Application of oligonucleotides to construct a conditional targeting vector for porcine *IκBα*

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Abstract. Conditional gene targeting at porcine *IκBα* may be a solution to delayed xenograft rejection, the main barrier to xenotransplantation. An oligonucleotide-based method was applied to construct the vector for conditional targeting of porcine *IκBα*. This method was free from PCR amplification during the assembling of the different vector elements, avoiding introduction of unwanted mutations. With the help of short double-stranded DNA fragments produced by annealing oligonucleotides, nondirectional cloning has also been avoided. By making the best of directional cloning, a highly complex targeting vector was built within 3 weeks. The present study also explained why the two recombination-based methods (recombineering and gateway recombination), although having demonstrated to be highly efficient in constructing ordinary targeting vectors, were not appropriate in this context. The description in the present study of an additional method to efficiently construct targeting vectors is suggested to introduce more flexibility in the field therefore helping to meet the different needs of the researchers.

Introduction

Transplantation is currently facing a problem of organ shortage (1). Therefore, xenotransplantation has been intensively pursued as a promising supplement to allotransplantation (2). Pigs have been identified as potential donors as they possess a similar biochemical profile to that of humans. However, the immunological barriers must be overcome prior to the achievement of successful xenotransplantation. The biggest hurdle in xenotransplantation is humoral rejection (1), which exists in two forms, namely hyperacute rejection and delayed xenograft rejection. Hyperacute rejection can be prevented by the knockout of the α-1,3-galactosyltransferase gene (1), so delayed xenograft rejection appears to be the most direct barrier.

Activation of transcription factor nuclear factor (NF)-κB serves a significant function in delayed xenograft rejection (3). Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α (IκBα) is the crucial inhibitor of NF-κB (4). However, IκBα can be phosphorylated and then degraded following stimulation with inflammatory agents (5), leading to NF-κB activation. To avoid the phosphorylation, a mutant version of IκBα lacking the phosphorylation sites was designed (6). Since NF-κB signaling is crucial for the growth and development of vertebrates (7), it is not feasible to inhibit this pathway in the embryonic period of donor animals by simply replacing *IκBα* with its mutant type. Therefore, it has been suggested that conditional gene targeting at porcine *IκBα* may be a proper choice: First the genomic *IκBα* locus could be targeted with a construct consisting of the wild type and the mutant version of *IκBα*, then the wild-type *IκBα* could still be expressed at the period of development, but the mutant could substitute for the wild type when the donor is mature. To achieve the controlled expression of the two types of porcine *IκBα*, a blueprint of the conditional gene targeting was designed (see Fig. 1). The first step of the process involved the engineering of a targeting vector from the available plasmid pFPC-1 (8). The present study demonstrated how to construct a highly complex targeting vector by a classic but useful method.

Materials and methods

Oligonucleotides. The sequences and uses of the oligonucleotides (PAGE purified) (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) are presented in Table I.

Cloning of the homologous arms. For cloning, 2.0- and 6.0-kb porcine genomic fragments (5' and 3' arms, respectively) were amplified by polymerase chain reaction (PCR) using the LA-Taq™ DNA polymerase (Takara Biotechnology Co., Ltd., Dalian, China) and genomic DNA from porcine iliac

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Table I. Oligonucleotides.

