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# Construction of engineered murine Embryonic stem cells with conditional knockout of FGFR2 depending on Cre-loxP

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**Key words:** fibroblast growth factor receptor-2 (FGFR2), conditional knockout, homologous recombination, Cre, ES cells

**ABSTRACT: Objective:** To investigate the functions of Fibroblast Growth Factor Receptor-2 (FGFR2) at different stages of cell differentiation. The engineered murine embryonic stem (ES) cells with conditional knockout of FGFR2 were developed depending on Cre-loxP. **Methods:** Cre-loxP system was used in a conditional targeting vector. The competent AM-1 bacteria, which expressed Cre-recombinase, was used to confirm the Cre-mediated deletion of the floxed exons 7 and 8 of FGFR2. The targeting vector was electroporated into the ES cells, and the transfected ES cells were screened with G418 and Ganciclovir. Finally, the ES clones with correct targeting events were identified by Southern Blot and PCR. **Results:** The targeting vector with conditional knockout of murine FGFR2 was successfully constructed and confirmed by PCR and digestion analysis in bacteria. 86 ES clones were collected by selective culture with G418 and Ganciclovir. Four of the 86 ES clones were found containing the targeting gene sequence in genomic DNA proved by Southern Blot with a 5'-end flank probe. Two of the four ES clones had the correct targeting events that included the insertion of the targeting gene sequence in genomic DNA and were checked by Southern Blot with a 3'-end flanking probe. Finally, the insertion of loxP (loxP3) between exons 8 and 9 in genomic DNA was identified in one of the two ES clones by Southern Blot and PCR. **Conclusion:** FGFR2 conditional knockout depending on Cre-loxP can be successfully used in ES cells.

## Introduction

The Cre-loxP system is a tool for tissue-specific knockout of such genes which are floxed by loxP (Kos, 2004). If the gene is excised, the function of the gene will be downregulated. Sometimes, the function of the gene will also be downregulated if loxP is inserted into the promoter of the gene. Then, the function of the gene can be activated again if the loxP is removed. The DNA excising between loxPs is depended on Cre-recombinase expression, which can be expressed in specific tissue drived by tissue-specific promoter. In addition, Cre-loxP system can also be used for time-specific DNA excising if Cre-recombinase is expressed at specific time.

The Cre-loxP system was firstly used for the site-specific recombination in bacteriophage P1 *in vitro* in 1985 (Abremski and Hoess, 1985). It is also possible to perform the control of conditional gene expression at different stages of stem cells or in gene therapy (Coumoul *et al*., 2004; Greco *et al*., 2006). At different stages from embryonic stem cells to specific tissue cells, cells have different functions. But we know little about the process of these functional changes. After the engineered ES cells with Cre-loxP system were developed, the specific gene expression can be controlled by Cre-recombinase.

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Fibroblast Growth Factor Receptor 2 (FGFR2) is a kind of tyrosine kinase (Muenke and Schell, 1995). It can bind to many fibroblast growth factor ligands by its varying affinity and specificity. Previous research results (Ornitz and Marie, 2002; Wilkie, 2001; Chen *et al*., 2003), show that FGFR2 has important effects on organs or tissues during formation and development of head, limbs, lung, kidney, skin, thymus, etc. Although the FGFR2 is an important gene in early stages of embryo development, little is known about the function of FGFR2 at different stages of ES cell differentiation. To study the physiological and pathological effects of FGFR2 during different stages of ES cell differentiation development, the embryonic stem cell modification with FGFR2 conditional knockout by Cre-loxP system was developed in present study.

## Materials and Methods

### *Materials*

pBluescript II SK plasmid was bought from Stratagene Inc. Gene targeting vector ploxP II-neo and ES cells were obtained from Dr. Chuxia Deng (NIH, USA). To amplify above plasmids, DH5α bacteria was used, which was keeping in our lab. AM-1 bacteria, which expressed Cre-recombinase, was obtained from Dr. Rucker (Missouri University, USA) (Rucker and Piedrahita, 1997). Mouse Embryo Fibroblast (MEF) with G418-resistance was obtained from Prof. Yang Xiao (Academy of Military Medical Science, China). Engineering enzymes were bought from New England, Roche and TaKaRa Inc. The λ/HindIII marker and the DL2000

