Indian Journal of Animal Sciences **87** (8): 939–943, August 2017/Article

TALEN construction for porcine IκBα gene and the detection of knockout activity

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Received: 8 May 2016; Accepted: 16 March 2017

ABSTRACT

Xenotransplantation often causes severe immunological rejection. The IκBα (inhibitor of nuclear factor kappa B alpha) gene plays an important role in delayed immunological rejection. Research shows that IκBα mutations can mitigate delayed immunological rejection. In the present study, the first exon sequence of the porcine IκBα gene was used to construct a transcription activator-like effector nuclease (TALEN) plasmid pair, and we used the double-strand break repair via single strand annealing (SSA) method to examine the knockout efficiency of the TALEN plasmid pair. The results showed that the knockout activity was 3.955, and the difference was extremely significant compared with the control group. A TALEN plasmid pair targeting the first exon sequence of the IκBα gene, was successfully constructed for the first time, and this study lays the foundation for knocking out the porcine IκBα gene to mitigate the delayed immunological rejection induced by xenotransplantation.

Key words: IκBα gene, Porcine, Knockout, SSA, TALEN

Owing to severe lack of donor organs, development of allotransplantation is severely limited, while xenotransplantation may open new avenues to solve the scarcity of same-species organs (Satyananda *et al.* 2013). Pigs are very similar to humans with respect to anatomy, physiology and biochemical indicators; therefore, they are considered the most ideal donor animal for xenotransplantation (Ekser *et al.* 2009). Xenotransplantation often causes severe immunological rejection, and delayed immunological rejection is an important form of immunological rejection (Le Bas-Bernardet *et al.* 2009, Griesemer *et al.* 2014). Studies showed that inhibition of nuclear transcription factor NF-κB (nuclear factor-kappa B) activity can effectively alleviate the delayed rejection response. The NF-κB inhibitory factor IκBα is an important inhibitory factor of NF-κB. Under stimulation by inflammatory mediators, IκBα is readily phosphorylated and degraded, thereby resulting in NF-κB activation. Some studies have shown that IκBα mutants can effectively resist phosphorylation (Majdalawieh *et al.* 2010, Ferreiro *et al.* 2010). Therefore, alteration of the IκBα gene has potential significance in the study of mitigating the delayed rejection response induced by xenotransplantation (Lee *et al.* 2013).

In addition to zinc-finger nuclease (ZFN) technology, the transcription activator-like effector nuclease (TALEN) is another new technology that can make effective targeted modifications to the genome. The transcription activator-like effector family includes a protein (TALEs) that can recognize and bind to DNA (Boch *et al.* 2009, Zhang *et al.* 2011). When Xanthomonas bacteria infect plants, they can secrete TALEs into the nucleus of the plant cell through a type III secretion pathway, and the TALEs can recognize and bind to specific DNA sequences (Christian *et al.* 2010, Li *et al.* 2011). The specific binding of the TALE to a DNA sequence is mainly mediated by a conserved 34-amino acid sequence in the TAL structure. TALEs and the cleavage domain of the FokI endonuclease are connected to form TALENs, thereby allowing targeted modification of genomic double-stranded DNA (Moscou *et al.* 2009, Morbitzer *et al.* 2010, Mahfouz *et al.* 2011, Miller *et al.* 2011).

Double-strand break (DSB) repair via single strand annealing (SSA) is a commonly used method to examine the enzymatic activity of DNA cleavage *in vitro.* It is an automatic DNA recombination repair system that exists *in vivo*. This system consists of 3 segments of sequences: 2 identical homologous sequences and the middle cleavage recognition region. When the cleavage recognition region is not cut in the middle, the gene in which the SSA system is blocked and cannot be translated into a functional protein. After cleavage, the SSA system automatically merges the homologous sequences, and the overlapping sequences become a single sequence. After merging, the gene undergoes recombination repair, and it can be read through and translated to generate a functional protein. Normally, luciferase driven by the CMV promoter is used as the reporter gene. The reporter vector contains 2 luciferase coding fragments, but the coding regions are incomplete

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and contain overlapping homologous sequences that are separated by a stop codon and TALEN target sequence. If TALEN can cause this target sequence to generate a DSB, then the DSB can induce the two homologous sequences to undergo recombination, generating a complete functional luciferase coding sequence and restoring the luciferase activity (Cradick *et al.* 2014). By comparing the changes in luciferase activity before and after the addition of TALEN, the activity of TALEN can be evaluated.