Name	Sequence	Use
5A-F	5'-GGCGCGCCTGTTCGTGCTTTCTGATTTCC-3'	Forward primer with AscI site for 5' arm of *IκBα*
5A-R	5'-TTAATTAATGAGGTCGTGTTCCTCCATT-3'	Reverse primer with PacI site for 5' arm of *IκBα*
3A-F	5'-GTCTTCCTCCACTGTTCTTGCCTCCTTTGT-3'	Forward primer for 3' arm of *IκBα*
3A-R	5'-TGTCCCTCTTCTGTTGGTAGACTCCACTCC-3'	Reverse primer for 3' arm of *IκBα*
LoxP-A	5'-CTAGCATAACTTCGTATAGCATACATTATACGA AGTTATCG-3'	Annealed to LoxP-B to yield the LoxP fragment
LoxP-B	5'-GATCCGATAACTTCGTATAATGTATGCTATACG AAGTTATG-3'	Annealed to LoxP-A to yield the LoxP fragment
NTEC-A	5'-AGCTGCGGCCGCACGCGTCTTAGAGCATGCT GGGGATGCGGTGGGCTCTATGG-3'	Annealed to NTEC-B to yield the NTEC fragment
NTEC-B	5'-AATTCCATAGAGCCCACCGCATCCCCAGCATG CTCTAAGACGCGTGCGGCCGC-3'	Annealed to NTEC-A to yield the NTEC fragment
NHCL-A	5'-AGCTTGCTAGCCTTAGAGCATGCTGGGGATG CGGTGGGCTCTATGGTATCGATG-3'	Annealed to NHCL-B to yield the NHCL fragment
NHCL-B	5'-AATTCATCGATACCATAGAGCCCACCGCATCC CCAGCATGCTCTAAGGCTAGCA-3'	Annealed to NHCL-A to yield the NHCL fragment
pUC1-A	5'-GATCCATCGATGTCGACACGCGTTTTAAACGG CCGGGCCGGCCATGCAT-3'	Annealed to pUC1-B to yield the pUC1 fragment
pUC1-B	5'-AGCTATGCATGGCCGGCCCGGCCGTTTAAAAC GCGTGTCGACATCGATG-3'	Annealed to pUC1-A to yield the pUC1 fragment
pUC2-A	5'-AATTGCGGCCGCGTTTAAACAAGCTTGAATT CGATATCGCTAGCG-3'	Annealed to pUC2-B to yield the pUC2 fragment
pUC2-B	5'-GATCCGCTAGCGATATCGAATTCAAGCTTGTT TAAACGCGGCCGC-3'	Annealed to pUC2-A to yield the pUC2 fragment

endothelial cells (PIEC; Shanghai Cell Bank of Chinese Academy of Sciences, Shanghai, China) as a template. The primers used were 5A-F and 5A-R for the 5'arm and 3A-F and 3A-R for the 3'arm. The thermocycling conditions for the 25 μl PCR reaction was as follows 95˚C for 5 min, 30 cycles of 60˚C for 30 sec and 72˚C for 2 min, and finally 72˚C for 10 min. A total of 50 ng genomic DNA was used as the template. The 5'arm with Asc*I*/Pac*I* sites was subcloned into pMD18-T vector, yielding pMD-5'arm. The 3'arm was subcloned into pCR®-XL-TOPO® (Invitrogen; Thermo Fisher Scientific, Inc.), yielding pCR-XL-TOPO-3'arm.

Construction of the Cre/LoxP system. To generate a Cre/LoxP system (pUC19-IκBα-TKneo-IκBαM; Fig. 2E) for controlled expression of the two types of porcine *IκBα*, an oligonucleotide-based method was deployed. There were three steps to the whole process. All the plasmids were purchased from Transgene (Beijing, China).

First, a series of suitable restriction sites were designed and generated for assembling various DNA fragments. DNA fragments (54- and 53-mer; NHCL and NTEC) were produced by annealing oligonucleotides NHCL-A/NHCL-B and NTEC-A/NTEC-B respectively (Table I). The fragments NHCL and NTEC were then inserted at the *Hind*III/*Eco*RI sites of pUC19 to respectively get pUC19-NHCL (Fig. 2B) and pUC19-NTEC (Fig. 2C). Then 49- and 45-mer DNA fragments (pUC1 and pUC2) were constructed by annealing oligonucleotides pUC1-A/pUC1-B and pUC2-A/pUC2-B respectively. The fragments pUC1 and pUC2 were sequentially inserted at the *Bam*HI/*Hind*III and *Eco*RI/*Bam*HI sites of pUC19, yielding pUC19-2 (Fig. 2D). The intermediate product, pUC19-1, can also be used to subclone the 3'arm (as mentioned below).