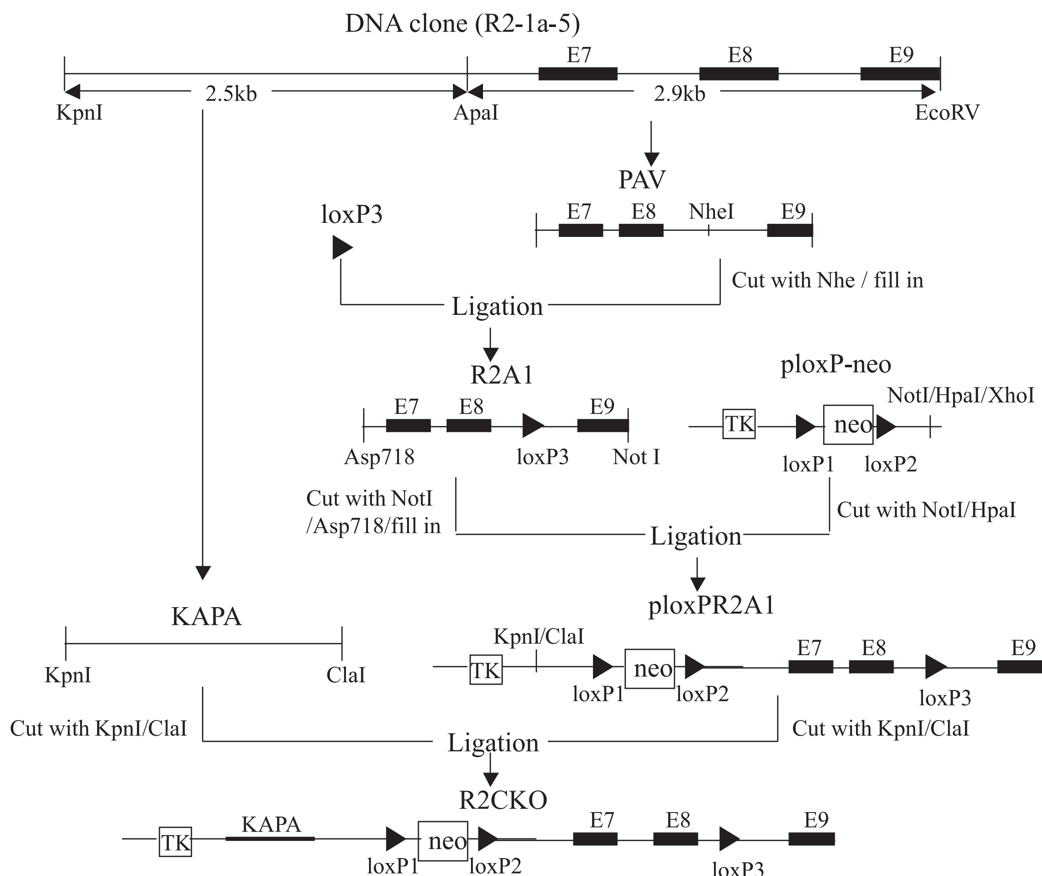


**FIGURE 1.** A scheme of the gene targeting strategy used in our present study.

marker were bought from TaKaRa Inc. Plasmid and Gel extraction kits were bought from Omega and Qiagen Inc respectively. DNA sequence was identified and all primers were synthesized by Shenergy Biocolor BioScience & Technology Inc (Shanghai, China). BioRad Gene Pulser II System£¨BioRad Company, USA) was used for electroporation. LIF (Leukocyte inhibitory factor), DMEM (Dulbecco's Modified Eagle's Medium), L-Glutamine, non-necessary amino acid, solution of penicillin and streptomycin, β-methylsulfonyl ethanol, fetal bovine serum, Mitomycin-C, DMSO (Dimethyl Sulfoxide), PBS buffer (pH 7.2), EDTA-Trypsin, Ganciclovir, G418 and Gelatin were bought from Gibco BRL,Sigma and HyClone Inc.

*Design and construction of the targeting vector*

For the construction of the targeting vector, the outline is shown in figure 1. The DNA colony (R2-1a-5), which includes the DNA sequence from intron 6 to exon 9 of FGFR2, was screened from the sv129 mouse genomic library by bacterial colonies *in situ* hybridization. A 2.9kb fragment was obtained after Apa I and EcoR V digestion. This 2.9kb fragment, which starts at