Therefore, we plan to take advantage of the high knockout efficiency of TALEN to modify the first exon of IκBα gene, and lay the foundation for the preparation of IκBα modified pigs and researching the immune rejection induced by xenotransplantation.

MATERIALS AND METHODS

The genomic DNA samples of 4 different types of pigs, large white, Landrace, Wuzhishan, and Bama, which were used to exclude single nucleotide polymorphism (SNP) sites in the present study, were from Nankou Wuzhishan species conservation base, Shunyi base, and Changping base of the Institute of Animal Sciences of Chinese Academy of Agricultural Sciences. Trans1-Blue competent cells were purchased from Transgen Biotech. Four basic unit module vectors, pTALE-A, pTALE-G, pTALE-C and pTALE-T, and the expression vectors JSD70 and JSD71 were purchased from Addgene. The restriction endonucleases *Spe*I, *Nhe*I, *Hind*III, *Kpn*I and *Bam*HI-HF were products of NEB.

Primer design: Specific primers were designed according to the porcine IκBα gene sequence to generate the recombinant plasmid TALEN-1L and TALEN-1R sequences, and the plasmids were synthesized by the Beijing branch of Invitrogen. The sequences of the primers are listed in Table 1.

Table 1. PCR primer information

Primer name	Primer sequence (5'→3')	Application
IκBαF1733	5' GCAAACCAGCAGTT CTCCATCCT 3'	PCR amplification
IκBαR2479	5' GCGACACTTACGAGT CTCCGTCC 3'	PCR amplification
JDS2978	5' TTGAGGCGCTGCT GACTG 3'	Sequencing
JDS2980	5' TTAATTCAATATATTC ATGAGGCAC 3'	Sequencing
JDS2778	5' CTGGCGCAAT GCGCTCAC 3'	Sequencing
JDS2979	5' AAGCAATGGCGA CCACCTGTTC 3'	Sequencing

Target site design: The porcine IκBα gene sequence was obtained by searching the NCBI database, and the first exon sequence of the IκBα gene was used as the target sequence. The online target site design software at http: // boglabx.plp.iastate.edu/TALENT/TALENT/was used to design the following site:

TGGATGACCGCCACGACA*GCGGCCTGGACTCC* ***ATGAAGGACGAGGAGTACGA.***

SNP site exclusion: The primers IκBαF1733 and IκBαR2479 were used to amplify the genomic DNAs of the four types of porcine samples (large white, Landrace, Wuzhishan, and Bama). The amplified products were sent for sequencing. DNAMAN was used to compare the sequences to maximally ensure that there were no SNPs in the recognition region.