Meanwhile, the fragments lp-IκBαM-pA and CMV-lp-IκBα-pA were constructed (Fig. 2A) on the basis of pcDNA3.1(+) (Invitrogen; Thermo Fisher Scientific, Inc.). To introduce a LoxP site in front of *IκBα* or *IκBαM* (mutant *IκBα*) cDNA, a 41-merDNA fragment (LoxP), assembled by annealing oligonucleotides LoxP-A and LoxP-B, was inserted at the *Nhe*I/*Bam*HI sites of pcDNA3.1(+) to get a plasmid pc3.1-lp. Second, the *IκBα*/*IκBαM* cDNA fragment was excised by the restriction enzymes *Nhe*I and *Pme*I and subcloned into pc3.1-lp, yielding pc3.1-lp-IκBα/IκBαM.

The second step was to prepare respectively the three major fragments (CMV-lp-IκBα-pA, TKneo and lp-IκBαM-pA) flanked by suitable restriction sites according to the distribution of restriction sites in the MCS of pUC19-2. For this purpose, TKneo fragment was excised by the endonucleases *Eco*RI/*Sca*I from the plasmid pFPC-1, and then inserted at the *Eco*RI/*Eco*RV sites of pUC19-2, yielding pUC19-TKneo (Fig. 2E). Meanwhile, fragments lp-IκBαM-pA and CMV-lp-IκBα-pA were excised by the endonucleases *Nhe*I/*Sph*I and *Mlu*I/*Sph*I respectively from the plasmids pc3.1-lp-IκBαM and pc3.1-lp-IκBα, and then subcloned into the vectors pUC19-NHCL and pUC19-NTEC, yielding pUC19-lp-IκBαM-pA (Fig. 2B) and pUC19-CMV-lp-IκBα-pA (Fig. 2C).