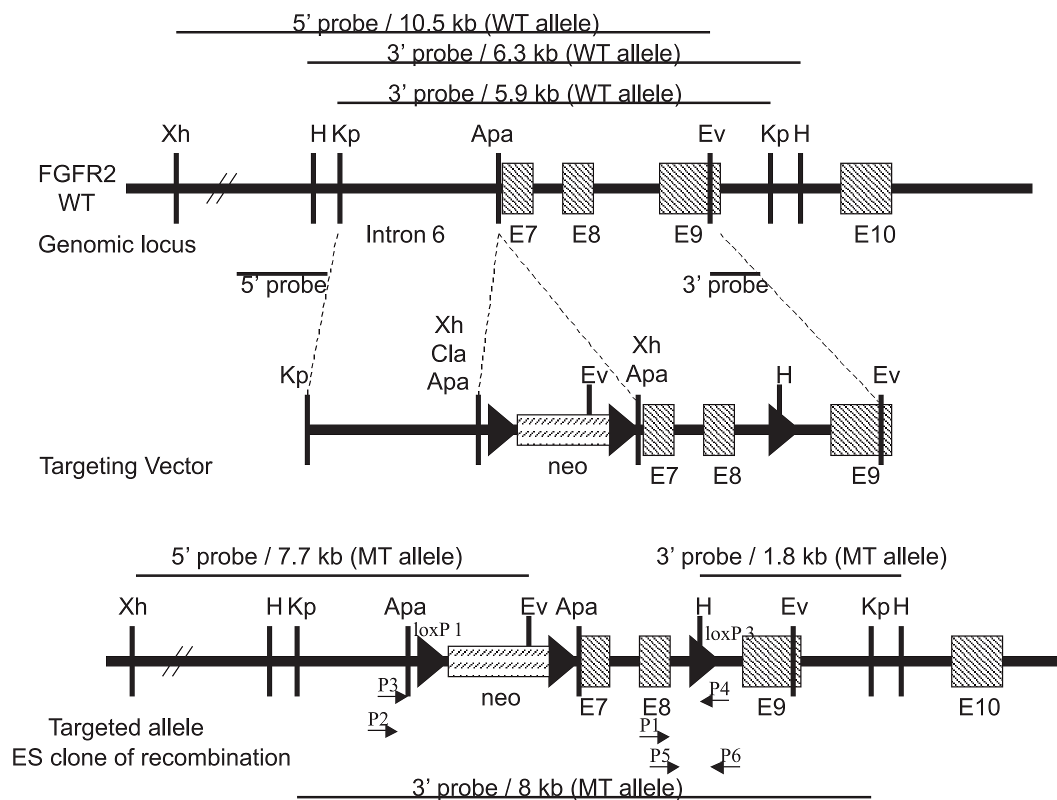


**FIGURE 2.** Homologous recombination between targeting vector and FGFR2 genomic DNA. 5'probe is probe site for Southern blot in 5'-end, the size is 3.1 kb; 3'probe is probe site for Southern blot in 3'-end, the size is 1.5 kb; 5'probe/10.5kb (WT allele) and 5'probe/7.7kb (MT allele) represent the site and size of Southern Blot fragment by using 5'-probe after Xho I and EcoR V digestion in wild type and mutant type, respectively. 3'probe/6.3kb (WT allele) and 3'probe/1.8kb (MT allele) represent the site and size of Southern blot fragment by using 3'-probe after Hind III digestion in wild type and mutant type, respectively. 3'probe/5.9kb (WT allele) and 3'probe/8.0kb (MT allele) represent site and size of Southern blot fragment by using 3'-probe after Kpn I digestion in wild type and mutant type, respectively. Frames represent exons (sign is E) and neo gene; arrows are site and direction of loxPs, which are loxP 1 ~ 3 from left to right. Xh: Xho I; H: Hind III; Kp: Kpn I; Apa: Apa I; Cla: Cla I; Ev: EcoR V; E7: Exon 7; E8: Exon 8; E9: Exon 9; E10: Exon 10; P1 and P2 are site and direction for two sequecing primers; P3 ~ P6 are site and direction of PCR primers.

the ApaI site of intron 6 and ends at the EcoRV site of exon 9, was inserted into pBluescript II SK to get the PAV plasmid. The PAV plasmid was digested with NheI and a loxP sequence (loxP3) was inserted at the NheI site to get the R2A1 plasmid. The R2A1 plasmid was digested with Not I and Asp718 to get a fragment (R2A1 fragment) with exons 7,8, 9 and loxP3. Complete fill in was performed at Asp718 site to get a blunt end. The R2A1 fragment as one homologous arm of the targeting vector (3'-end long arm) was inserted into the ploxP II-neo plasmid to get the ploxPR2A1 plasmid. In addition, a 2.5kb fragment with intron 6 of FGFR2 was also cut from R2-1a-5 colony by digestion with KpnI and ApaI. In order to amplify this 2.5kb fragment, it was inserted into the pBluescript II SK to get the KAPA plasmid. The KAPA plasmid was digested with KpnI and ClaI to get the KAPA fragment. The KAPA fragment as another homologous arm of the targeting vector (5'-end short arm) was then inserted into the ploxPR2A1 plasmid to get the conditional targeting vector R2CKO.

*Structural identification of the R2CKO targeting vector*

*Identification by digestion*

To identify the reading frame of the vector, the conditional knockout vector R2CKO was digested with NotI, NotI+XhoI, KpnI+ClaI, respectively.

*Identification by sequencing*

The partial sequence of the targeting vector was identified by ABI 3700 Genetic Analyzer. The sequencing primers are P1 (5'-GATTGGACTGCACACTTCCC-3') and P2 (5'-TAGGTAGTCCATAACTCGG-3'). The location of P1 and P2 is showed in figure 2.

*Functional identification of the R2CKO targeting vector by Cre-mediated loxP recombination*

*The transfected AM-1 bacteria with R2CKO plasmid was prepared by electroporation*

40μl of the competent bacteria AM-1 with 200 ng of the conditional targeting vector DNA were electroporated using Biorad Gene Pulser II Electroporation with parameters set to 25KV, 200Ω and 2.5μF. After incubation with shaking for 1 h at 37ºC, the mixture was seeded into LB culture medium containing Amp for overnight at 37ºC. 40 positive colonies with Amp-resistance were selected for the functional identification of Cre-mediated recombination.

*Screening of recombination bacteria by PCR*

Bacterial colonies with correct recombination, in which the DNA sequence including exon 7, exon 8 and neo gene in R2CKO plasmid were deleted, were collected by PCR. Primers were primer P3 (5'-GTGAATAAGAGGCCTTATACC-3') and primer P4 (5'-CCTCCAGTGTCACAGTCACTGG-3'). P3 was located before loxP1 in the short arm, and P4 was located behind loxP3 in the long arm. (Fig. 2).

*Identification of positive colonies with the loxP recombination by digestion*

After the selected AM-1 bacteria with R2CKO plasmid were amplified, the plasmids in the AM-1 bacterial colonies were extracted and digested with EcoR V for further identification of the recombination.

