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Research Paper

Generation of *Fgfr3* Conditional Knockout Mice

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Abstract

Fibroblast growth factor receptor 3 (FGFR3), highly conserved in both humans and murine, is one of key tyrosine kinase receptors for FGF. FGFR3 is expressed in different tissues, including cartilage, brain, kidney, and intestine at different development stages. Conventional knockout of *Fgfr3* alleles leads to short life span, and overgrowth of bone. In clinic, human FGFR3 mutations are responsible for three different types of chondrodysplasia syndromes including achondroplasia (ACH), hypochondroplasia (HCH) and thanatophoric dysplasia (TD). For better understanding of the roles of FGFR3 in different tissues at different stages of development and in pathological conditions, we generated *Fgfr3* conditional knockout mice in which loxp sites flank exons 9-10 in the *Fgfr3* allele. We also demonstrated that *Cre*-mediated recombination using *Col2a1-Cre*, a *Cre* line expressed in chondrocyte during bone development, results in specific deletion of the gene in tissues containing cartilage. This animal model will be useful to study distinct roles of FGFR3 in different tissues at different ages.

Key words: FGFR3; conditional knock out; *Cre-Loxp*; gene targeting

Introduction

Fibroblast growth factor receptor 3 (FGFR3) is one of the four membrane-spanning receptor tyrosine kinases that serve as high affinity receptors for multiple fibroblast growth factors [1]. *Fgfr3* is mainly expressed in cartilage, and negatively regulates bone growth [2, 3]. Gain-of-function mutations in human FGFR3 lead to three types of chondrodysplasia syndromes including achondroplasia (ACH), hypochondroplasia (HCH) and thanatophoric dysplasia (TD) [4-9]. Recently partial loss-of-function mutation of FGFR3 was found to cause camptodactyly, tall stature, scoliosis, and hearing loss (CATSHL) syndrome [10]. The expression of *Fgfr3* is also detected in other organs such as kidney, intestine, brain, spinal cord and so on [11-14], which indicates that FGFR3 could regulate the development and physiological and pathological functions of these cells.

To explore the roles of FGFR3 in development and diseases, mouse models with genetic modification of FGFR3 have been generated [1-3, 15-18]. Deng and Colvin, *etc* have independently generated conventional *Fgfr3* knock out mouse models [2, 3] or

model with deficiency in a specific isoform [18], which have been used to study the role of FGFR3 in a variety of developmental and disease processes [2, 19-21]. In these conventional knock out mice, *Fgfr3* is disrupted in all cells, which prevents the effort for further studying of the role of FGFR3 in specific types of cells and their corresponding cellular and molecular mechanisms. For example, it is difficult to know if the decreased bone mass in *Fgfr3* null mice [20] is secondarily related to the altered chondrogenesis and/or directly related to changes in osteogenesis [2, 3], because both chondrogenesis and osteogenesis were found changed in *Fgfr3* knock out mice [16, 20]. Furthermore, in certain genetic background, the lifespan of *Fgfr3* null mice is relatively shorter than their normal littermates [20], which prevents us from studying the role of FGFR3 in later development and diseases in adult stages such as aging related diseases. In addition, in conventional *Fgfr3* knock out mouse models, the morphology structure of tissue has significant changes during early development stage. It is impossible to exclude the potential role of already changed tissue in the pathogenesis of regeneration and healing. To overcome these disadvantages, we have generated a *Fgfr3* conditional null allele, the $Fgfr3^{flox}$ allele.

The mouse *Fgfr3* gene contains 19 exons and spans about 25 kb on chromosome 5. The protein product of FGFR3 is encoded by exons 2-18 [22]. As for FGFR3, alternative splicing of the second half of the third Ig domain produces two isoforms, IIIb (encoded by exons 7 and 8) and IIIc (encoded by exons 7 and 9) [23]. The transmembrane domain is encoded by exon 10. Deletion of exons 9 and 10 is expected to cause loss of function of both IIIb and IIIc isoform of FGFR3.

