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

Xavier Coumoul, Vivek Shukla, Cuiling Li, Rui-Hong Wang and Chu-Xia Deng*

Genetics of Development and Disease Branch, 10/9N105, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland, MD 20892, USA

Received March 10, 2005; Revised May 27, 2005; Accepted June 4, 2005

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 into cultured cells, allowing mRNA down-regulation within 24–72 h (1–5). Plasmids can be stably integrated into host genome and allow mRNA down-regulation within 24–72 h (1–5). Plasmids can be stably integrated into host genome and allow mRNA down-regulation within 24–72 h (1–5). Plasmids can be stably integrated into host genome and allow mRNA down-regulation within 24–72 h (1–5).

Recent 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 intramembranous 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...
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 malformed formation of the egg cylinder (21). Mutant embryos homozygous for Fgfr2-IgIII domain deletion (Fgfr2<sup>ΔIII/ΔIII</sup>) 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<sup>ΔIII/ΔIII</sup> 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 knockdown this gene in mice 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-Fgr2 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’-CGAAGATTTACTAGTCCGAC-3’ and 5’-AAACAAAGGGTCCTTCTCAGAG-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 Fgr2-RNAi transgene**

Mice carrying the U6-ploxPneo-Fgr2-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’-CCCT GTT TTG CAC GTP CAC CG-3’ and Cre-3: 5’-ATG CTT CTG TCC GTC CG-3’). Pregnant EIIa-Cre;U6-ploxPneo-Fgr2 and Ap2-Cre;U6-ploxPneo-Fgr2 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’-GACGCCGCACTCCTAGG-3’ and 5’-AGGCT-TTCTCAGGGATT-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 CO₂. 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)₄₈ 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: Fgr1-F: 5’-TTCTGGGCTGTGTGTCAC-3’ and Fgr1-R: 5’-CGGAACCTGTAGCTCAGA-3’; Fgr2-F: 5’-AAGGT-TTACAGCGATGCTAC-3’ and Fgr2-R: 5’-ACACCATG-CAAGGATCAA-3’; Fgr3-F: 5’-CTAGTTGTCGTCGTTTG-GGT-3’ and Fgr3-R: 5’-TTCTTATCTCCTGCAGCTCC-3’; Fgr4-F: 5’-CTGTGAGC-TCTTTCAGG-3’ and Fgr4-R: 5’-CGTTAGCCACCGTCCTCACC-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 Fgrf1, Fgrf3 and Fgrf4. Fgrf2 primers were described as Fgrf2-Up: 5’-TCGCCCTACCTCAAGGTTTG-3’; and Fgrf2-Down: TAGAATTTACCCCGCAAGC-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’-GAGTCATCAAAGAGAG-3’; and β-actin-Down: 5’-GATGCCACAGGATTCCATA-3’.

The following primers were used for genomic real-time PCR: Neo-U: 5’-TGCTGACATCAAAGAGAG-3’; and Del-1: 5’-AGAG-ATCATCCTGATC-3’. The following primers were used as control for both RT–PCR and genomic PCR, following pairs of primer were used: Neo-1: 5’-AGAG-GCTATTTCCGGTATGCT-3’; Neo-2: 5’-GTCGCCACAGGATACG-3’; Fgr1-F: 5’-CC-CATTCCCATTTCTCTAC-3’; and Fgr1-D: 5’-TTCTGGTGTGGAAACAG-3’.

For amplification of neo gene and neo deletion by genomic PCR, following pairs of primer were used: Neo-1: 5’-AGAG-GCTATTTCCGGTATGCT-3’; Neo-2: 5’-TGCTGACATCAAAGAGAG-3’; Del-1: 5’-CCACAGAATCGTGGGAGAA-3’; and Del-2: 5’-CACAATTTATTTAAGGTCAG-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-N² membrane.
Schleicher & Schuell, Keene N.H.) and was cross linked by using the auto-crosslink function of a Strat linker. The membrane was hybridized overnight to a [γ-32P]labeled DNA probe corresponding to the 21mer antisense strand of the Fgfr2 shRNA (GGCCTCAGCTTCCCTGAGCA). 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,

![Diagram of U6-ploxPneo-Fgfr2 transgene before and after deletion of ploxPneo and EIIa-Cre](image)

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 Elia-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 Elia-Cre transgenic mice, we obtained two live bigenic (U6-Fgfr2;Elia-Cre) mice (Table 1, crosses between U6-ploxPneo-Fgfr2 and Elia-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-thirds 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;Elia-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 Elia-Cre mice and dissected females at days 11.5 through 13.5 during their pregnancy. Our data indicated that U6-Fgfr2;Elia-Cre embryos were present at a Mendelian ratio (Table 1, crosses between U6-ploxPneo-Fgfr2 and Elia-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, Elia-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;Elia-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 Elia-Cre or AP2-Cre transgenic mice |
|---|---|---|---|---|---|---|
| Ages | Total | WT | Elia-Cre | U6-ploxPneo-Fgfr2 | U6-Fgfr2;Elia-Cre | AP2-Cre | U6-Fgfr2;AP-2Cre |
| Crosses between U6-ploxPneo-Fgfr2 and Elia-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 |

aSmall size and limb abnormalities.
bAll 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 Fgfr2−/− 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;Elia-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.
Ella-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; Ella-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; Ella-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; Ella-Cre embryos examined, while no obvious alteration was observed in U6-ploxPneo-Fgfr2 and Ella-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; Ella-Cre, but not in any other control embryos (Figure 3B). These observations provide a molecular basis for the observed embryonic lethality of U6-Fgfr2; Ella-Cre embryos.