*Preparation of mouse embryo fibroblast and the culture of ES cells*

After being expanded, the primary mouse embryo fibroblast (MEF) were treated with Mitomycin C for 2-3 h (the concentration of Mitomycin C is 10 μg/ml). Then, MEF were stored in -80°C.

*Electroporation of ES cells with the targeting vector*

ES cells were cultured on the G418-resistance MEF feeder. When the number of ES cells in culture was more than $2 \times 10^7$, the ES cells were collected and resuspended in 1ml PBS. 50μg linearized targeting DNA vector and 1ml ES cells were mixed by electroporation. The parameters of the electroporation were 600V, 25μF, 200Ω, and 32ms. After electroporation, ES cells were cultured for 24 h. Then, the medium was changed into the selective culture medium (containing G418, 280μg/ml and Ganciclovir, 2μM).

*Selection and amplification of ES clones*

On the 8$^{th}$ day after electroporation, the resistant ES cell clones were collected. Each ES cell clone was divided equally into two samples. One was stored in -80°C and the other was amplified for extraction of genomic DNA.

*Identification of the FGFR2 targeted ES cells*

*Identification through Southern Blot*

After the ES cells were amplified, genomic DNA was extracted and digested with corresponding enzymes. The Southern Blot was carried out by using flanking probes of 5'-end and 3'-end arm, respectively.

*Identification of loxP3 through PCR*

To detect whether loxP3 is located in intron 8, PCR was performed by using primer P5 (5'-ATAACTTCGTATAATGTATGC-3') and P6 (5'-GAATATACGTGCTTGGCGG-3') which are located on the two flanks of loxP3 (Fig. 2).

## Results

*Construction of the FGFR2 conditional targeting vector*

There is a short arm of 2.5kb and a long arm of 2.9kb in the targeting vector for the FGFR2 conditional knockout. The long arm (3'-end arm) contains exons 7, 8, 9 and loxP3; the short arm (5'-end arm) contains intron 6. The original two loxPs (loxP1 and loxP2) from the ploxP II-neo vector are located in the two flanks of the neo gene respectively, and a new inserted loxP (loxP3) sequence is located at intron 8. The exons 7, 8 and neo gene can be deleted through Cre-mediated loxP recombination. Primary digestion sites in the targeting vector are shown in figure 3.

*Identification of the FGFR2 conditional targeting vector by restriction enzyme digestion*

The digestion gel display was used for the identification of the obtained FGFR2 conditional targeting vector. The vector was linearized by Not I digestion, and digested into 3 bands of 1.9kb, 2.9kb and 5.5kb with Not I and Xho I, or into 2 bands of 2.5kb and 10kb with Kpn I and Cla I. These results (Fig. 4A) are consistent with our original design.

*Identification of the FGFR2 conditional targeting vector by sequencing*

The R2CKO conditional targeting vector was partially sequenced. The sequenced regions include the two boundary regions between two ends of neo gene and two homologous arms. In addition, neo gene, HSV-thymidine kinase (TK) gene and a part of intron 8 containing loxP3 were also sequenced. The results are shown in figure 5. The sequence of the partial long arm (1-80,146-612) and loxP3 (81-145) is shown in figure 5A. The sequence of 3'-end of the short arm (1-112), loxP1 (113-157) and the 5'-end of neo gene (158-610) is shown in figure 5B. The results show that the sequence, localization and direction of the primary regions in the targeting vector are correct.

*Identification of the loxP recombination function of the targeting vector in AM-1 competent bacteria*

Because the expression of Cre recombinase in AM-1 bacteria and recombination of loxPs in plasmids, the DNA sequence including exon 7, exon 8 and neo gene were deleted in plasmids.

The picked bacterial colonies were firstly detected by PCR with P3 and P4 primers. The results show two different kind of lanes. One is from positive colony (Fig. 4B-lane 2), and the other is from negative colony (Fig. 4B-lane 1). In positive colony, a 280bp band reveals that exons 7, 8 and neo gene were deleted. In negative colonies, this band is absent because no recombination

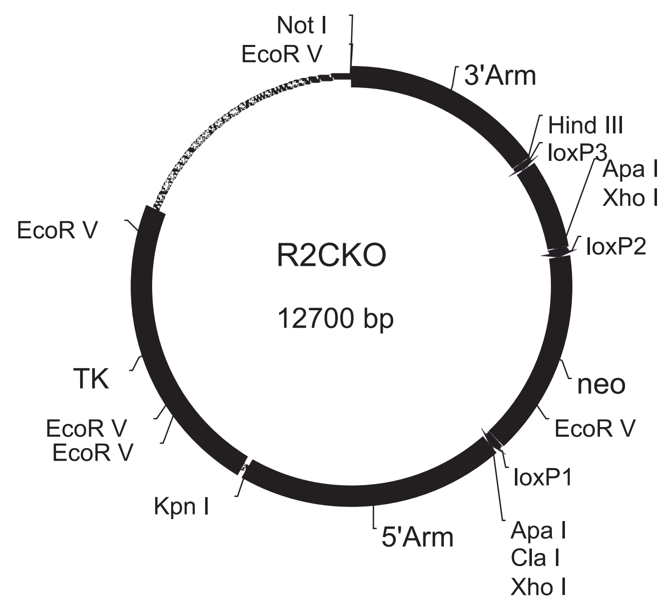


**FIGURE 3.** The structure of targeting vector for FGFR2 conditional gene knockout. Thin line represents genomic sequence, loxP3 is located in the long arm (3'-end arm), trigon represents sequence and direction of loxP, neo gene is located between long arm (3'-end) and short arm (5'-end), and HSV-thymidime kinase (tk) gene is located in the external short arm (5'-end).

occurred and exons 7, 8 and neo gene remain so that the size of PCR product is too large to amplify efficiently.