Materials and Methods

Generation of the $Fgf3^{floxneo}$ allele

The targeting vector was constructed using *Fgfr3* genomic DNA isolated previously [3]. The *ploxPneo* vector was described previously [24]. Briefly, in the targeting vector, the 3.8-kb fragment including intron 10 to part of exon 19 was inserted into *ploxPneo* vector using *Not I* and *Sma I* to generate the 3′ homology recombination arm. The 5′ homology recombination arm in the targeting vector was generated by inserting the 5.5-kb *Cla I-Xba I* fragment including part of intron 2 to intron 10 and the third *Loxp* was inserted into intron 8 of the *Fgfr3* locus. The finished targeting construct, $Fgfr3^{floxneo}$, is shown in Figure 1a.

Electroporation of ES cells and generation of germline chimeras

TC1 embryonic stem (ES) cells [3] were transfected with *Not* I-digested *ploxPneoFgfr3* by electroporation and selected using G418 and -(2-deoxy-2-fluoro-β-D-avabinofuranosyl-*S*-iodouracil (FIAU). The homologous recombination events within the *Fgfr3* locus from both G418- and FIAU-resistant ES clones were analyzed by Southern blot using the 5′ external probe with *Spe I*-digested DNA. The 5′ probe is a 1.9-kb *BamH I-Sac I* fragment specific to the *Fgfr3* sequence. The third *Loxp* was identified by PCR using primer p1 (5′-GATGCCTCAACAATACTGGTAG CCC-3′) and p2 (5′-CCAGACAGATGGATGGACA GGAA-3′). Cells heterozygous for the targeted mutation were microinjected into blastocysts from C57/B6 mice to obtain germ-line transmission following standard procedures.

Genotype analysis

The chimeric males were bred to C57BL/6 females, and F1 agouti offspring were analyzed by PCR for the presence of the $Fgfr3^{floxneo}$ allele. Genotypes of the mice bearing the $Fgfr3^{neofloxed}$, $Fgfr3^{flox}$, $Fgfr3^{null}$ and wild-type allele were determined by PCR analysis as illustrated in Figure 2 and 3. The primers are p3 (5′-GCTCCCTGTCCTGCCTCGTG-3′), p4(5′-CAGCT CATTCCTCCCACTCATGAT-3′) and p5 (5′-TGTAAAAGGGGTGGGGTGGTAG-3′).

Skeletal preparation and histology.

Mice at P15 were sacrificed. The femurs were fixed overnight in 4% paraformaldehyde at 4°C and rinsed in PBS and decalcified in 15% EDTA (pH 7.4) for 10-15 days before embedding in paraffin as described previously. Six-micron thick sections were used for H.&E. staining. High-resolution X-ray of femurs from 3-month-old mice were obtained using a Faxitron MX20.

RNA extraction and RT-PCR

RNA was extracted from brain of mice using Trizol (Invitrogen) reagent according to the manufacturer's instructions. Single strand cDNA was synthesized from 1 μg of total RNA using RNA reverse transcriptase kit (Takara). The mRNA level of *Fgfr3* was detected by primer FGFR3-F(5′-CCACCGA CAAGGAGCTAGAGG-3′) and FGFR3-R (5′-CGGTGACAGGCTTGGCAGTA-3′). The β-actin gene detected by primer β-actin-F (5′-TTGTTACCAACTGGGACGACATGG-3′) and β-actin-R (5′-GATCTTGATCTTCATGGTGCTAGG -3′) was an internal control.

