diphtheria toxin cassette, at the terminus of the targeting vector, was introduced for negative selection against random integration of the targeting vector into the ES cell genome. The design of the *Jag2^floxneo^* allele permits removal of the PGK*neo* cassette by mating to a Flpe deleter mouse line (generating the *Jag2^flox^* allele), or removal of *Jag2* genomic sequence (including promoter sequences and exons 1 and 2) and the PGK*neo* cassette by mating to a Cre deleter line. To distinguish the *Jag2* mutant allele generated by Cre-mediated deletion of the $Jag2^{flox}$ or $Jag2^{floxneo}$ alleles from the $Jag2^{del1}$ allele, our previously published *Jag2* targeted null mutant allele (Jiang *et al.*, 1998), we designate the *Jag2* null allele generated by Cre recombinase-mediated deletion of the $Jag2^{flox}$ or $Jag2^{floxneo}$ alleles the $Jag2^{del2}$ allele.

The $Jag2^{floxneo}$ targeting construct was electroporated into R1 ES cells (Nagy et al., 1993), and three correctly targeted clones (Fig. 1b) were injected into C57BL/6J blastocysts. Chimeras were mated to C57BL/6J female mice, and germline transmission was obtained from one clone. $Jag2^{floxneo}/+$ heterozygous mice were mated to a deleter line expressing the Flpe recombinase (Farley *et al.*, 2000) to excise the PGK*neo* cassette and generate the $Jag2^{flox}$ allele. Mice heterozygous for the $Jag2^{del2}$ null allele were generated by mating $Jag2^{flox}/+$ heterozygous mice to *Meox2-Cre* mice which, in addition to other tissues, express Cre recombinase in the germline (Tallquist and Soriano, 2000). As with the $Jag2^{del1}$ allele, heterozygous $Jag2^{del2}/+$ mice were viable, fertile and displayed no obvious phenotypic abnormalities.

Cre-mediated excision of the $Jag2^{flox}$ allele to generate the $Jag2^{del2}$ allele deletes predicted *Jag2* promoter sequences, the ATG translation start site and exons 1 and 2, which we predict would create a *Jag2* null allele. In order to assess the functionality of the $Jag2^{del2}$ allele, embryos and neonatal mice homozygous for the $Jag2^{del2}$ allele were obtained by intercrossing $Jag2^{del2}/+$ heterozygous mice. We assessed *Jag2* RNA expression in $Jag2^{del2}/Jag2^{del2}$ and $Jag2^{del2}/+$ littermate control embryos at E10.5 by whole mount in situ hybridization. *Jag2* RNA was not expressed at levels detectable by in situ hybridization in $Jag2^{del2}/Jag2^{del2}$ homozygous mutant embryos (Fig. 2). We also assessed *Jag2* RNA expression in homozygous $Jag2^{del2}/Jag2^{del2}$ mice by quantitative RT-PCR. Using a primer set that spanned exons 3 and 4, $Jag2^{del2}/Jag2^{del2}$ homozygous mutants expressed 11.5 ± 1.3% of wildtype *Jag2* transcript levels. Utilizing a primer set spanning exons 6–8 of the *Jag2* gene, $Jag2^{del2}/Jag2^{del2}$ mutant embryos expressed 21.8± 3.6% of wildtype *Jag2* transcript levels. However, these levels of the $Jag2^{del2}$ mutant transcript do not appear to have any functional consequences, since $Jag2^{del2}/Jag2^{del2}$ homozygous mutant mice exhibited a phenotype identical to that exhibited by $Jag2^{del1}/Jag2^{del1}$ null mutant mice (Fig. 3). $Jag2^{del2}/Jag2^{del2}$ neonates died the first day of birth, exhibiting syndactyly of the fore- and hindlimbs and palate-tongue fusions that caused palatal clefting (Fig. 3). These data demonstrate that the $Jag2^{del2}$ allele is a *Jag2* null allele, functionally equivalent to the $Jag2^{del1}$ allele, and that the extent of Cre-mediated deletion of the $Jag2^{flox}$ allele can be assessed by in situ hybridization. The neonatal lethality of *Jag2* null mice has precluded analysis of *Jag2* function in postnatal and adult mice. Utilization of the $Jag2^{flox}$ conditional null allele will enable the investigation of *Jag2* function in a tissue-specific manner throughout the mouse life span.