The targeting vector was also checked by restriction enzyme digestion. The result, show that the plasmid in positive colonies was digested into 3 bands of 4.1kb, 2.9kb and 1.6kb with EcoR V (Fig. 4C-lane 1), and the plasmid in negative colonies was digested into 4 bands of 4.5kb, 2.9kb, 2.5kb and 1.6kb with EcoR V (Fig.4C-lane 2). This result confirms that the exon 7, 8 and neo gene of the targeting vector can be effectively deleted by Cre-mediated loxP recombination. Therefore, our targeting vectors of FGFR2 conditional knockout are correctly designed, and can be used for transfection of ES cells.

*Screening of the targeted ES cells*

After the targeted ES cells were selectively cultured with G418 and Ganciclovir, 86 resistant ES clones were collected on the 8$^{th}$ day of the electroporation. The collected ES clones were then divided equally into two samples. One was amplified for the extraction and identification of genomic DNA; the other was stored in -80°C for next revitalization.

After the ES genomic DNA was digested by using Xho I and EcoR V, Southern Blot with a probe designed from the 5'-end flank of short arm (the location of the probe and the sites of Xho I and EcoR V are shown in Fig. 2) shows that a non-specific 22kb band and a10.5kb band were found in the wild type (Fig. 6A- lane 1), and a 7.7kb band was additionally found in the mutant type (Fig. 6A- lanes 2-5). The presence of this 7.7kb band in the mutant type is because the FGFR2-CKO targeted allele contains an EcoR V site from neo gene. For the 5'-end probe hybridization, the DNA fragment from Xho I to EcoR V is 10.5kb in wild type (See FGFR2 WT in Fig. 2), and it is 7.7kb because of an EcoR V site presence on the neo gene in mutant type (See targeted allele in Fig. 2).

The Southern Blot result shows that four of 86 resistant ES clones were found as positive ES cell clones.

*Revitalization of the positive ES cell clones and further identification*

After the screening of the targeted ES cells, the four positive ES clones were resuscitated by rapid thawing and amplified to further identify.

