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Conditional knockdown of *Fgfr2* in mice using Cre-LoxP induced RNA interference

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ABSTRACT

RNA interference (RNAi)-mediated gene knockdown is a potent approach for studying gene function. We have previously reported a plasmid-based, tamoxifen-inducible gene knockdown system in cultured cells using a combined RNAi and Cre-LoxP system. Here, we validate this system in mouse and show that it can be used to suppress the expression of an endogenous gene (*Fgfr2*) with high efficiency. We show that transgenic mice carrying the *U6-ploxPneo-Fgfr2* RNAi construct are normal, displaying *Fgfr2* transcripts equivalent to those of wild-type controls, indicating that the U6 promoter is inactive *in vivo* due to the presence of the *neo* in the promoter. After excision of the *neo* by crossing with transgenic mice that express Cre in the mouse germline, the U6 promoter is activated, leading to over 95% reduction of *Fgfr2* transcripts, and consequently, embryonic lethality. On the other hand, activation of the U6 promoter using transgenic mice that express Cre in the progress zone of the limb results in live mice with malformation of digits of both the forelimbs and hindlimbs. This method provides a fast, yet efficient way to decipher gene functions *in vivo* in a tissue-specific manner.

INTRODUCTION

RNA interference (RNAi) is widely used to target and degrade specific mRNA (1). Small interfering RNA or short hairpin RNA (shRNA)-producing vectors can be transfected in cultured cells, allowing mRNA down-regulation within 24–72 h (1–5). Plasmids can be stably integrated into host genome and provide several advantages compared with transfected siRNA oligos (1–5). In order to provide a controllable RNAi, several studies have developed inducible regulation of RNAi in mammalian cells using either tetracycline or ecdysone-responsive systems (6–9).

Recently, we have reported a tamoxifen-inducible gene knockdown system in cultured cells using a combination of the RNAi and the Cre-LoxP system (3). In this method, we inserted a neomycin cassette (*neo*) (10) into the RNA polymerase III (U6) promoter to disrupt its activity. Because the *neo* is flanked by loxP sites (*ploxPneo*), it can be excised upon the Cre-mediated recombination, thus restoring the activity of the U6 promoter (3). Using a tamoxifen-inducible Cre (11), we have demonstrated that this process indeed works with high efficiency in the suppression of two endogenous genes, fibroblast growth factor receptor 2 (*Fgfr2*) and Survivin, in mouse embryonic stem (ES) cells (3). The Cre-LoxP system has been widely utilized for conditional gene knockout in mice (12–14), and numerous transgenic mouse strains expressing Cre at specific tissues/organs have been developed to achieve spatial and temporal control of gene expression (15). We postulate that if our *U6-ploxPneo-RNAi* vector works in mouse, it will eventually allow the utilization of a large collection of Cre transgenic mice and significantly enhance our ability to use RNAi system to decipher gene function *in vivo*.

FGFR2 is one of the four high affinity FGF receptors that mediate the signaling of, at least, 22 FGF ligands (16,17). Full-length form of FGFR2 contains a hydrophobic leader sequence, three immunoglobulin (Ig)-like domains, an acidic box, a transmembrane domain and a divided tyrosine kinase domain. Many isoforms can be generated through alternative splicing and internal poly-adenylation. For example, alterations in exons 7–9 that encode the IgIII yield b (FGFR2b) and c (FGFR2c) isoforms (18,19). It has been shown that missense mutations of FGFR2 in humans result in at least seven distinct types of craniosynostosis syndromes, primarily affecting bones formed through intramembraneous ossification [reviewed in (17)]. Some patients also display limb abnormalities at birth, such as short fingers, broad thumbs, bigger toes and/or soft-tissue syndactyly [reviewed in (20)]. Gene targeting in mouse

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revealed that Fgfr2 plays important roles in embryonic development and organogenesis. It has been demonstrated that Fgfr2-null embryos die a few hours after implantation owing to abnormalities during the implantation process and malformation of the egg cylinder (21). Mutant embryos homozygous for Fgfr2–IgIII domain deletion ($Fgfr2^{\Delta III/\Delta III}$) die at embryonic day (E) 10.5–11.5 owing to failure of placenta formation (22). After rescue of placenta defects with wild-type tetraploid cells, the $Fgfr2^{\Delta III/\Delta III}$ embryos survive up to birth; however, they display abnormalities in multiple organs/tissues (23).

Considering embryonic lethality associated with the loss of function mutations and the potential critical roles of FGFR2 in postnatal development (17), we decided to knock-down this gene in mouse to test the efficiency of our Cre-mediated RNAi strategy. By using two different transgenic mice that express the Cre in the germline and the progress zone of the limb, respectively, we have generated mouse models with dramatically reduced expression of *Fgfr2* that can be used to study functions of this gene in a tissue-specific manner.