**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 alcin 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 × 10⁹ 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
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

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.
mice survived to adulthood (Table 1, crosses between U6-ploxPneo-Fgfr2 and Ella-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 Ella-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 Ella-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 Ella-Cre mice, as this cross should generate one-fourth of U6-ploxPneo-Fgfr2 transgenic mice (F2), which were further crossed with Ella-Cre mice to generate F3, then F4 and so on. At the same time, these crosses should also generate one-fourth of U6-Fgfr2: Ella-Cre mice at each generation that can be used for phenotypic analysis. During the course of this work, some U6-Fgfr2: Ella-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.

ACKNOWLEDGEMENTS

The authors thank H. Lu for pronuclear injection, Dr S. Lee and members of Deng laboratory for critical discussion and comments of the manuscript. Funding to pay the Open Access publication charges for this article was provided by National Institutes of Health, USA.

Conflict of interest statement. None declared.

REFERENCES

1. Novina,C.D. and Sharp,P.A. (2004) The RNAi revolution. Nature, 430, 161–164.
2. Yu,J.Y., DeRuiter,S.L. and Turner,D.L. (2002) RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. Proc. Natl Acad. Sci. USA, 99, 6047–6052.
3. Coupouli,X., Li,W., Wang,R.H. and Deng,C. (2004) Inducible suppression of Fgfr2 and Survivin in ES cells using a combination of the RNA interference (RNAi) and the Cre-loxP system. Nucleic Acids Res., 32, e85.
4. Elbashir,S.M., Lendeckel,W. and Tuschl,T. (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev., 15, 188–200.
5. Elbashir,S.M., Harborth,J., Lendeckel,W., Yalcin,A., Weber,K. and Tuschl,T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature, 411, 494–498.
6. Wang,J., Tekle,E., Oubrahim,H., Mieyal,J.J., Stadman,E.R. and Chock,P.B. (2003) Stable and controllable RNA interference: Investigating the physiological function of glutathionylated actin. Proc. Natl Acad. Sci. USA, 100, 5103–5106.
7. Czauderna,F., Santel,A., Hinz,M., Fechner,M., Durieux,B., Fisch,G., Leenders,F., Arnold,W., Giese,K., Klippel,A. et al. (2003) Inducible shRNA expression for application in a prostate cancer mouse model. Nucleic Acids Res., 31, e127.
8. Gupta,S., Schoer,R.A., Egan,J.E., Hannon,G.J. and Mitali,V. (2004) Inducible, reversible, and stable RNA interference in mammalian cells. Proc. Natl Acad. Sci. USA, 101, 1927–1932.
9. Matsukura,S., Jones,P.A. and Takai,D. (2003) Establishment of conditional vectors for hairpin siRNA knockdowns. Nucleic Acids Res., 31, e77.
10. Yang,X., Lu,C., Xu,X. and Deng,C. (1998) The tumor suppressor SMAD4/DPC4 is essential for epiblast proliferation and mesoderm induction in mice. Proc. Natl Acad. Sci. USA, 95, 3667–3672.
11. Metger,D., Clifford,J., Chiba,H. and Champon,P. (1995) Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase. Proc. Natl Acad. Sci. USA, 92, 6991–6995.
12. Branda,C.S. and Dymecki,S.M. (2004) Talking about a revolution: the impact of site-specific recombinases on genetic analyses in mice. Dev. Cell, 6, 7–8.
13. Le,Y. and Sauer,B. (2000) Conditional gene knockout using cre recombinase. Methods Mol. Biol., 136, 477–485.
14. Capecci,M.R. (1989) Altering the genome by homologous recombination. Science, 244, 1288–1292.
15. Nagy,A. (2000) Cre recombinase: the universal reagent for genome tailoring. Genesis, 26, 99–109.
16. Ornitz, D.M. and Itoh, N. (2001) Fibroblast growth factors. Genome Biol., 2, REVIEW530005.

17. Coumoul, X. and Deng, C.X. (2003) Roles of FGF receptors in mammalian development and congenital diseases. Birth Defects Res Part C Embryo Today, 69, 286–304.

18. Hou, J.Z., Kan, M.K., McKeohan, K., McBride, G., Adams, P. and McKeehan, W.L. (1991) Fibroblast growth factor receptors from liver vary in three structural domains. Science, 251, 665–668.

19. Werner, S., Duan, D.S., de Vries, C., Peters, K.G., Johnson, D.E. and Williams, L.T. (1992) Differential splicing in the extracellular region of fibroblast growth factor receptor 1 generates receptor variants with different ligand-binding specificities. Mol. Cell. Biol., 12, 82–88.