METHODS

Construction of the $Jag2^{floxneo}$ Allele

To construct the $Jag2^{floxneo}$ targeting vector, the 5′ LoxP site was cloned into the *BclI* site in the *SacI* digested fragment from the bMQ-136I11 strain 129 BAC clone, approximately 1.1

kb 5′ of the translational start site of the JAG2 protein. The polylinker sequences adjacent to the 5′ LoxP site introduce new *SacI* and *HindIII* sites, which are used for differentiation between the targeted and wildtype alleles by Southern blot analysis. An FRT-PGKneo-FRT-LoxP cassette with a *ScaI* site for Southern blot screening was inserted 3′ of exon 2 by bacterial recombineering (Warming et al., 2005). These fragments were assembled in a modified pBluescript II SK vector (Promega) containing a diphtheria toxin negative selection cassette.

Electroporation of ES cells and Generation of $Jag2^{floxneo}$ Mice

The $Jag2^{floxneo}$ targeting vector was linearized with SpeI and electroporated into R1 ES cells (Nagy et al., 1993). G418-resistant colonies were screened for homologous recombination by Southern blot hybridization. 10 μg of genomic DNA was digested with restriction enzymes, fractionated in 0.8% agarose gels, transferred to nylon membranes, and hybridized with P^{32}-labelled probe. ES cells containing the expected recombination events were injected into C57BL/6J blastocysts. Male chimeric mice were bred with C57BL/6J females to obtain germ line transmission of the $Jag2^{floxneo}$ targeted allele.

Generation of the $Jag2^{flox}$ and $Jag2^{del2}$ Alleles

$Jag2^{floxneo}$/+ heterozygous mice were mated to a deleter line expressing the Flpe recombinase (Farley *et al.*, 2000) to excise the PGK*neo* cassette and generate the $Jag2^{flox}$ allele. Excision of the PGK*neo* cassette was determined by PCR using the primers J2NeoF (CACCCCTCGAGCCCTTAAT) and J2NeoR (GCTGATCCGGAACCCTTAAT). $Jag2^{flox}$/+ heterozygous mice were mated to the *Meox2-Cre* deleter line (Tallquist and Soriano, 2000) to excise *Jag2* genomic sequences between the loxP sites and generate the $Jag2^{del2}$ null allele. Excision of *Jag2* genomic sequences was determined by PCR using the primers J2FlxF (GGCAGAGTAGCACATCACCA) and J2FlxR2 (ACATGCCTGCACAGCCTAC).

Mouse and Embryo Genotyping

$Jag2^{floxneo}$ mice were genotyped using 2 primers (J2FlxF, J2FlxR1: CAAGCGCACAGGTTGAGTAG) spanning the 5′ loxP site with a 401bp WT band and a 453 Flox band. $Jag2^{flox}$ mice were genotyped using primers spanning the deleted FNF cassette yielding a 202-bp mutant allele (J2NeoF and J2NeoR). The $Jag2^{del2}$ allele was monitored using primers spanning the entire deleted region between the two LoxP sites, yielding a 302-bp band (J2FlxF and J2FlxR2). All PCR reactions were performed using the following standard cycling conditions: 94°C 3 min, (94°C 30 sec, 58°C 45 sec, 72°C 45 sec) X 39 cycles, 72°C 5 min.

Histological Analysis and In Situ Hybridization

We considered the day of vaginal plug as E0. E16.5 embryos were dissected and DNA was prepared from the tails for genotyping by PCR. Embryos were fixed in 10% formalin buffer. Feet were dissected and put into 30% glycerol for photography. For histological analysis, heads of the fixed embryos were dehydrated through graded alcohols, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin. Whole mount in situ hybridization of E10.5 embryos was performed as described previously (Jiang *et al.*, 1998). The *Jag2* in situ hybridization probe was generated from a 1.4 kb partial *Jag2* cDNA clone (Jiang *et al.*, 1998), encoding EGF repeats 1–10 of the mouse JAG2 protein.

Reverse Transcription and Quantitative RT-PCR Analysis

For RT-PCR, total RNA was extracted from the E10.5 embryos, using the Trizol Plus RNA Purification System (Invitrogen, USA) and following the manufacturer's instructions. 500 nanogram RNA was reverse-transcribed using the MessageSensor RT Kit (Ambion, USA).