MATERIALS AND METHODS

Generation of transgenic mice

pBS/U6-ploxPneo-Fgfr2 vector used in this study was described previously (3). The vector was digested by KpnI and AflIII restriction enzymes and purified from agarose gel after electrophoretic migration using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The fragment (2745 bp) was resuspended in Tris (1 mM)–EDTA (0.1 mM) buffer and adjusted to a final concentration of 2 ng/µl and injected into oocytes isolated from FVB/N mice through pronuclear injection. Offspring from the injection were genotyped using PCR with the following pair of primers (5′-CGAAGTTATCTAGAGTCGAC-3′ and 5′-AAACAAGGCTTTTCTCCAAGG-3′) that amplifies 102 bp from the U6 promoter and the connecting *neo* gene. PCR-positive mice were crossed with wild-type FVB/N mice to obtain germline transmission. Three chimeric mice passed the transgene through germline.

Functional analysis of *Fgfr2-RNAi* transgene

Mice carrying the *U6-ploxPneo-Fgfr2-RNAi* transgene were crossed with *EIIa-Cre* (24) and *Ap2-Cre* (25) transgenic mice to assess efficiencies of *Fgfr2-RNAi* transgene. The *EIIa-Cre* mice and *Ap2-Cre* mice were genotyped using the following pair of primers (Cre-1: 5′-CCT GTT TTG CAC GTT CAC CG-3′ and Cre-3: 5′-ATG CTT CTG TCC GTT TGC CG-3′). Pregnant *EIIa-Cre;U6-ploxPneo-Fgfr2* and *Ap2-Cre;U6-ploxPneo-Fgfr2* mice were killed at different stages of pregnancy to check embryonic development. Cre mediated recombination was verified by using the following pair of primers (5′-GACGCCGCCATCTCTAGG-3′ and 5′-AGGCTTTTCTCCAAGGGATATT-3′). This pair of primers flanks the *ploxPneo* in the U6 promoter and amplifies 278 bp after Cre-mediated recombination. Animals were handled in accordance with guidelines of the NIDDK Animal Care and Users Committee.

Whole-mount skeletal preparation

Animals were euthanized by asphyxiation with CO_2. After removing the skin, carcasses were eviscerated, fixed in 95% ethanol, stained with Alizarin red S and alcian blue, cleared by KOH treatment, then stored in glycerol as described previously (26).

PCR, RT–PCR and real-time PCR

RT–PCR was performed on total RNA extracted from embryos using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX) and reverse-transcription was performed according to Ambion's instructions using 'Cells-to-cDNA™ II kit (Catalog nos 1722, 1723)'. Two micrograms of RNA were used for cDNA synthesis using 0.5 µg of oligo$(dT)_{18}$ primer, 1 µl of RNase inhibitor, 1 µl of M-MLV reverse transcriptase and 2 µl of 10× RT buffer (Ambion) in a final volume of 20 µl. The reaction was carried out for 45 min at 42°C and for 10 min at 95°C and then cooled to 4°C. An aliquot of 1 µl of each RT reaction product was amplified to a 24 µl-volume PCR premix, containing 1 pmol of each primer, 2.5 mM dNTP mix and 1 U of *Taq* polymerase. PCR was carried out in a PCR thermal cycler (Gene Amp PCR Systems 9700). The samples were heated to 94°C for 2 min, and run through 22–31 cycles (95°C for 10 s, 60°C for 20 s and 72°C for 30 s) to monitor linearity of the amplification. The PCR was kept in 72°C for 10 min and then stopped at 4°C.

RT–PCR was performed using the following primers: Fgfr1-F: 5′-TTCTGGGCTGTGCTGGTCAC-3′ and Fgfr1-R: 5′-GCGAACCTTGTAGCCTCCAA-3′; Fgfr2-F: 5′-AAGGTTTACAGCGATGCCCA-3′ and Fgfr2-R: 5′-ACCACCATGCAGGCGATTAA-3′; Fgfr3-F: 5′-CTAGTGTTCTGCGTGGCGGT-3′ and Fgfr3-R: 5′-TTCTTATCCATTCGCTCCGG-3′; Fgfr4-F: 5′-CTGTTGAGCATCTTTCAGGG-3′ and Fgfr4-R: 5′-CGTGGAAGGCCTGTCCATCC-3′.

We measured relative gene expression by real-time RT–PCR as suggested by the manufacturer (ABI PRISM 7500 Realtime System; Applied Biosystems) using the above primers for *Fgfr1*, *Fgfr3* and *Fgfr4*. *Fgfr2* primers were described as Fgfr2-Up: 5′-TGCCCTACCTCAAGGTTCTG-3′; and Fgfr2-Down: TAGAATTACCCGCCAAGCAC-3′.

The following pair of primers for β-actin was used as control for both RT–PCR and real-time PCR: β-actin-Up: 5′-TGCGTGACATCAAAGAGAAG-3′; β-actin-Down: 5′-GATGCCACAGGATTCCATA-3′.