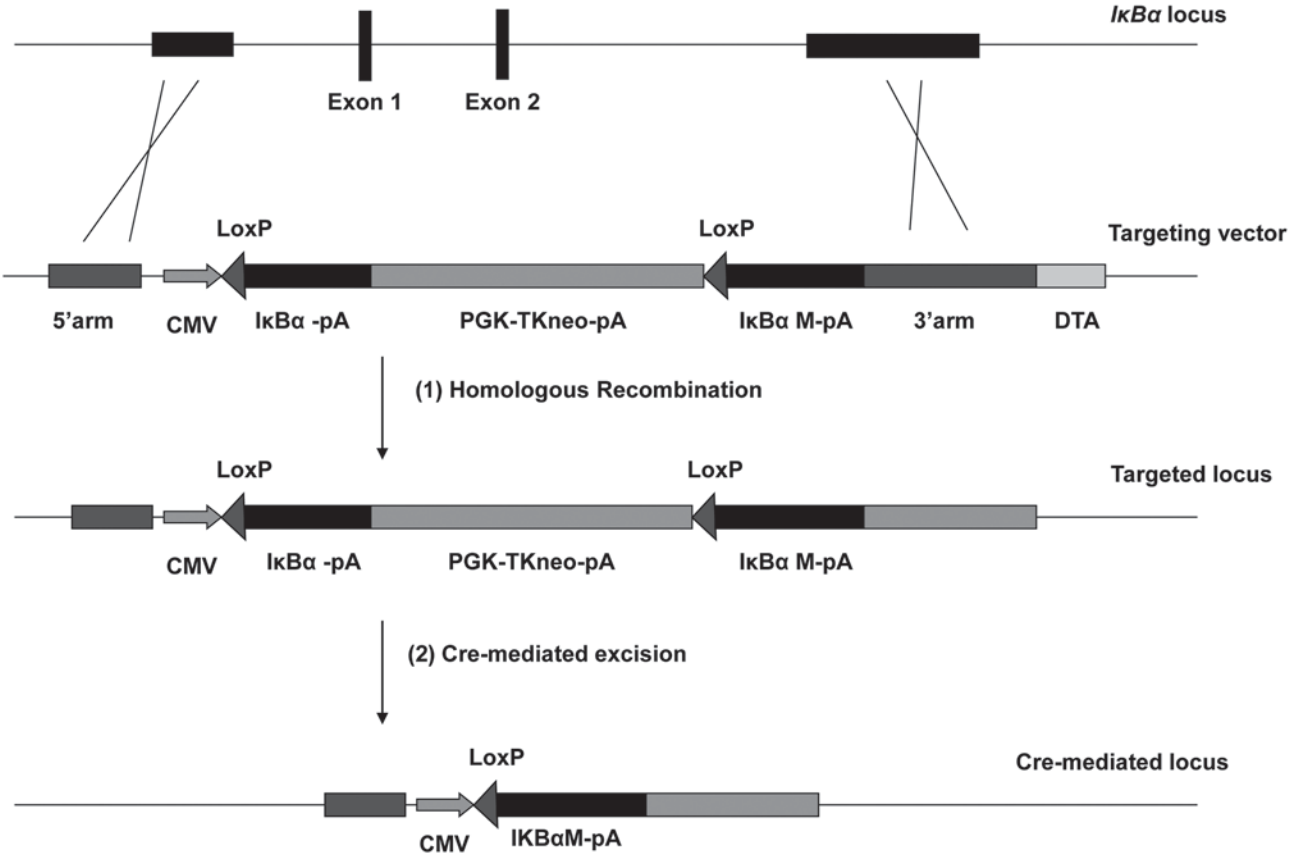

Figure 1. Blueprint of conditional gene targeting at porcine *IκBα*. The porcine *IκBα* gene is composed of 6 exons, located on chromosome 7. The targeting process consisted of two steps: i) First, targeting genomic *IκBα* locus with a construct consisting of the wt *IκBα* and its mutant *IκBαM*, to still be able to express the wt *IκBα* during the development of the animal and ii) substituting the mutant for the wt by Cre-mediated excision when the donor is mature. CMV, human cytomegalovirus immediate-early promoter/enhancer; PGK, promoter of mouse phosphoglycerate kinase-1 gene; TKneo, a fusion of thymidine kinase and neomycin resistance genes; DTA, a gene coding for fragment A of diphtheria toxin; wt, wild type; IκBα, nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α.

The third step was to assemble the three major fragments (CMV-lp-IκBα-pA, TKneo and lp-IκBαM-pA) together to get the expected Cre/LoxP system (Fig. 2E). The fragments lp-IκBαM-pA and CMV-lp-IκBα-pA were respectively excised by the endonucleases *Nhe*I/*Cla*I and *Not*I/*Eco*RI from the vectors pUC19-lp-IκBαM-pA and pUC19-CMV-lp-IκBα-pA, and then sequentially inserted into pUC19-TKneo, yielding pUC19-IκBα-TKneo-IκBαM.

Construction of the conditional targeting vector. A fragment containing the3'arm was excised by the endonucleases *Mlu*I and *Eag*I from pCR-XL-TOPO-3'arm, and then inserted into pUC19-1, yielding pUC19-3'arm (Fig. 3A).

To generate the targeting vector (Fig. 3B), 5'arm, the fragment containing the Cre/LoxP system (6.4kb), and 3'arm were respectively excised by the endonucleases *Asc*I/*Pac*I, *Not*I/*Fse*I and *Cla*I/*Fse*I from the vectors pMD-5'arm, pUC19-IκBα-TKneo-IκBαM and pUC19-3'arm, and then sequentially inserted into the plasmid pFPC-1, yielding pLHG-1 (19.1kb; Fig. 3C).

Results and Discussion

A possible solution to delayed xenograft rejection. There are numerous examples of how to inhibit NF-κB signaling in cells (6,9,10) and animals (11-14) by means of overexpressing different types of *IκBα*. A point mutant of *IκBα* in which serines 32 and 36 are substituted by alanine residues is no longer phosphorylated in response to diverse stimuli (6). This mutant behaves as a potent dominant negative IκB protein (6,11), inhibiting NF-κB activation. The present study used the cDNA of such a mutant *IκBα* (*IκBαM*) to construct a vector. As NF-κB signaling is crucial for the development process of mammals (7), a Cre/LoxP system was designed and generated (see Fig. 2E), aimed at controlling expression of the two types of porcine *IκBα*, the wild and the mutant types. This Cre/LoxP system may provide a solution to delayed xenograft rejection.