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Two nanograms of cDNA were used for real-time PCR amplification for each well, using primers from Primerbank. All reactions were performed in triplicate using Power SYBR Green PCR Master Mix on a 7500 Real Time PCR system (Applied Biosystems). Data were normalized to *Actb* levels. *Hey1* gene expression was used as an internal control. Primer sequences: *Actb*, F (forward): GGCTGTATTCCCCTCCATCG, R (reverse): CCAGTTGGTAACAATGCCATGT, product size 154 bp; *Hey1*, F: GCGCGGACGAGAATGGAAA, R: TCAGGTGATCCACAGTCATCTG, 231 bp; *Jag2 Exons3–4*, F: CAATGACACCACTCCAGATGAG, R: GGCCAAAGAAGTCGTTGCG, 203 bp; *Jag2 Exons6–8*, F: TGTGACGAGTGTGTCCCCTA, R: GTTCCATCCTGACGGACAGT, 318 bp.

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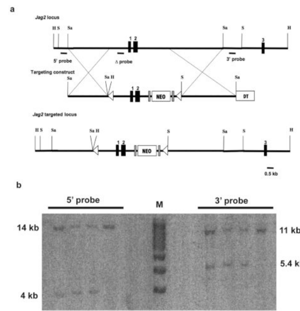

FIG. 1.

Generation of a *Jag2* conditional null allele. (**a**) Schematic representation of a portion of the wildtype *Jag2* allele, the *Jag2*^{floxneo} targeting vector, and the targeted *Jag2*^{floxneo} allele. Exons are indicated by boxes with coding sequences designated by dark shading. LoxP sequences are marked by triangles and FRT sites by gray rectangles. The FRT-PGK*neo*-FRT-LoxP- cassette was inserted between exons 2 and 3 and the second LoxP site was inserted 5′ to exon 1, approximately 1.1 kb upstream of the *Jag2* translational start site. A diphtheria toxin (DT) cassette was included for negative selection of randomly integrated clones. The hybridization probe used for Southern blot analysis is indicated. Abbreviations: H, *HindIII*; S, *ScaI*; Sa, *SacI*. (**b**) Southern blot analysis of targeted ES cells. *HindIII* or *ScaI* digested ES cell genomic DNA was hybridized with the 5′ or 3′ probe respectively as indicated in **a**, yielding 14 kb wildtype and 4 kb mutant bands for the 5′ probe, and 11 kb wildtype and 5.4 kb mutant bands for the 3′ probe. M: markers.

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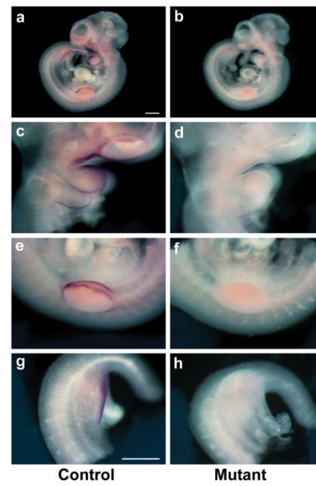

FIG. 2.

Absence of *Jag2* RNA expression in $Jag2^{del2}/Jag2^{del2}$ embryos. (**a,b**) Whole mount in situ hybridization for *Jag2* transcripts in $Jag2^{del2}/+$ control littermate (**a,c,e,g**) and $Jag2^{del2}/Jag2^{del2}$ (**b,d,f,h**) embryos at E10.5. *Jag2* RNA was not detectable by in situ hybridization in the mutant embryo. (**c–h**) Higher magnification views. (**c,d**) Branchial arches. (**e,f**) Apical ectodermal ridge of forelimb bud. (**g,h**) Hindlimb bud. Scale bars**:** **a,b**, 500 μm; **c–g**, 500 μm.

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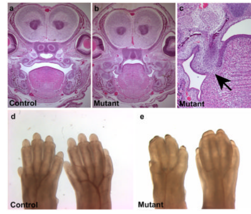

FIG. 3.

Phenotype of *Jag2*^{del2} homozygous mutant embryos. (**a–c**) Cleft palate in *Jag2*^{del2}/*Jag2*^{del2} homozygotes. Coronal sections of E16.5 embryos show that the *Jag2*^{del2}/*Jag2*^{del2} homozygote has cleft palate and fused tongue. (**c**) Fusion of the tongue with the unelevated palatal shelves in the *Jag2*^{del2}/*Jag2*^{del2} homozygote (black arrow). (**d,e**) Limb defects in *Jag2*^{del2}/*Jag2*^{del2} homozygotes. A forefoot (left) and hindfoot (right) of E16.5 mouse are shown in each panel. *Jag2*^{del2}/*Jag2*^{del2} homozygous mutant embryos (**e**) exhibit syndactyly of both fore- and hindlimbs.