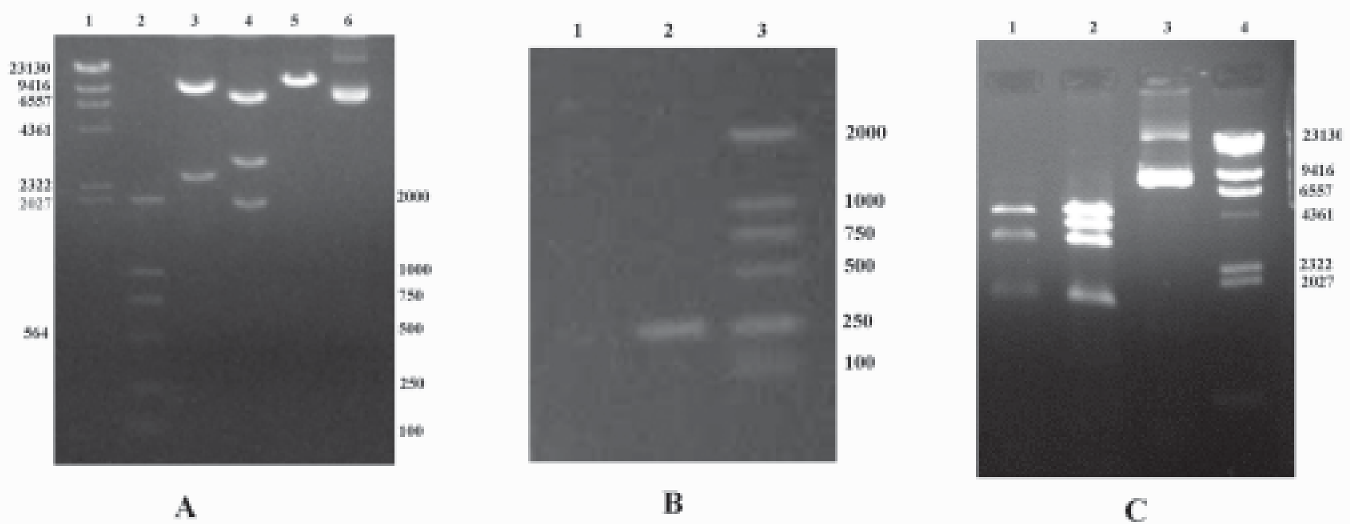


**FIGURE 4.** Identification for the R2CKO vector and its recombination.
**A.** Identification of primary digestion sites for R2CKO. Lane 1 and 2 are λ/HindIII and DL2000 marker; lane 3 is KpnI and ClaI digestion; lane 4 is NotI and XhoI digestion; lane 5 is NotI digestion; lane 6 is control for R2CKO without digestion.
**B.** PCR identification for the recombination of conditional gene targeting vector in AM-1-Cre bacteria. Lane 1 shows that exon 7, 8 of R2CKO and neo gene have not been deleted; Lane 2 shows that exons 7, 8 of R2CKO and neo gene have been deleted. Lane 3 is DL 2000 marker.
**C.** Digestion identification for the recombination of conditional gene targeting vector in AM-1-Cre bacteria. Lane 1 shows the digestion result of recombination R2CKO vector with EcoR V that exons 7, 8 and neo gene have been deleted; Lane 2 showed the digestion result of R2CKO without recombination with EcoR V ; lane 3 is R2CKO plasmid without digestion; Lane 4 is λ/Hind III marker.

After the derived genomic DNA was digested with Xho I and EcoR V, Southern Blot with the 5'-end flanking probe shows the same result as the screening of the targeted ES cells (Fig. 6A).

After the genomic DNA was digested with Kpn I, Southern Blot with the 3'-end flanking probe reveals that a 5.9kb band was found in the wild type (Fig. 6B- lane 1); a 5.9kb and a 8.0kb band were found in the mutant type (Fig. 6B- lane 2 and 3). The presence of the additional 8.0kb band in the mutant type is because neo gene and loxP sequence were inserted into the FGFR2-CKO targeted allele, so that the size of DNA fragment between Kpn I site was increased from 5.9kb to 8.0kb.

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  1 AATCTCCAATATTGGATAGGATCCGGTGTGGAACCAAACCAAAGAACAAA
 51 ACTTTCATTAGGTGAATCCTATCATGCTAGAAGCTGGAACCCTTAATATA
101 ACTTCGTATAATGTATGCTATACGAAGTTATTAGGATCCAAGCTTCTAGC
151 ACTTCTCTACATAACCAGCGTAAAAAGAAATGTGAGTCCTGCTTCATAAT
201 CAGATAGTGCAGACTCAACAGGCATGCAAATGCAAGGTCCCTGAGCACTC
251 AGACACAGTGATCATGGGAAGTGTGCAGTCCAATCAAGACAGACCAGAAA
301 GAAGCGCTTGAAAACAGGGTTAGGGACGGAGGATAATCTTCAAGCACTTT
351 GAACATTGTGGGCATTTTTCCATGGCCACTGGCATGACGCCAGCTGCATC
401 ACCAAAGAAAGATCATTATAAATAAACCAAGGCCATGAGAGTTACTCTAG
451 AAGCTGCCTGCTGCCTCCCAAAGCACCGAGTCTTTTAAACTCCCTATATC
501 CAGTTGTCTTAACAACAAAAACACAAACAAAAAACAAAAACACACAAACA
551 CAACAAACGTCGTTACCTTGCTGTTTGGGCAGGACAGTGAGCCAGGCAGG
601 ATGGTTGGCCTG
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5A