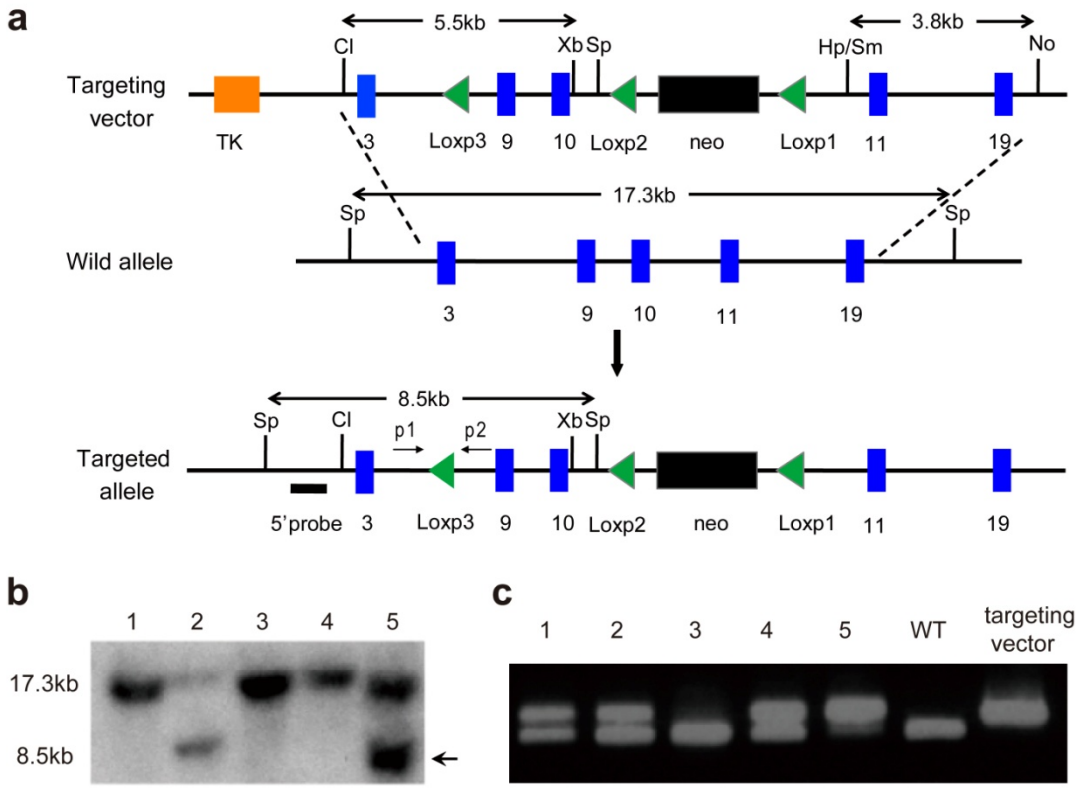

Figure 1. Generation of the *Fgfr3*^{floxneo} allele. (a) Strategy for generating the *Fgfr3* targeting vector and *Fgfr3*^{floxneo} (targeted) allele. Blue boxes represent exons. The 5' external probes for Southern Blot is indicated by thick lines. The predicted length of Southern fragments are indicated with double arrow lines. Cl, Cla I; No, Not I; Sm, Sma I; Sp, Spe I; Xb, Xba I; Hp, Hpa I; p, primer. (b) Targeted events were identified by Southern analysis of *Spe I*- digested genomic ES cell DNAs with a 5'flanking probe. (c) The third *Loxp* in targeted allele was confirmed by PCR.