The following primers were used for genomic real-time PCR: Neo-U: 5′-AGAGGCTATTCGGCTATGACTG-3′; Neo-D: 5′-GTGGCCAGCCACGATAGC-3′; Fgfr1-U: 5′-CCCCATCCCATTTCCTTACCT-3′; and Fgfr1-D: 5′-TTCTGGT GTGTCTGAAAACAGCT-3′.

For amplification of *neo* gene and *neo* deletion by genomic PCR, following pairs of primer were used: Neo-1: 5′-AGAGGCTATTCGGCTATGACTG-3′; Neo-2: 5′-TTCGTCCAGATCATCCTGATC-3′; Del-1: 5′-CGCACAGACTTGTGGGAGAA-3′; and Del-2: 5′-CACAATTACTTTACAGTTAG-3′. Fifty nanograms of DNA was used in the reaction.

Northern blotting

For analysis of the shRNA expression, 20 µg of total RNAs were resolved in a 15% (wt/vol) polyacrylamide–8 M urea gel, transferred by electroblotting onto Nytran-N^+ membrane

(Schleicher & Schuell, Keene N.H.) and was cross linked by using the auto-crosslink function of a Stratlinker. The membrane was hybridized overnight to a [γ-^{32}P]labeled DNA probe corresponding to the 21mer antisense strand of the *Fgfr2* shRNA (GGCCTCAGCGTTCCTGAGCGA). The hybridization (Sigma, Hybridization buffer, PerfectHyb™ Plus) and wash steps were carried out at 37°C. The membrane was then exposed on a Kodak film for overnight before developing the image.

RESULTS

Expression of *U6-Fgfr2* results in embryonic lethality

To study the potential effects of RNAi of *Fgfr2 in vivo*, we generated transgenic mice carrying *U6-ploxPneo-Fgfr2* (Figure 1A) through pronuclear injection. After breeding with FVB/N mice, three offspring from the injection passed the *U6-ploxPneo-Fgfr2* transgene through germline. All the transgenic mice were normal owing to the presence of the *neo*,