Construction of the recombinant plasmids (TALEN-1L and TALEN-1R): The nucleic acid recognition unit of TAL is the di-residue pairs that are spaced by a constant sequence of 32 amino acids. The di-residues have a constant corresponding relationship with A, G, C, T, namely, NI recognizes A, NG recognizes T, HD recognizes C and NN recognizes G. The pTALE-A, pTALE-G, pTALE-C and pTALE-T plasmids are single module vectors, and each one respectively encodes the DNA plasmids that contain the nucleic acid recognition units for the above four TALs. The 5' ends of the encoding DNA have an *Spe*I enzyme recognition sequence, and the 3' ends have consecutive *Nhe*I and *Hind*III enzyme cleavage recognition sequences. Using the *Spe*I, *Nhe*I and *Hind*III enzyme cleavage sites on the unit module vector, the TAL units corresponding to the target sequence can be connected in tandem and cloned, though the right sight target sequence needs to construct the module in reverse. Structural maps of the four plasmids and a diagram of the working principle are shown in Fig. 1A and 1B. According to the selected target site, pTALE-A, pTALE-G, pTALE-C and pTALE-T were used to construct the recombinant plasmid TALE-1L, which recognizes 18 nucleotides (5'-TGGATGACCGCCACGACA-3'). The recombinant plasmid TALE-1L was ligated to the expression vector JDS70 to construct the recombinant plasmid TALEN-1L, and the construction procedure is shown in Fig. 2A. According to the selected target site, pTALE-A, pTALE-G, pTALE-C and pTALE-T were used to construct the recombinant plasmid TALE-1R, which recognizes 18 nucleotides (5'-GAAGGACGAGG

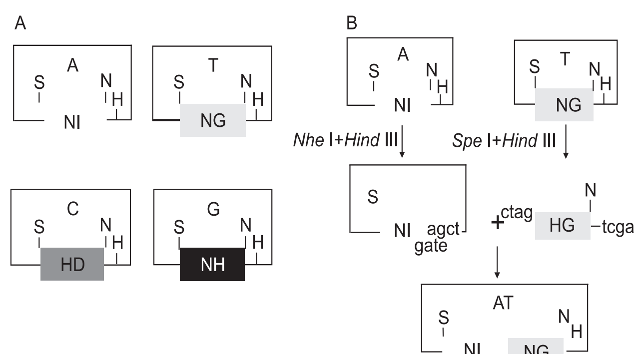

Fig.1. Structures of the 4 plasmids and a diagram illustrating the working principle. (A) The construction of the four TALEN module vectors NI, NG, HD, and NN, which correspond to A, T, C, and G, respectively. (B) Using the restriction sites on the module vectors, 4 different modules were assembled in accordance with the target sequence.