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  1 CTTTTTCTGATTCTCTAAATAAGTTATCCTTTACAAGGGTTGTGCTTCAG
 51 TGACTCACTTTGAAGTCATTTGTGTTGTGCATGGGGCCCCCCCTCGAGGT
101 CGACGGTATCGATGGTCGACTCTAGATAACTTCGTATAGCATACATTATA
151 CGAAGTTATATTGCTAGAGTCAGCTTCTGATGGAATTAGAACTTGGCAAA
201 ACAATACTGAGAATGAAGTGTATGTGGAACAGAGGCTGCTGATCTCGTTC
251 TTCAGGCTATGAAACTGACACATTTGGAAACCACAGTACTTAGAACCACA
301 AAGTGGGAATCAAGAGAAAAACAATGATCCCACGAGAGATCTATAGATCT
351 ATAGATCATGAGTGGGAGGAATGAGCTGGCCCTTAATTTGGTTTTGCTTG
401 TTTAAATTATGATATCCAACTATGAAACATTATCATAAAGCAATAGTAAA
451 GAGCCTTCAGTAAAGAGCAGGCATTTATCTAATCCCACCCCACCCCCACC
501 CCCGTAGCTCCAATCCTTCCATTCAAAATGTAGGTACTCTGTTCTCACCC
551 TTCTTAACAAAGTATGACAGGAAAAACTTCCATTTTAGTGGACATCTTTA
601 TTGTTTAATA
```

5B

**FIGURE 5.** Partial sequence identification of the targeting vector. The result in Fig. 5A was sequenced with primer P1, and the result in Fig.5B was sequenced with primer P2. loxP sequence is marked by frame lines in Fig. 5A and Fig. 5B. It shows that the location and direction of loxP are correct.

With Hind III digestion, the result of Southern Blot with the 3'-end flanking probe shows that a 6.3kb band was found in the wild band (Fig. 6C- lane 1); a 6.3kb and a 1.8kb band were found in the mutant type (Fig. 6C- lane 2). The reason of the presence of the additional 1.8kb band in mutant type is that a loxP3 sequence with a Hind III site was inserted into FGFR2-CKO targeted allele, so that the size of the DNA fragment between Hind III was declined from 6.3kb to 1.8kb.

Through above detection of Southern Blot with 3'-probe, we found that two of the four ES clones had the correct targeting events, which included the insertion of the targeting gene sequence in genomic DNA.

Additionally, the presence of loxP3 sequence was confirmed by PCR with primer P5 and P6 that are located in the two flanks of loxP3. The result showed that there is a 250bp band in the wild type (Fig.6D- lane 3), a 250bp and a 310bp band in the mutant type (Fig. 6D-lane 1). The presence of the 310bp band is because the inserted loxP in mutant type is about 63bp. The confirmation of loxP3 presence is only from one of the two ES clones, so that we finally obtained one ES clone with the insertion of loxP (loxP3) between exons 8 and 9 in genomic DNA.

## Discussion

### *The strategy of the modified ES cell construction*

The ploxP II-neo targeting vector used in present study contains a positive (Neo) and a negative (TK) selective gene. There are two loxPs located in the two flanks of 3'-end and 5'-end of neo gene respectively, so that the neo gene can be deleted by Cre-mediated loxP recombination in order to exclude the influence of neo gene on the targeted gene expression (Alexandra, 2000). Depending on our experience, it is better that the total length of homologous arms is between 5.0kb and 8.0 kb, and each arm is longer than 0.5 kb for the design of the targeting vector. The two homologous arms of FGFR2 targeted vector are 2.5 kb and 2.9 kb, respectively, in the present study. To delete the exons 7 and 8 of FGFR2 by conditional knockout, the other loxP sequence (loxP3) was inserted into intron 8. When FGFR2 conditional gene knockout mice are crossed with Cre-recombinase transgene mice that express Cre-recombinase in special tissues or cells, the exons 7 and 8 of FGFR2 will be deleted selectively in those tissues or cells where Cre-recombinase is expressed specially.

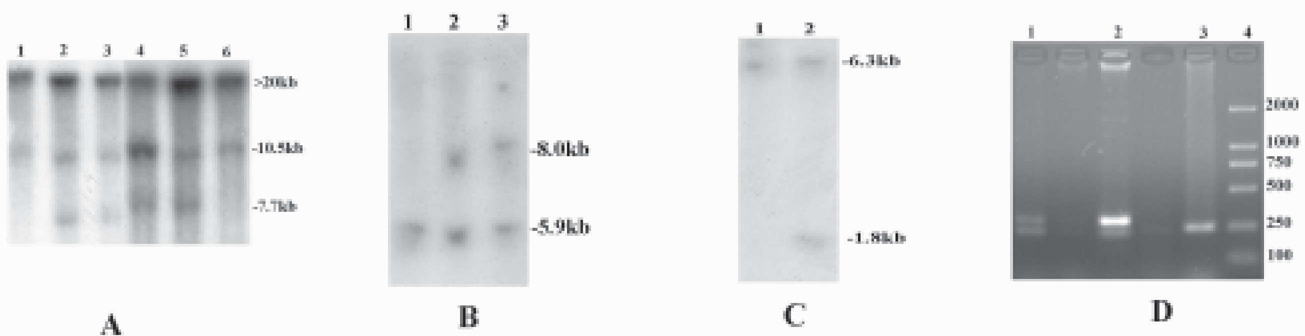


**FIGURE 6.** Identification of digestion and PCR for targeted ES cells.
**A.** Digestion with both XhoI and EcoR V, and detection by 5'-probe. Lane 1 and 6 are negative control from genomic DNA of wild type; lane 2 ~5 are Southern blot results from genomic DNA of ES cell, coding number are G11, B11, G4 and H4, respectively.
**B.** Digestion with KpnI, and detection by 3'-probe. Lane 1 and 6 are negative control from genomic DNA of wild type; lane 2 and 3 are Southern blot results from genomic DNA of ES cell, coding number are G11 and B11, respectively.
**C.** Digestion with Hind III, and detection by 3'-probe. Lane 1 is negative control from genomic DNA of wild type; lane 2 is Southern blot results from genomic DNA of B11ES cells.
**D.** PCR detection in loxP3 flanks. Lane 1 is from genomic DNA of positive ES cells; lane 2 is positive control from R2CKO targeting vector; lane 3 is negative control from genomic DNA of wild type; lane 4 is DL2000-DNA marker.

Of course, the loxP-recombination can also be induced by Cre-expression plasmids in local tissue or *in vitro* (Arrigoni *et al*., 2005).

It is a crucial step to identify for the targeting vector (Xu *et al*., 2002). In the present study, therefore, the targeting vector was identified in both vector structure and recombination function. For structure identification, the targeting vector was identified with primary site digestion. Meanwhile, the sequence and direction of three loxPs were also determined by sequencing. For recombination function identification, the AM-1 bacteria expressing Cre-recombinase were used. Due to the Cre-mediated homologous recombination occurred between two loxP sequences on both same direction and same DNA chain, it was verified that the DNA sequence between loxP1 and loxP3 in our targeted vector can be deleted successfully in AM-1 bacteria expressing Cre-recombinase. The results suggested that the structure of the targeting vector was correct and the recombination function of loxPs was effective, and the FGFR2 conditional knockout mediated by Cre-recombinase occurred successfully in the specific bacteria through our FGFR2 conditional targeted vector.