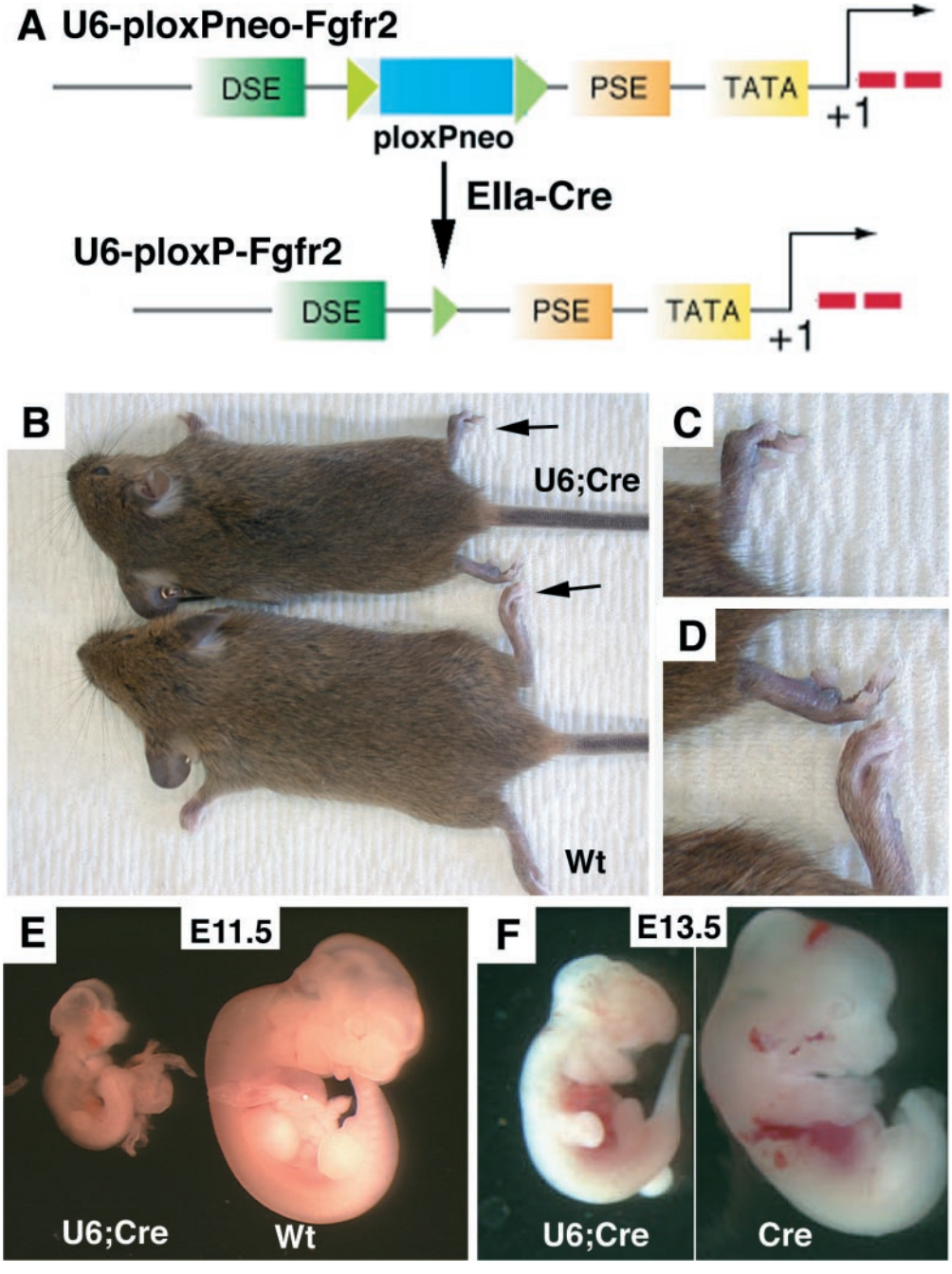

Figure 1. Expression of *U6-Fgfr2* results in embryonic lethality. (**A**) Structure of *U6-ploxPneo-Fgfr2* transgene before and after deletion of *ploxPneo*. (**B**) P19 *U6-Fgfr2;EIIa-Cre* and control mice. Abnormal limbs (arrows) were magnified in (**C** and **D**). (**E** and **F**) E11.5 (E) and E13.5 (F) mutant and control embryos. Wt, wild type; U6, *U6-ploxPneo-Fgfr2*; Cre, *EIIa-Cre*; and U6;Cre, *U6-Fgfr2;EIIa-Cre*.

which blocks U6 promoter activity (3). Next, we crossed these mice with *EIIa-Cre* transgenic mice that express Cre in germ cells of the mouse (24). After screening 81 mice generated from breeding of one founder line (Line A) with *EIIa-Cre* transgenic mice, we obtained two live bigenic (*U6-Fgfr2;EIIα-Cre*) mice (Table 1, crosses between *U6-ploxPneo-Fgfr2* and *EIIa-Cre* mice). Both mice appeared unhealthy at birth and were much smaller than their littermate control (Figure 1B) with a body weight of about two-third of their littermates at postnatal day 19 (P19). Both mice also displayed abnormal limbs (Figure 1C and D). Screening of 40 offspring from other two founder lines failed to obtain live *U6-Fgfr2;EIIα-Cre* mice at birth, suggesting that they were also embryonic lethal. Altogether, these observations suggested that the expression of *U6-Fgfr2* RNAi might result in severe developmental defects, leading to embryonic lethality of majority of mutant mice. To confirm this, we crossed the *U6-ploxPneo-Fgfr2 mice* (Line A) with *EIIa-Cre* mice and dissected females at days 11.5 through 13.5 during their pregnancy. Our data indicated that *U6-Fgfr2;EIIα-Cre* embryos were present at a Mendelian ratio (Table 1, crosses between *U6-ploxPneo-Fgfr2* and *EIIa-Cre* mice). However, ~60% (8/14) of them were developmentally abnormal or partially resorbed (Figure 1D and E), while no obvious differences were found among control (wild type, *EIIa-Cre* or *U6-ploxPneo-Fgfr2*) embryos in the same litters.

Our previous study performed in ES cells indicated that Cre-mediated deletion of the *ploxPneo* restored the original distance between the distal sequence element and the proximal sequence element in the U6 promoter. It allowed the expression of *Fgfr2* RNAi, and, consequently, led to a markedly reduction of *Fgfr2* transcripts (3). To determine whether this occurred *in vivo*, we performed the following three experiments using Line A transgenic mice: we first determined the number of copies of the *U6-ploxPneo-Fgfr2* transgene by real-time PCR. Our analysis using a pair of primers located in the *neo* gene indicated that the transgenic mice contained five copies of the transgene (Figure 2A). This analysis also revealed that the intensity of the *neo* decreased to ~20% in *U6-Fgfr2;EIIa-Cre* embryos compared with the embryos without Cre (Figure 2A). The similar decrease of *neo* intensity was also revealed by genomic PCR analysis using primers against the *neo* (Figure 2B). To confirm that this reduction is caused by *neo* deletion, we performed PCR using primers flanking the *ploxPneo*. Our analysis detected Cre-mediated deletion (del) product in DNA isolated from *U6-Fgfr2;*

Table 1. Offspring and genotypes of crosses between *U6-ploxPneo-Fgfr2 mice* (Line A) with *EIIa-Cre* or *AP2-Cre* transgenic mice

Ages	Total	WT	*EIIa-Cre*	*U6-ploxPneo-Fgfr2*	*U6-Fgfr2;EIIa-Cre*	*AP2-Cre*	*U6-Fgfr2;AP-2Cre*
Crosses between *U6-ploxPneo-Fgfr2* and *EIIa-Cre* mice							
After birth	81	27	25	27	2[a]		
E11.5–E13.5	51	10	10	17	14		
Crosses between *U6-ploxPneo-Fgfr2* and *AP2-Cre* mice							
After birth	62	21		10		15	16[b]

[a]Small size and limb abnormalities.
[b]All displayed limb defects.