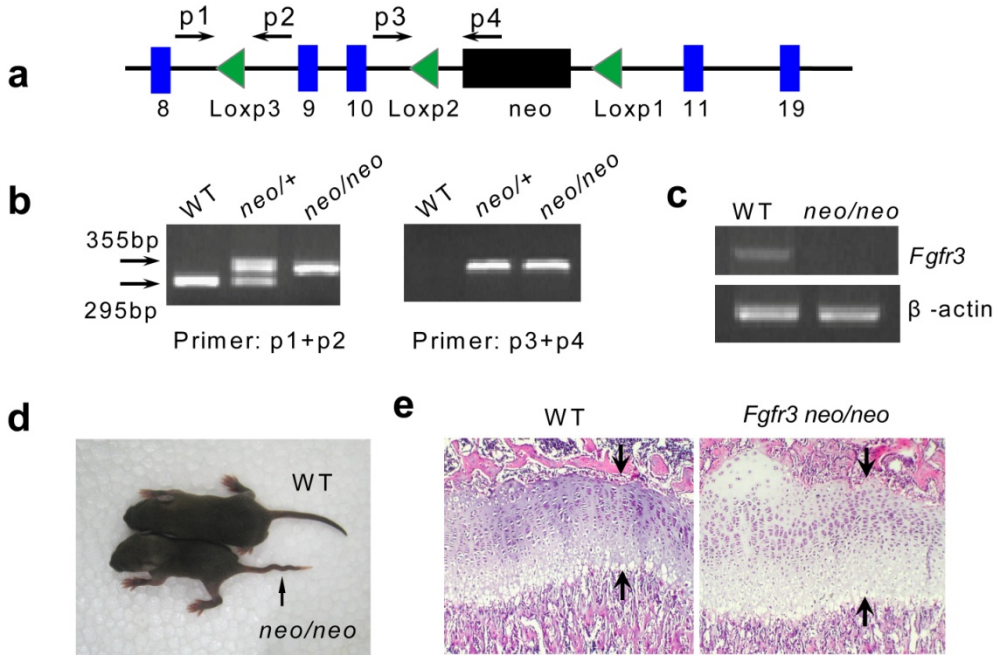

Figure 2. Generation of *Fgfr3*$^{floxneo/floxneo}$ mice. (a) Map of *Fgfr3*^{floxneo} allele. The position of primers was marked in the map. (b) Genotype of *Fgfr3*$^{floxneo/floxneo}$ mice was identified by PCR (primer p1, p2, p3 and p4). (c) There is no expression of *Fgfr3* in *Fgfr3*$^{floxneo/floxneo}$ mice (d) *Fgfr3*$^{floxneo/floxneo}$ mice showed kinky tails, which is also found in *Fgfr3* knock out mice. (e) Increased expansion of proliferating and hypertrophic chondrocytes in the growth plate in *Fgfr3*$^{floxneo/floxneo}$ mice on P15.