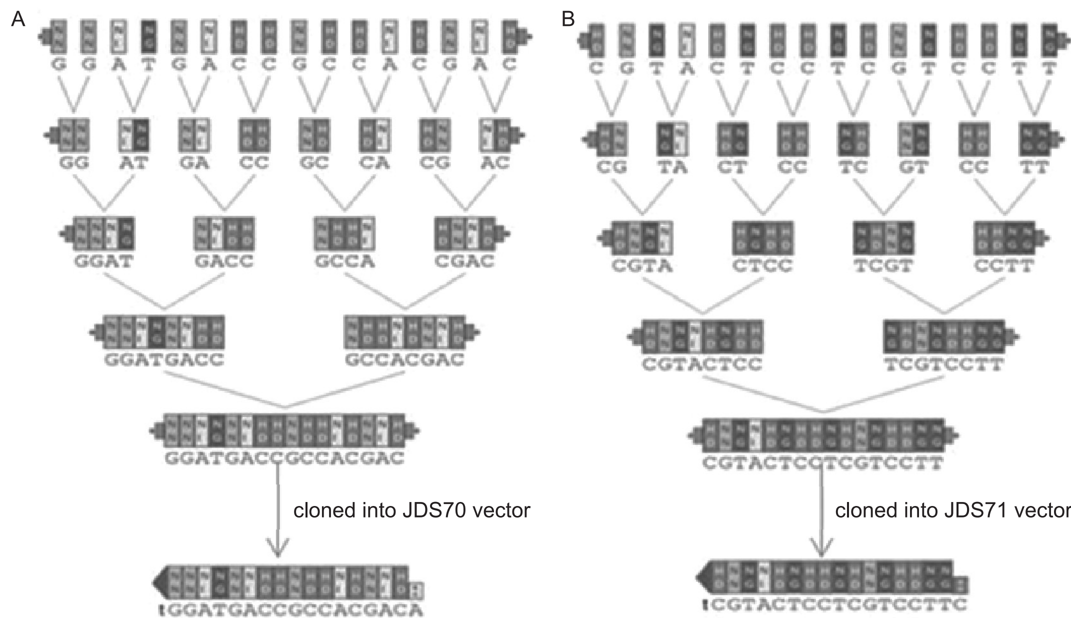

Fig. 2. The procedure for constructing the recombinant plasmids TALEN-1L and TALEN-1R. The TAL units that recognize the 18 nucleotides of the target sequence were connected in tandem to construct the recombinant plasmids TALE-1L and TALE-1R, which were cloned into JDS70 and JDS71, respectively, to form the recombinant plasmids TALEN-1L and TALEN-1R. (A) The procedure for constructing TALEN-1L. (B) The procedure for constructing TALEN-1R.

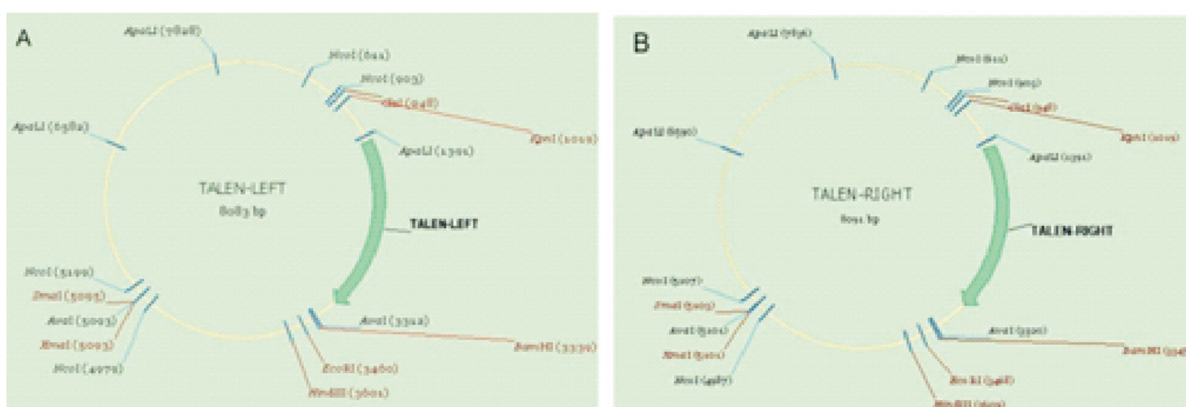

Fig. 3. Structures of the TALEN expression plasmids. (A) The structure of TALEN-1L. (B) The structure of TALEN-1R.

AGTACGA-3'). The recombinant plasmid TALE-1R was ligated to the expression vector JDS71 to construct the recombinant plasmid TALEN-1R, and the construction procedure is shown in Fig. 2B. The recombinant plasmids TALEN-1L and TALE-1R were verified by *Kpn*I and BamHI-HF enzyme digestion. A band at approximately 2.1 kb on the electrophoresis gel provided an initial positive confirmation. Fig. 3A and 3B are the structural maps of the TALENs.

Detection of TALEN activity by the SSA method: The fragment containing the first exon of the porcine IκBα gene was synthesized. *Aat*II was used for enzymatic digestion of the synthesized fragment and the pSSA vector, and then the digested fragment and the vector backbone were ligated to obtain the recombinant plasmid pSSA-IκBα (reporter plasmid). The following 2 groups of experiments were conducted. Each experiment was repeated three times, and each repeat was performed in triplicate. The average of the 3 repeats was taken as the result. The transfection reagent used was DNA Fect Transfection Reagent (CWBIO). The amount of transfection reagent used in each treatment was 6 μl, and the operation followed the manufacturer's instructions. In the experimental group, 0.4 μg of Renilla luciferase plasmid, 2.0 μg of recombinant plasmid pSSA-IκBα, 4 μg of recombinant plasmid TALEN- 1L and 4 μg of TALEN-1R plasmid were co-transfected into PIEC cells using liposomal Lip2000. In the control group, 0.4 μg of Renilla luciferase plasmid and 2.0 μg of recombinant plasmid pSSA-IκBα were co-transfected into PIEC cells with liposomal Lip2000. At 24 h after transfection, the cells from each group were lysed to detect the luminescence of the luciferase. In addition, 8 μg of pEGFP-C1 plasmid was transfected into PIEC cells with liposomal Lip2000 as a reference to examine the transfection efficiency.