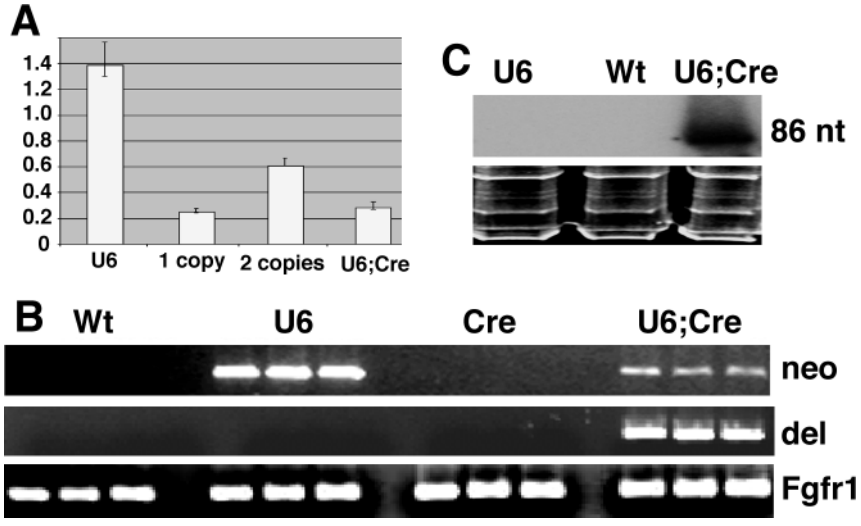

Figure 2. Cre-mediated deletion of the *neo* from *U6-ploxPneo-Fgfr2* transgene allows expression of *Fgfr2* RNAi. (**A**) Assessment of copy number of *U6-ploxPneo-Fgfr2* transgene by real-time PCR using primers for *neo* gene. DNA extracted from *Sirt6*+/− and *Fgfr1*−/− ES cells are used as controls for 1 copy and 2 copies of *neo* gene, respectively. Primers for *Fgfr1* genomic DNA are used as analysis control. (**B**) Assessment of *neo* gene deletion by genomic PCR using primers for *ploxPneo* (*neo*) and primers that flanking the *neo*. PCR using *neo* primers is stopped at 25 cycles and all others are stopped at 30 cycles. (**C**) Northern blot analysis showing that *U6-Fgfr2* transgene is only expressed in *U6-Fgfr2;EIIa-Cre* mice. Equal RNA loading was assessed by ethidium bromide staining of the upper portion of the gel (lower). All samples, except for those from ES cells, are from E12.5 embryos.

EIIa-Cre embryos, but not in DNA isolated from embryos with other genotypes (Figure 2B). Then, we performed northern blot analysis using RNAs extracted from *U6-Fgfr2; EIIa-Cre*, wild type and *U6-ploxPneo-Fgfr2* whole embryos. Our analysis detected a strong band of predicted size of 86 nt (63 bases for the two RNAi oligos and hairpin sequences and 23 bases from transcription initiation site to the beginning of the first oligo) in *U6-Fgfr2;EIIa-Cre*, but not in wild-type and *U6-ploxPneo-Fgfr2* embryos (Figure 2C). These data provide *in vivo* evidence that the presence of the *neo* gene efficiently silenced the U6 promoter and its deletion restored the promoter activity.

Next, we determined if the expression of *Fgfr2* RNAi could efficiently knockdown *Fgfr2 in vivo* using RT–PCR. Our data revealed a dramatic (>95%) down-regulation of *Fgfr2* mRNA levels in all three E12.5 *U6-Fgfr2;EIIa-Cre* embryos examined, while no obvious alteration was observed in *U6-ploxPneo-Fgfr2* and *EIIa-Cre* embryos compared with their wild-type littermates (Figure 3A). Fgf receptors exist as a gene family of four members. Our analysis revealed that *U6-Fgfr2* RNAi-mediated suppression is specific to *Fgfr2*, but not other members (Figure 3A). Finally, our real-time PCR analysis confirmed dramatic down-regulation of *Fgfr2* transcripts in the *U6-Fgfr2;EIIa-Cre*, but not in any other control embryos (Figure 3B). These observations provide a molecular basis for the observed embryonic lethality of *U6-Fgfr2;EIIa-Cre* embryos.