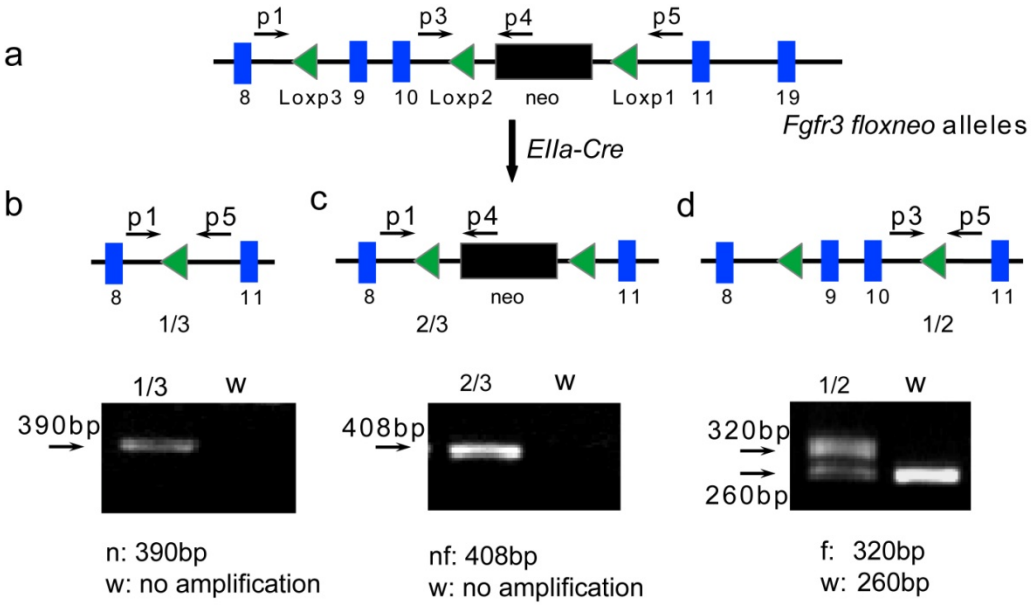

Figure 3. Validation of exons 9-10 and *neo* gene in *Fgfr3*^{floxneo} alleles deleted by *Cre* recombinase. (a) Mice containing *Fgfr3*^{floxneo} alleles were crossed with *EIIa-Cre* transgenic mice resulting in three kinds of deletion between three *Loxp* sites (b-d). Position of primers was also marked. (b) Primer p1 and p5 amplify fragments of 390 bp from *Fgfr3*^{null} allele with deletion exons 9-10 and *neo* between *Loxp*1 and 3 (1/3), no amplification for the wild type (WT). (c) Primer p1 and p4 amplify fragments of 408 bp from *Fgfr3*^{neofloxed} allele with deletion exons 9-10 between *Loxp*2 and 3 (2/3), no amplification for WT. (d) Primer p3 and p5 amplified fragments of 320 bp from *Fgfr3*^{flox} allele with deletion neo between *Loxp*1 and 2 (1/2), but only 260bp from WT. n, *Fgfr3*^{null} allele; nf, *Fgfr3*^{neofloxed} allele; f, *Fgfr3*^{flox} allele; w, wild type.

Results and Discussion

To construct the *Fgfr3*^{floxneo} allele, we have made a targeting vector that contains two *Loxp* sites flanking the entire exons 9 and 10. In this way, existence of *Cre* recombinase would lead to deletion of exons 9 and 10, and subsequent inactivation of FGFR3. We inserted a 5.5-kb fragment including exons 3-10 and the third *Loxp* (located in intron 8) into *ploxPneo* vector [24] as the 5′ homology recombination arm, then put a 3.8-kb fragment containing exons 10 to 19 into *ploxPneo* to form 3′ homology recombination arm (Fig. 1a). The *Not I*-linearized targeting vector *Fgfr3*^{floxneo} was transfected into TC1 ES cells by electroporation. Two targeted ES clones were detected by Southern blot using a 5′ flanking external probe (Fig. 1a and b). The third *Loxp* in the ES clones was identified by PCR (Fig. 1a and c), which confirmed that *Fgfr3*^{floxneo} allele in the two targeted ES clones containing the third *Loxp*. These two positive ES clones were microinjected into blastocysts harvested from C57/BL6 mice according to standard protocols.

We first assessed if the recombination of *Fgfr3* in mice is correct. Since we previously found in the *Fgfr3*$^{neoG369C/+}$ mice that the presence of Neomycin gene (*neo*) at the same site of intron 10 blocked the normal splicing of the *Fgfr3* mRNA, and homozygous *Fgfr3*$^{neoG369C/neoG369C}$ mice showed phenotypes similar to those of *Fgfr3*-null (*Fgfr3*$^{-/-}$) mice [16]. We generated *Fgfr3*$^{floxneo/floxneo}$ mice by crossing *Fgfr3*^{floxneo} heterozygous mice. PCR was used to identify *Fgfr3*$^{floxneo/floxneo}$ mice (Fig. 2a and b). RT-PCR analysis revealed that there was no *Fgfr3* expression in *Fgfr3*$^{floxneo/floxneo}$ mice (Fig. 2c). We also found that *Fgfr3*$^{floxneo/floxneo}$ mice indeed showed skeleton phenotype including crooked tail and increased height of growth plates (Fig. 2d and e), which is similar to the phenotype of *Fgfr3*$^{-/-}$ mice [2, 3]. These results indirectly demonstrated the correct recombination of the targeting construct.

To test whether *neo* gene and exons 9-10 in *Fgfr3*^{floxneo} allele can be deleted by the *Cre* recombinase, heterozygous (*Fgfr3*$^{floxneo/+}$) mice were crossed with *EIIa-Cre* transgenic mice that express *Cre* recombinase in germline [25, 26]. The offspring were identified by PCR with primers shown in Figure 3a. As expected, we found that this crossing breeding generated three types of alleles due to *Cre*-mediated recombination among three *Loxp* sites. A 390bp fragment was amplified from *Fgfr3*^{null} allele with deletion of *neo* gene and exons 9-10 between *Loxp1* and *Loxp3* (confirmed by sequencing) (Fig. 3b). *Fgfr3*^{neofloxed} allele with deletion of exons 9-10 was detected by producing an amplified 408bp fragment (Fig. 3c). The PCR-amplified 320 fragment indicated the excision of the *neo* gene, which

produced a *Fgfr3*^{flox} allele (conditional knockout allele) in *Fgfr3*$^{flox/+}$ heterozygous mice (Fig. 3d).