*The significance of modified ES cell with FGFR2 conditional knockout*

Fibroblast Growth Factor Receptors (FGFRs), a class of transmembrane tyrosine kinase receptors, are closely involved in mammalian development of many vital organs and congenital diseases (Muenke and Schell, 1995). These receptors have three immunoglobulin (Ig)-like domains, an acidic box, a transmembrane region, and a divided tyrosine domain. FGFRs have high affinity for FGFs (Eswarakumar *et al*., 2002; Ornitz *et al*., 1996). All FGFs share a core domain containing conserved structural motifs of Ig II and Ig III of FGFRs. Alternative splicing events of FGFR1, FGFR2, and FGFR3 generate numerous isoforms (Ornitz and Marie, 2002; Yayon *et al*., 1992). In FGFR2, the carboxyl-terminal of Ig-III is encoded by exons 7 and 8 or exons 7 and 9. The Ig-IIIa/IIIb of variant KGFR (FGFR2b) is encoded by exons 7 and 8. The Ig-IIIa/IIIc of variant BEK (FGFR2c) is encoded by exons 7 and 9. The resource, distribution and relative ligands with high affinity are different between KGFR and Bek (Chen and Deng, 2005). KGFR (keratinocyte growth factor receptor) is expressed and localized in epithelial cells, and its primary ligands with high affinity are FGF1, 3, 7 and 10, which are from mesenchymal cells. Bek fibroblast growth factor receptor is expressed and localized in mesenchymal cells, and its primary ligand with high affinity is other FGFs (such as FGF2), which are from epithelial cells. KGFR and Bek correspond and regulate each other to induce the growth and development of organs, especially those organs containing epithelial cells (such as lungs, kidney, liver and skin) (Chen and Deng, 2005).

To investigate the function of FGFR2 *in vivo*, the mouse model with FGFR2 gene knockout has been established (Yu *et al*., 2003). Xu *et al*. (1998) found that on 10.5 day of embryo, the mice with FGFR2 gene knockout in exon 7, 8 and 9 had smaller embryos and showed abnormal development in limbs and placenta comparing to the normal mice. The two-thirds mutant mice died on 10 ~ 11.5 day of embryo due to abnormal placenta development and yolk sac fusion. All mutant mice died on 11.5 day of embryo. Other investigations (Chen and Deng, 2005; Xu *et al*., 1998; Wilkie and Wall, 1996), also found that mice that are homozygous for null alleles of Fgfr2 die on the early stage of embryo development. Thus, it is impossible to investigate the physiological and pathophysiological effects of FGFR2 in adult mice with FGFR2 complete knockout.

The advent of conditional gene knockout technique provides an alternative way to investigate gene functions in adult mice. Embryo death caused by gene knockout in all tissues of FGFR2 expression can be avoided through conditional knockout (Yu *et al*., 2003). In FGFR2 conditional knockout mice constructed by Yu *et al*. the floxed DNA sequence covered the coding region of IgG IIIb, IIIc and transmembrane domain. In our present study, only IgG IIIb and IIIc region (containing exon 7 and 8) were deleted so that the ligand binding region can be deleted and it can be expected to exclude the FGFR 2 functions completely, including KGFR and Bek.

Although studying gene function *in vivo* is the best method (Xu *et al*., 2002), it brings overwhelming work with slow progress. On the contrary, performing studies *in vitro* is a more simple way. Therefore, studying *in vitro* at cell level provides a more effective approach to understand the function of most of the genes. Especially, the study at single cell level can be only carried out *in vitro*. Of course, the function of FGFR2 at different stages of embryo development can be investigated in FGFR2 conditional knockout mice. However, the observation of embryo development in uterus is difficult. We established the engineered ES cells so that ES cell development and differentiation can be observed directly, and ES cell function at different stages can be investigated easily.

In addition, the development of engineered ES cells with Cre-loxP has also provided an example for tissue-specific gene therapy. Embryonic stem cells are important for cell therapy. Stem cell therapy has been used for diabetes mellitus, myocardial infarction, skin damage, etc (Hussain and Theise, 2004; Shi *et al*., 2006; Janssens *et al*., 2006). However, the low efficiency and poor direction are major limitation of cell therapy. Some genetic modification of stem cells has improved the efficiency of cell therapy (Mangi *et al*., 2003; Dzau *et al*., 2005). For example, adult bone-marrow-derived mesenchymal stem cells (MSCs) overexpressing the anti-apoptotic gene Akt1 (Akt-MSCs) became more resistant to apoptosis *in vitro* and *in vivo*. Moreover, the application of Cre-loxP system would improve the targeting of gene therapy. For example, a novel adenovirus-based gene therapy system, including Cre-loxP system together with the astrocytoma-specific promoter, has been used for the targeting astrocytoma (Maeda *et al*., 2006). In this system, the herpes simplex virus thymidine kinase (HSV-TK) gene was used as a suicide mechanism. The promoter driving the HSV-TK was inactivated by two loxPs with a stuffer sequence. After the expression of Cre recombinase was induced by tissue- or cell-specific molecular, the stuffer sequence was deleted and HSV-TK gene was activated again in specific tissue or cells. Therefore, we believe that the Cre-loxP system would provide a more accurate and more effective strategy for gene therapy.

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