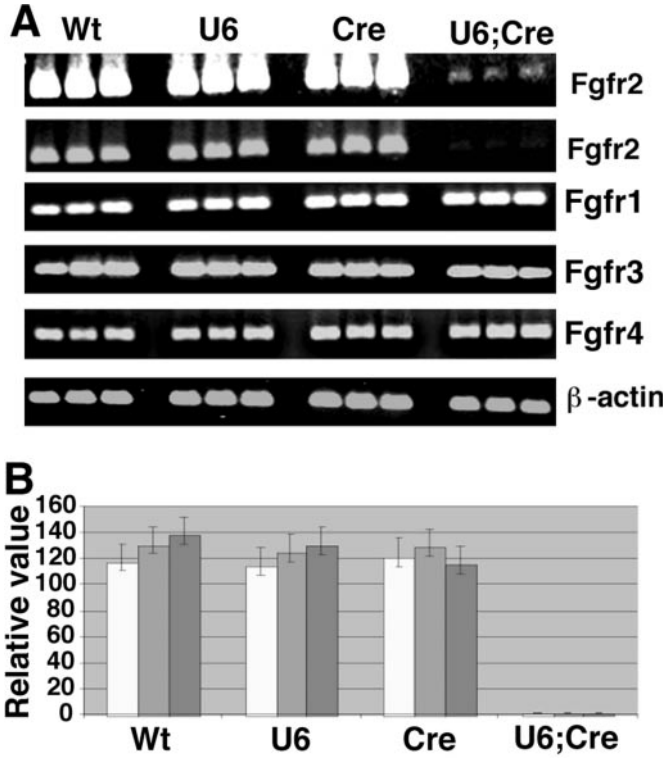

Figure 3. Analysis of *Fgfr1-4* expression. (**A**) RT–PCR analysis of *Fgfr1-4* expression in E12.5 embryos of different genotypes. Wt, wild type; U6, *U6-ploxPneo-Fgfr2*; Cre, *EIIa-Cre*; and U6;Cre, *U6-Fgfr2;EIIa-Cre*. The first row is a longer exposure of the second row. (**B**) Real-time PCR analysis of *Fgfr2* mRNA levels. Three embryos for each genotype were shown. The three mutant embryos used here were less affected and had similar size compared with the littermate controls. The experiments were repeated at least three times and similar data were obtained on each time.

Limb distal mesenchyme-specific knockdown of *Fgfr2* results in abnormal digit formation

Thus, we have created a Cre-mediated conditional *Fgfr2* mutant strain using the RNAi technology. This strain of mice should be a useful tool for studying functions of Fgfr2 in the development and formation of multiple organs/tissues. To demonstrate this in principle, we performed limb-specific knockdown of *Fgfr2* by crossing the *U6-ploxPneo-Fgfr2* mice with *AP2-Cre* transgenic mice, which express *Cre* in the distal mesenchyme [also called progress zone (27)] right after limb bud initiation (25). After crossing with the two strains, we observed a normal distribution of double transgenic animals (*U6-Fgfr2;AP2-Cre*) at birth (Table 1, crosses between *U6-ploxPneo-Fgfr2* and *AP2-Cre* mice). However, all the *U6-Fgfr2;AP2-Cre* animals exhibited limb abnormalities characterized by the reduced number of digits in both the forelimbs (Figure 4A) and hindlimbs (Figure 4B). Alizarin red- and alcian blue-stained whole-mount skeleton imaging revealed that all mutant forelimbs only had two relatively normal digits (digits 4 and 5), while the anterior digits were either missing or developmentally retarded (Figure 4C) in comparison with controls (Figure 4D). On the other hand, all the mutant hindlimbs did not have the third digit (Figure 4E, arrow), while other digits present, however, appeared abnormal (Figure 4F). Furthermore, mutant hindlimbs showed delayed ossification of tarsal bones and distortion in the ankles (Figure 4E and F). In contrast, all control animals (wild type, *U6-ploxPneo-Fgfr2* and *AP2-Cre*) appeared normal (Figure 4C and D). Collectively, these results demonstrate that RNAi can be used to achieve spatial and temporal knockdown of transcripts in mouse.

DISCUSSION

The 3×10^9 base sequence of mouse genome encodes at least 30 000 genes. Despite great efforts having been directed at deciphering functions of these genes by gene targeting, published knockouts represent only ~10% of mouse genes (28). One of the obvious rate limiting factors is the generation of mutant mice, especially those carrying conditional mutations, which requires multi-step modifications (29). In this paper, we have described a method that can be used for conditional RNAi *in vivo* on endogenous genes, using the widely used Cre-LoxP system (15). We showed that the expression of *U6-Fgfr2* RNAi in mouse germline suppressed *Fgfr2* expression with high efficiency, as only <5% of *Fgfr2* transcripts were detected compared with those of controls. We have also performed progress-zone-specific knockdown of *Fgfr2* and demonstrated, in principle, that our strategy can be applied for tissue-specific targeted gene suppression. Given the critical roles of Fgfr2 in mammalian development (17), the *U6-ploxPneo-Fgfr2* mice should be useful in assessing functions of this gene in multiple organs/tissues. We are in the process to cross this strain with transgenic mice that express Cre in a number of other tissues. Because the ratio of RNAi to targeted mRNA varies from one cell type to another, the efficiency of