To further determine the correction of *Fgfr3* recombination in mice, we generated homozygous *Fgfr3* null mice. RT-PCR analysis showed that there was no expression of *Fgfr3* in the brain (Fig. 4a). These mice showed increased length of long bone and decreased bone mineral density (Fig. 4b and c), which is also similar to the phenotypes of *Fgfr3* knock out mice [3, 20]. Finally we crossed the *Fgfr3*$^{flox/+}$ mice with mice that carry a *Col2a1-Cre* transgene to assess the activity of *Fgfr3* conditional knockout allele. The result indicated that the Cre-mediated deletion occurs in several tissues that contain cartilage, including the tail, and knee joint, but not in other tissues where the *Col2a1-Cre* transgene is not expressed, such as the lung, liver, and spleen (Fig. 4d). These data indicate that the *Fgfr3*^{flox} allele can be recombined to delete exons 9-10 in a tissue-specific fashion, thus the Fgfr3 conditional mouse model should be very useful for studying *Fgfr3* functions during later stages of development.

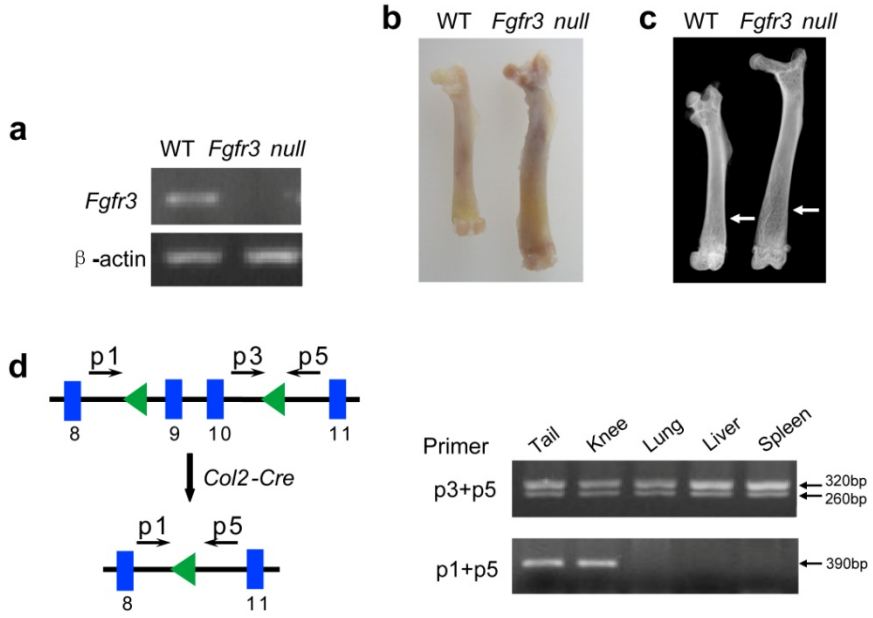

Figure 4. Identification of *Fgfr3* null mice and validation of *Fgfr3* conditional knockout allele. (a) There was no expression of *Fgfr3* in brain RNA of *Fgfr3* null mice (homozygous mice with both exons 9-10 and *neo* deleted in *Fgfr3).* (b) The length of femur in 3-month-old *Fgfr3* null mice was longer than that in wild-type mice. (c) X-ray analysis showed increased length and decreased bone mineral density of femur from *Fgfr3* null mice (Arrows). (d) Tissue-specific inactivation of the *Fgfr3* conditional allele by a *Col2a1-Cre* transgene in tissues containing cartilage was revealed by PCR analysis using primer pair p3/p5, which amplifies about 320 bp from the unrecombined allele, and primer pair p1/p5, which amplifies about 390 bp from the recombined allele.

Conclusion

The results indicate that we successfully generated a mouse model for conditional deletion of *Fgfr3*, which will help to explore the role of FGFR3 in development and the related skeletal diseases.

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Conflict of Interest

All the authors declare the absence of conflicts of interests and the absence of financial interests.

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