20. Muenke, M. and Schell, J.U. (1995) Fibroblast-growth-factor receptor mutations in human skeletal disorders. Trends Genet., 11, 308–313.

21. Arman, E., Haffner-Krausz, R., Chen, Y., Heath, J.K. and Lonai, P. (1998) Targeted disruption of fibroblast growth factor (FGF) receptor 2 suggests a role for FGF signaling in pregastrulation mammalian development. Proc. Natl Acad. Sci. USA, 95, 5082–5087.

22. Xu, X., Weinstein, M., Li, C., Naski, M., Cohen, R.I., Ornitz, D.M., Leder, P. and Deng, C. (1998) Fibroblast growth factor receptor 2 (FGFR2)-mediated reciprocal regulation loop between FGF8 and FGF10 is essential for limb induction. Development, 125, 753–765.

23. Li, C., Guo, H., Xu, X., Weinberg, W. and Deng, C.X. (2001) Fibroblast growth factor receptor 2 (Fgfr2) plays an important role in eyelid and skin formation and patterning. Dev. Dyn., 222, 471–483.

24. Lakso, M., Pichel, J.G., Gorman, J.R., Sauer, B., Okamoto, Y., Lee, E., Alt, F.W. and Westphal, H. (1996) Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. Proc. Natl Acad. Sci. USA, 93, 5860–5865.

25. Nelson, D.K. and Williams, T. (2004) Frontonasal process-specific disruption of AP-2 alpha results in postnatal midfacial hypoplasia, vascular anomalies, and nasal cavity defects. Dev. Biol., 267, 72–92.

26. McLeod, M.J. (1980) Differential staining of cartilage and bone in whole mouse fetuses by alcian blue and alizarin red S. Teratology, 22, 299–301.

27. Summerbell, D., Lewis, J.H. and Wolpert, L. (1973) Positional information in chick limb morphogenesis. Nature, 244, 492–496.

28. Austin, C.P., Battey, J.F., Bradley, A., Bucan, M., Capechi, M., Collins, F.S., Dove, W.F., Duyk, G., Dymecki, S., Eppig, J.T. et al. (2004) The knockout mouse project. Nature Genet., 36, 921–924.

29. Deng, C.X. and Xu, X. (2004) Generation and analysis of Brca1 conditional knockout mice. Methods Mol. Biol., 280, 185–200.

30. Weinstein, M., Xu, X., Ohyama, K. and Deng, C.X. (1998) Fibroblast growth factor receptor-3 and receptor-4 function cooperatively to direct alveogenesis in the murine lung. Development, 125, 3615–3623.

31. Xu, X., Wagner, K.U., Larson, D., Weaver, Z., Li, C., Ried, T., Hennighausen, L., Wynshaw-Boris, A. and Deng, C.X. (1999) Conditional mutation of Brca1 in mammary epithelial cells results in blunted ductal morphogenesis and tumour formation. Nature Genet., 22, 37–43.

32. Kioussis, D. and Festenstein, R. (1997) Locus control regions: overcoming heterochromatin-induced gene inactivation in mammals. Curr. Opin. Genet. Dev., 7, 614–619.

33. Frazar, T.F., Weissman, J.L., Anderson, S.M., Cline, A.P., Garrett, L.J., Felsenfeld, G., Gallagher, P.G. and Bodine, D.M. (2003) Variegated expression from the murine band 3 (AE1) promoter in transgenic mice is associated with mRNA transcript initiation at upstream start sites and can be suppressed by the addition of the chicken beta-globin 5’ HS4 insulator element. Mol. Cell. Biol., 23, 4753–4763.

34. Li, W., Qiao, W., Chen, L., Xu, X., Yang, X., Li, D., Li, C., Brodie, S.G., Meguid, M.M., Hennighausen, L. et al. (2003) Squamous cell carcinoma and mammary abscess formation through squamous metaplasia in Smad4/Dpc4 conditional knockout mice. Development, 130, 6143–6153.

35. Xu, X., Li, C., Garrett-Beal, L., Larson, D., Wynshaw-Boris, A. and Deng, C.X. (2001) Direct removal in the mouse of a floxed neo gene from a three-loxP conditional knockout allele by two novel approaches. Genesis, 30, 1–6.

36. Holzenberger, M., Lenzner, C., Leneuve, P., Zaoui, R., Hamard, G., Vaulont, S. and Bouc, Y.L. (2000) Cre-mediated germline mosaicism in mice generates offspring from alleles of a target gene. Nucleic Acids Res., 28, E92.

37. Deng, C.X. (2002) Tumor formation in Brca1 conditional mutant mice. Environ. Mol. Mutagen., 39, 171–177.

38. Ventura, A., Meissner, A., Dillon, C.P., McManus, M., Sharp, P.A., Van Parjis, L., Jaenisch, R. and Jacks, T. (2004) Cre-lox-regulated conditional RNA interference from transgenes. Proc. Natl Acad. Sci. USA, 101, 10380–10385.

39. Chang, H.S., Lin, C.H., Chen, Y.C. and Yu, W.C. (2004) Using siRNA technique to generate transgenic animals with spatiotemporal and conditional gene knockdown. Am. J. Pathol., 165, 1535–1541.