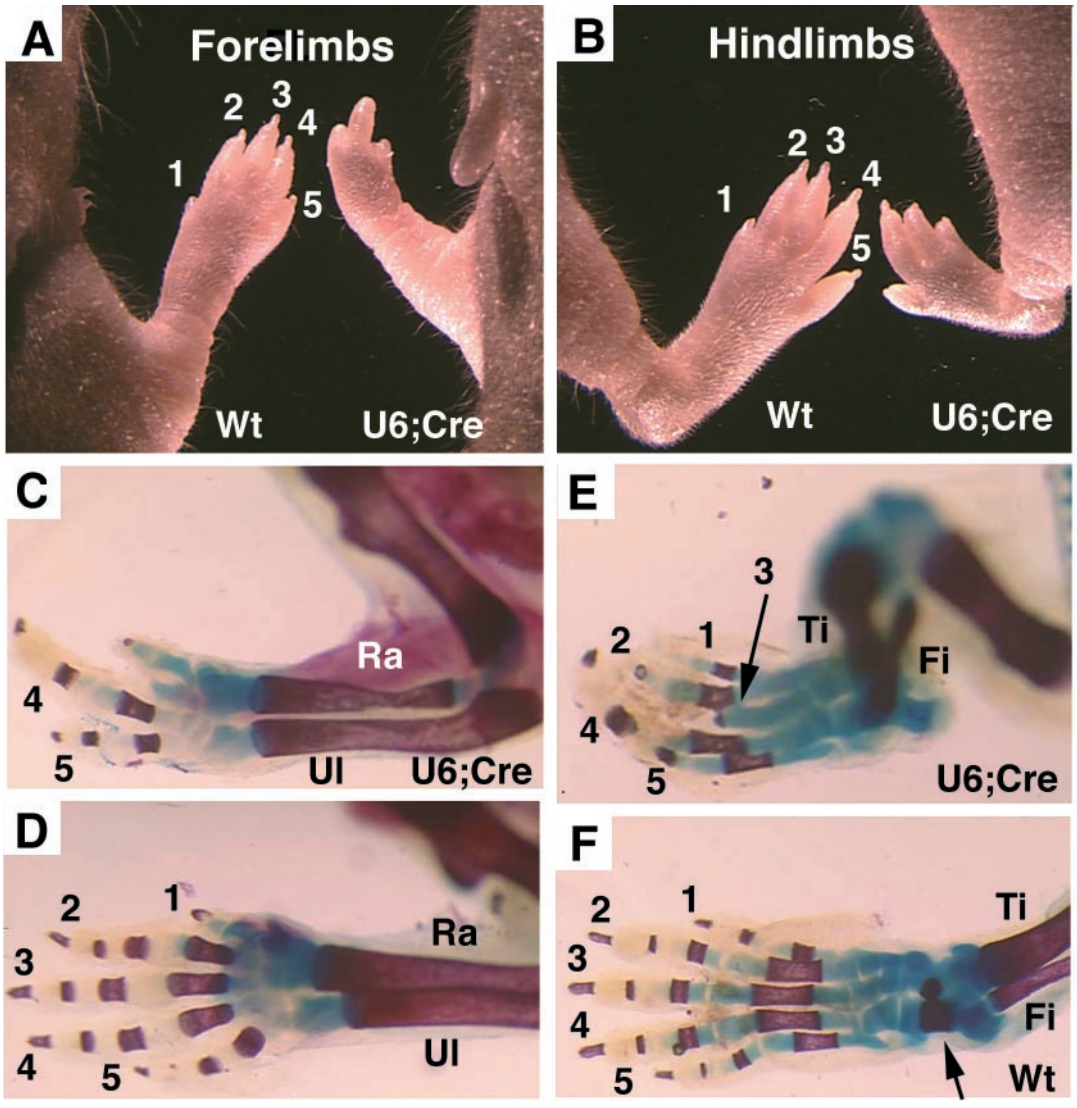

Figure 4. Abnormal digit formation and limb development in *U6-Fgfr2;Ap-2Cre* mice. (**A** and **B**) P7 *U6-Fgfr2;Ap-2Cre* and control forelimbs (A) and hindlimbs (B). (**C**–**F**) Alizarin red- and alcian-blue stained *U6-Fgfr2;Ap-2Cre* (C and E) and control (D and F) limbs. (C and D) were forelimbs, and (E and F) were hindlimbs. Arrow in (E) points to the third finger, which is truncated in all mutant limbs. Arrow in (F) points to calcified tarsal bones, which have not developed in mutant bones (E). Note, the tibia and fibula of mutant hindlimbs (E) were out of focus because of a distortion in the ankle. Ra, radius; Ul, ulna; Ti, tibia; Fi, fibula.

RNAi knockdown may change. We will carefully evaluate the efficiency of *Fgfr2* knockdown in different tissues.