As illustrated in Fig. 2E, the Cre/LoxP system consists of three major expression cassettes of *IκBα* gene, *TKneo* (a fusion of the thymidine kinase and neomycin resistance genes) and *IκBαM* cDNA (followed by a poly A sequence). A marked difference between this Cre/LoxP system and others (8,15,16) is that not only the positive selectable marker but also the *IκBα* cDNA is floxed (flagged by two LoxP sites). This is a prerequisite to achieve controlled expression of the two types of porcine *IκBα*. If the genomic *IκBα* locus was to be replaced *in vivo* by the Cre/LoxP system, wild type *IκBα* may be expressed during the developmental process. The *IκBα* cDNA may be ablated by Cre recombinase when the

donor is mature, and as a result, a new expression cassette, for *IκBαM,* would be generated. The expression of the mutant *IκBα* may result in an almost complete inhibition of NF-κB signaling (11) and subsequently a way of preventing delayed xenograft rejection. Prior to producing gene-targeted animals, it will be necessary to perform the controlled expression described above in a porcine vascular endothelial cell line as the vascular endothelium is the target of delayed xenograft rejection (3). The analysis of the phenotype of the targeted PIEC could, to a certain extent, be a good model of what would occur in an *in vivo* system.

In the last 20 years, gene targeting has been used as a powerful tool for studying gene function (15,17,18). In addition, the stable and site-specific modification of mammalian genomes has a variety of applications in biomedicine and biotechnology (19). Site-specific integration, rather than random integration, is the best way of achieving the stable long-term expression of an introduced gene (19). The final purpose of the present study was to apply the results of gene function studies to NF-κB signaling to inhibit delayed xenograft rejection, not simply to achieve the knockout of *IκBα*. Controlled expression of the wild type and the mutant of *IκBα* may provide an example of how specific biomedicine challenges may be overcome by gene targeting.

An oligonucleotide based method for vector construction. As illustrated in Figs. 2 and 3, the vector construction method is based on conventional ligation reactions and 5 pairs of oligonucleotides facilitate the 7 rounds of assembly of various fragments. This complicated targeting vector (pLHG-1; Fig. 3C) was constructed within 3 weeks. Unlike ordinary targeting vectors, this vector is used to introduce the wild type and the mutant of the same gene into the target genomic locus. Thus, the targeting vector should contain 7 extra fragments (CMV promoter, *IκBα* cDNA, *IκBαM* cDNA, 2 copies of BGH pA and 2 copies of LoxP), apart from the homologous arms and the selectable markers. This increases the difficulty of vector construction. In order to eliminate errors, maps and sequence files were generated for every cloning step designed for the targeting vector prior to starting bench work (16). The vector was constructed using 5 short double-stranded DNA fragments made by annealing oligonucleotides. The vector pLHG-1 was confirmed by various endonucleases (*Not*I, *Eco*RI, *Bam*HI and *Hind*III; data not shown) digestion and sequencing with 5 primer sequences (Table II). An alternative plasmid containing His-tagged *IκBαM* cDNA was also constructed as described above (pLHG-2; data not shown).

To avoid unwanted mutations, the use of PCR amplification in the assembly of various fragments was eliminated. With the help of the oligonucleotides, no n-directional cloning was also avoided when assembling the different elements of the vector. By making full use of directional cloning, satisfactory subcloning efficiency was obtained throughout the whole process of vector construction. In the last step of the assembly the efficiency of the subclone step was confirmed to be high. This result is comparable to the high efficiency of gateway system recombination reported previously (15,20). In addition, methylation effects should be avoided by proper design of the oligonucleotides when dam-/dcm-methylation-deficient competent *E. coli* cells were not used for plasmid transformation. To obtain high digestion efficiency, moderate guanine-cytosine content (40-70%) of the oligonucleotides is preferred.

Table II. Sequencing primers of pLHG-1.