RESULTS AND DISCUSSION

Exclusion of SNP site: The IκBα gene of the four types of pigs was sequenced, and no SNPs were found in the recognition region (Fig. 4).

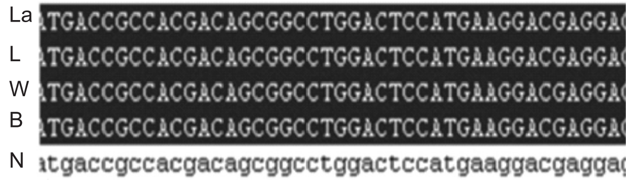

Fig. 4. Excluding the possibility of SNPs in the IκBα genes from 4 different pig breeds. PCR products of the IκBα genes from 4 pig breeds were sequenced. Using DNAMAN software, the sequenced results were compared with the sequence of the *S. scrofa* IkBα gene [Z35483.1] from the NCBI. The comparison indicated that there were no SNPs in the recognition region. La, L, W, B, and N refer to the Large white, Landrace, Wuzhishan, and Bama, and NCBI IkBα gene sequences, respectively.

*Recombinant plasmid detection: Kpn*I and BamHI-HF were used to perform enzymatic digestion of the constructed vectors TALEN-1L and TALEN-1R, and the electrophoresis result showed a band at approximately 2.1 kb (Fig. 5), confirming that the plasmids were positive.

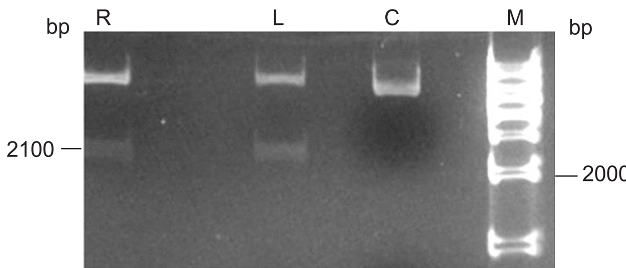

Fig. 5. Positive identification of TALEN-1L and TALEN-1R. Double digestion was performed on the constructed TALEN-1L and TALEN-1R plasmids, and the 2,100 bp target band was observed when the digestion products were examined by electrophoresis. R, L, C, and M refer to TALEN-1R, TALEN-1L, control, and marker, respectively.

Transfection results in PIEC cells: Twenty-four hours after the PIEC cells were transfected with pEGFP-C1 plasmid, the expression of green fluorescent protein was examined. The results showed that this plasmid was

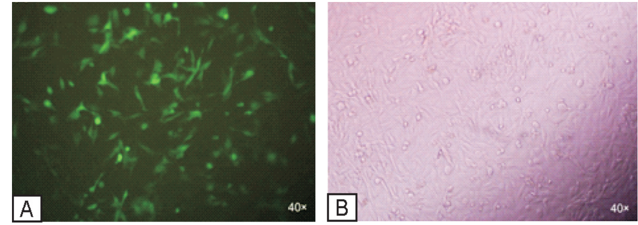

Fig. 6. Fluorescence detection results for PIEC cells transfected with pEGFP-C1.(A) An image of cells under UV light. (B) An image of cells under bright-field microscopy.

successfully transfected into the PIEC cells (Fig. 6A, B). By inference, the Renilla luciferase plasmid, pSSA-IκBα recombinant plasmid and TALEN plasmids were also successfully transfected into the PIEC cells.

Detection of activity of TALENs: Twenty-four hour after transfection, the cells in the experimental and control groups were lysed to detect the luminescence signal of luciferase, and the results are shown in Table 2.