Because our approach does not require constructing sophisticated gene-targeting vectors and ES cell manipulation, it is relatively fast to generate mutant mice carrying RNAi construct. In this study, we were able to generate the *U6-ploxPneo-Fgfr2* mice in 3 months, a significant gain of time compared with homologous recombination procedures for the conditional gene knockouts (12–24 months). Moreover, because RNAi acts dominantly, only one allele of transgene is needed to suppress the endogenous genes, and it reduces the time needed for breeding together two mutant alleles required by conditional knockout of genes with recessive nature. This advantage becomes more obvious when genetic interaction of two or more genes is studied (30,31). Thus, compared with the gene targeting technique, our approach is both fast and simple, and therefore should be widely accessible.

In our approach, *U6-ploxPneo-Fgfr2* construct was introduced through pronuclear injection to obtain mutant mice. Although our crosses between all three *U6-ploxPneo-Fgfr2* founder strains with *EIIa-Cre* transgenic mice resulted in similar embryonic lethality, the expression of transgenes, in theory, should subject to position effects. This is primarily caused by random integration of transgenes into different chromosomal sites that differentially regulate gene expression (32). Thus, the activity of U6 promoter may vary at different integration sites. Apparently, position independent and high levels of gene expression are essential for the success of our approach. It is reported recently that position effect can be minimized or overcome by using an insulator (33). This is a promising strategy that should be tested in the next generation of the RNAi constructs.

We found that the *U6-Fgfr2;EIIa-Cre* animals died at different stages of embryonic development and two mutant

mice survived to adulthood (Table 1, crosses between *U6-ploxPneo-Fgfr2* and *EIIa-Cre* mice). Because all these mice were derived from a single transgenic founder line, this variation was not due to the position effects on the transgene. Instead, it could be due to the fact that each animal contained varying degree of *Fgfr2* knockdown level, with most severe knockdown leading to earlier lethality. One of the factors that may affect the efficiency of *Fgfr2* knockdown is the extent of *ploxPneo* deletion. Based on our experience on conditional knockout of a number of genes, Cre-mediated deletion of floxed sequences is in a range of 60–90% and never reaches 100% even in the most efficient case (31,34). Our real-time PCR analysis indicated that the efficiency of the *ploxPneo* deletion by EIIa-Cre is ~80%, which is comparable with efficiencies of most conditional knockouts. Another factor that may affect Cre efficiency is the copy number of loxP. It was shown previously that EIIa-Cre could cause incomplete deletion of DNA sequences containing three loxP sites and create mosaicism (35,36). Thus, efficiency of Cre may be further reduced in situations when many copies of transgene were cumulated at one locus. In this case, weak Cre activity may result in a variable number of active transgene while in case of strong Cre, only one active transgene will be left. This could result in an additional level of phenotypic variation. One way to avoid high copy number of the transgene is to reduce concentration of DNA for pronuclear injection. Furthermore, phenotypic variation could also occur if the RNAi transgenic mice used for the crossing with Cre-transgenic mice still carried more than one transgene integration that had not yet segregated from each other. Although the integration of transgene into multiple loci is uncommon, it needs to take into account. One way to overcome this is to breed the transgenic mice with wild-type for a number of generations before crossing them with Cre transgenic mice. However, this may be both time and labor consuming. In this study, instead of using wild-type mice, we directly crossed F1 with *EIIa-Cre* mice, as this cross should generate one-fourth of *U6-ploxPneo-Fgfr2* transgenic mice (F2), which were further crossed with *EIIa-Cre* mice to generate F3, then F4 and so on. At the same time, these crosses should also generate one-fourth of *U6-Fgfr2; EIIa-Cre* mice at each generation that can be used for phenotypic analysis. During the course of this work, some *U6-Fgfr2;EIIa-Cre* mice have reached more than seven generations and they still showed same phenotype compared with earlier generations.

On the other hand, phenotype variation is often observed in mice generated by gene targeting, suggesting that it is an intrinsic feature associated with the loss of function mutation, perhaps due to a difference response of each individual to the mutation. Actually, this could be beneficial in some circumstances where variable phenotypes associated with some knockouts may reveal new and unexpected information. Thus, scientists intentionally generate hypomorphic alleles using sophisticated targeting modifications (37). This is a challenge for RNAi after minimizing the undesired position effects; however, it may be achieved in two ways: one is the actual copy number of the RNAi transgene (i.e. breeding of heterozygote to homozygote), and the other is using a suboptimal shRNA vector, as varying efficiency always occurs in culture cells when different sequences of the same gene are used.

During the course of this study, two recent investigations showed that lentivirus-based RNAi systems could be used to generate transgenic animals with Cre-loxP mediated gene knockdown (38,39). While it is not possible to have a direct comparison between different approaches, our work is the first demonstration that the plasmid-based RNAi, with its simplicity in construction, can be used to specifically knockdown an endogenous gene, *Fgfr2*, with high efficiency *in vivo*. Because all the techniques using RNAi to suppress endogenous genes are still in their early developing stages, our method should provide an additional choice for this scientific field.

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Conflict of interest statement. None declared.

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