Name	Sequence
3'arm-DTA	5'TGGCCTGGGAGCTTCTG3'
3'arm-lpmIKBpA	5'GAATGGACTTTAGTAAGGC ATC3'
Tkneo-lpmIKBpA	5'CCATCACGAGATTTCGATTC CACC3'
5'arm-CMVlpIKBpA	5'GAGACCTGGACATGGTGAA CCT3'
5'arm-Amp	5'GGGAACTGGTGGTCTTTAT TC3'

Previously, various methods and technologies, particularly recombination-based methods including recombineering and gateway recombination, have been developed to facilitate and simplify targeting vector construction. For recombineering, Zhang *et al* (21) combined genomic library screening and gene-targeting vector construction in a single step, then Liu *et al* (22) and Cotta-de-Almeida *et al* (23) managed to avoid the need to construct or screen genomic libraries by manipulating bacterial artificial chromosomes (BACs). Subsequently, recombineering was employed more and more extensively in targeting vector construction. For gateway recombination, Iiizumi *et al* (15) improved the commercially available Multi Site Gateway system and use it to facilitate targeting vector construction. Nyabi *et al* (24) also applied Multi Site Gateway system to build targeting vectors for transgenesis. To take advantage of these two recombination methods, Ikeya *et al* (20) tested a strategy of generating gene-targeting vectors by combining *in vivo* recombination (recombineering) with *in vitro* recombination (gateway system). Wu *et al* (16) described a module cloning protocol for constructing gene targeting vectors by combining the two recombination methods. This 'combined' strategy may possess potential in constructing gene targeting vectors, particularly complicated ones. However, the two recombination methods (recombineering and gateway recombination) exhibit their own shortcomings in constructing targeting vectors which are as complicated as the one designed in the present study. One of the problems of using recombineering is the difficulty of getting isogenic DNA for the cell line PIEC from commercially available BAC clones, as isogenic DNA is preferred to improving targeting efficiency (18). Additionally, repetitive sequences in the original vector pFPC-1 would lead to aberrant recombination, usually causing the failure to obtain consistent results (22). For gateway recombination, the destination vectors should be modified by restriction enzyme-based cloning to be compatible with targeting vector construction, and it is necessary to use restriction enzyme-based cloning during the process of constructing the desired targeting vector. The module cloning protocol (16) contains this conventional cloning