Student's t-test was performed on the above two groups of data, and the result was P = 0.009735 <0.01, a highly significant difference. The knockout efficiency of the TALENs {(the second group F/R): (the first group F/R)} was 3.955. In other words, the knockout activity of the recombinant plasmids TALEN-1L TALEN-1R on the target fragment was 3.955. Fig. 7 shows that the difference between the experimental and control groups was highly significant.

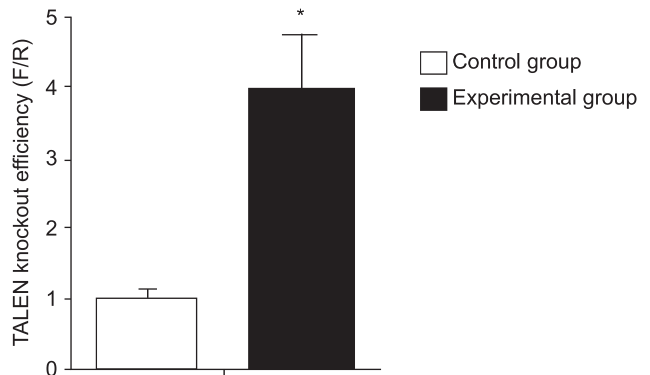

Fig. 7. Comparison of TALENs knockout efficiency. The knockout activity of the control group was 1.000667, while the experimental group activity was 3.957333. *Indicates very significant difference (P<0.01).

Table 2. Luminescence signal values of luciferase

	Experimental group			Control group		
	Firefly luminescence signal	Renilla luminescence signal	Firefly luminescence signal/Renilla luminescence signal	Firefly luminescence signal	Renilla luminescence signal	Firefly luminescence signal/Renilla luminescence signal
First repeat	1508	480.4	2.72	386.9	500.5	0.773
Second repeat	1622	430.1	3.772	632.4	600.9	1.052
Third repeat	2287	425.2	5.38	808.7	686.9	1.177
Average value of F/R of three repeats		3.957333			1.000667	

There is no doubt that xenograft animal organs have great potential as an alternative resource that can solve the shortage of clinical donor resources (Ju *et al*. 2015). However, the body's immunological rejection is the biggest barrier to xenotransplantation, and hyperacute immunological rejection and delayed immunological rejection are the two major forms of immunological rejection. Using targeted gene modification measures, hyperacute immunological rejection has been primarily resolved. Therefore, it is a reasonable concept to resolve the delayed immunological rejection by altering porcine genes. After reviewing the literature, we selected the IκBα gene, which plays a very important role in delayed immunological rejection (Romer *et al*. 2010).

In the present study, a pair of TALEN plasmids were designed near the 32AA site and the 36AA site of the first exon of the IκBα gene to cause a DSB in this region (Ruan *et al.* 2014). According to our requirements for the cleavage site, we used an online software to obtain the recommended TALEN recognition site and construction strategy. Subsequently, we performed SNP exclusion on this site and completed the construction of the vector recognition region based on the recommended strategy. Two TALE fragments were assembled that were then ligated one each into the TALEN expression vectors JDS70 and JDS71. Enzymatic digestion showed that there was an expected band at approximately 2.1 kb, and sequencing confirmed that the assembly was successful. Subsequently, we examined the activity of the constructed TALENs using the SSA method, and we obtained a knockout activity of 3.955. Compared with the control group, the difference in the experimental group was highly significant.

This study is the first report that successfully constructed a high-efficiency TALEN plasmid pair against the fist exon of IκBα gene. The result of this study will be useful for exploring novel gene modification technologies and for studying the delayed rejection response.

ACKNOWLEDGMENTS

This study is supported by grants from 948 program (2011-G11), National Nature Science Foundation of China (31172189), and the Agricultural Science and Technology Innovation Program (ASTIP-IAS05) of the Chinese Academy of Agricultural Sciences.

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