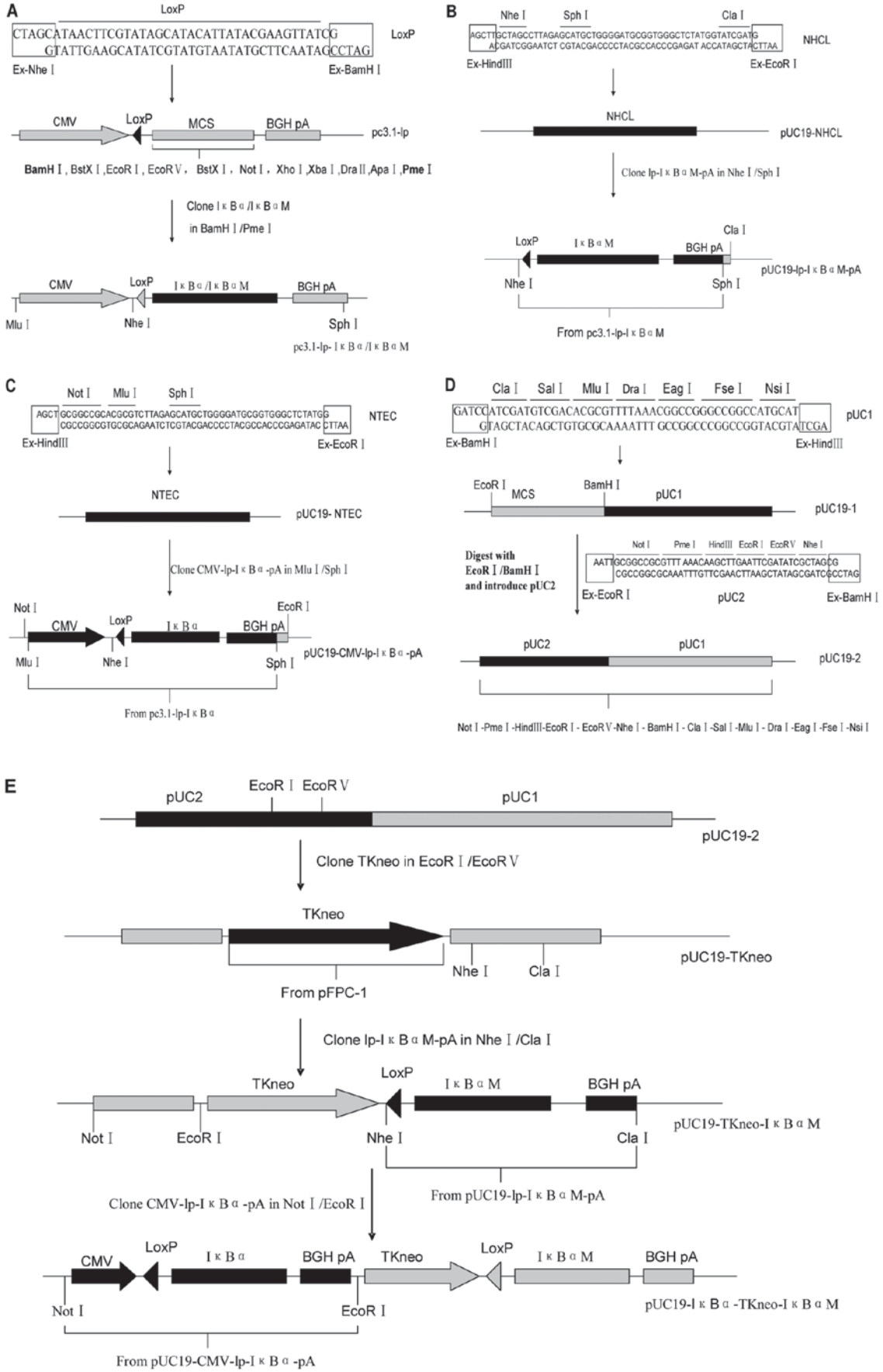

Figure 2. Construction of the Cre/LoxP system using a method based on oligonucleotides. (A) Construction of the fragments lp-IκBαM-pA and CMV-lp-IκBα-pA in the basis of pcDNA3.1(+). (B) Adding *Nhe*I/*Cla*I sites onto the 5' and 3'ends of the fragment lp-IκBαM-pA. (C) Adding *Not*I/*Eco*RI sites onto the 5' and 3'ends of the fragment CMV-lp-IκBα-pA. (D) Modifying the MCS of pUC19 to facilitate the final assembly step of the Cre/LoxP system through two steps of cloning. (E) Assembling the three fragments (CMV-lp-IκBα-pA, lp-IκBαM-pA and TKneo) to obtain the Cre/LoxP system. CMV, human cytomegalovirus immediate-early promoter/enhancer; MCS, multiple cloning site; TKneo, a fusion of thymidine kinase and neomycin resistance genes.

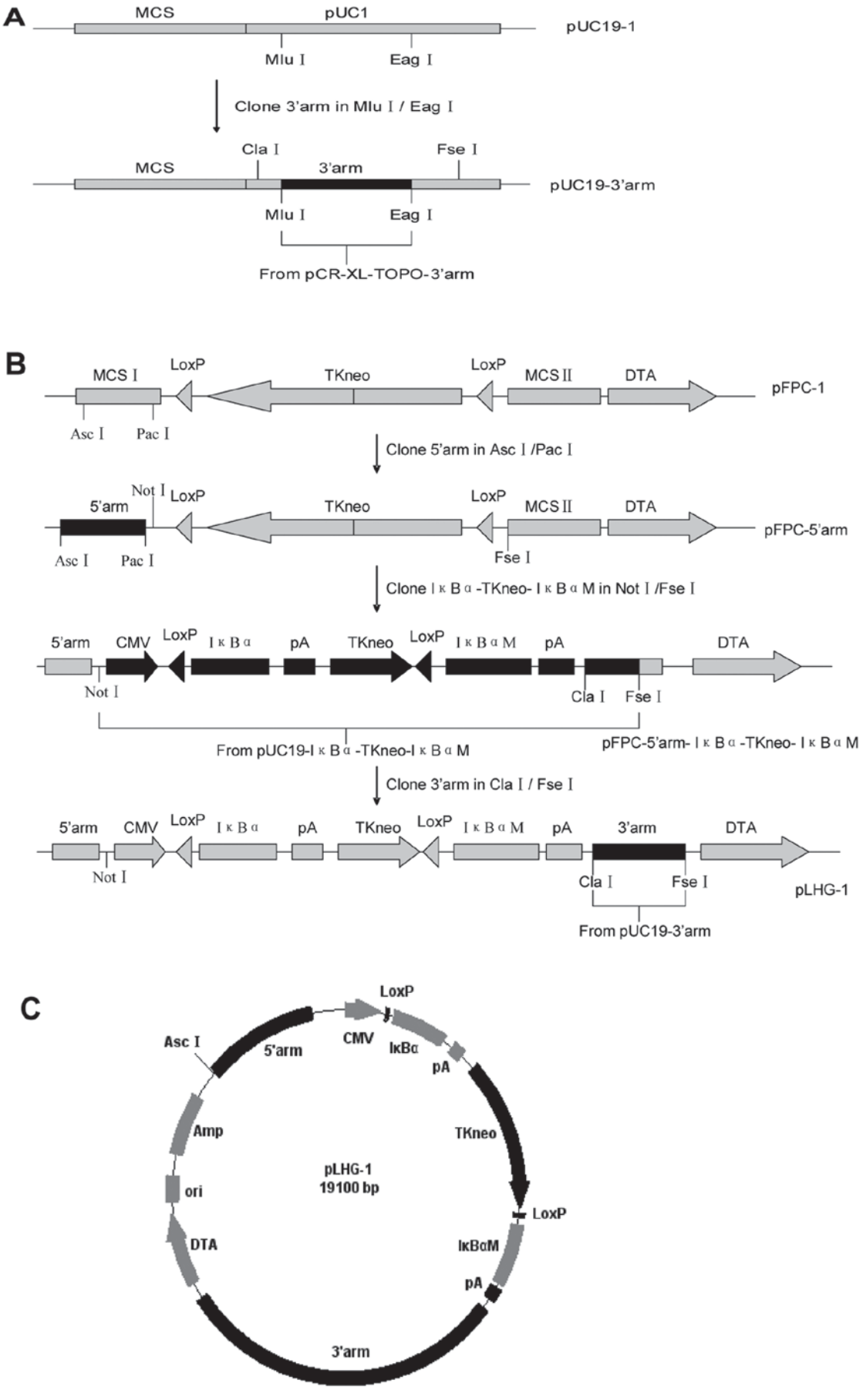

Figure 3. Construction of the conditional targeting vector. (A) Adding *Cla*I/*Fse*I sites onto the 5' and 3' ends of 3'arm. (B) Final step of the vector construction. 5'arm (2.0 kb), the fragment containing the Cre/LoxP system (6.4 kb) and 3'arm (6.1 kb) are sequentially inserted into the plasmid pFPC-1, yielding the conditional targeting vector pLHG-1. (C) Plasmid profile of pLHG-1. CMV, human cytomegalovirus immediate-early promoter/enhancer; MCS, multiple cloning site; TKneo, a fusion of thymidine kinase and neomycin resistance genes.

method. Furthermore, PCR amplification, which may introduce unwanted mutations, has to be extensively used in the gateway method. In addition, a MultiSite Gateway system remains too expensive to be used for normal laboratory practice. For the reasons described above, it was decided to construct the desired targeting vector by restriction enzyme-based cloning. The present study provided an example of constructing complicated targeting vector with restriction enzyme-based cloning methods. It is suggested that this strategy maybe employed in any normal laboratory. The description here of an additional method to efficiently construct targeting vectors will introduce more flexibility in the field, therefore helping to meet the different requirements of researchers.

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