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# Establishment of Conditional Transgenic Mice Model with Cavernous Hemangioma Using the Tet-On System

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## 1. Introduction

During the last two decades, fundamental insight into gene function has been efficiently provided through conventional knockout and transgenic experiments. Although genetically modified mice have provided important new information about the function of many genes, there are serious limitations to current animal models for a number of diseases. Introducing genetic changes to the germ line of a mouse may track down the effects of a particular gene but may also have severe developmental consequences, complicating or even precluding the desired experimental analysis. Good example illustrating these restrictions is embryonic lethality. In gene therapy, an ideal vector should include a regulatory system that is off in the resting state, exhibit tight regulation and allow for rapid and repeatable induction in response to a clinically approved inducer molecule. To overcome these undesired limitations and to precisely control gene expression, conditional mouse models are becoming increasingly popular. Although there are many kinds of inducible transgenic expression systems, tetracycline-controlled expression systems have been employed most frequently. The tet system developed by Gossen and co-workers includes two basic variants: one is the Tet-Off system (tTA system) and the other Tet-On system(rtTA system)(1.2). As a positive regulatory system, Tet-On system was used more widely in numerous transgenic animal models (3.4).

The original tet system contained two transcriptional units, transactivator and responder, on two plasmids. Currently available tetracycline-regulated transgenic methods require most of the generation of two transgenic strains, one carrying the transgene of interest under the control of the tet operator, and the other the reverse transactivator (rtTA). Crossing the lines generates progeny with both transgenes, allowing the regulation of the gene of interest through the administration of tetracycline. However, crossing and analysis of animals transgenic for the individual components of the system is costly and time consuming. Therefore, the generation of a vector containing the two units in a single plasmid may provide advantages for the simplified generation of conditional transgenic animals, as well as for gene therapy, and the successful use of a single construct containing the two elements has been reported (5.6.7). In addition, improvements of newly created tet-based systems should be focused on the elimination of background expression, increased sensitivity to tetracycline or its derivative doxycyline (Dox), and increased inducibility.

We designed and constructed two conditional transgenic vectors combining the two transcriptional units on a single plasmid. In addition, in order to eliminate the background expression of the gene of interest, the most powerful repressor domain containing a 'kruppel-associated box' (KRAB) of the zinc finger protein NK10 (8.9) was used and fused to tetR. The transactivator was placed under the control of CMV promoter, and the tet responsive element, driving the gene of interest, was inserted downstream into the same vector. To minimize any potential interference between the two elements, they were spaced by a 1.2Kb cHS4 insulator. To shield the transgene from the affection of chromosomal position effect and improve the transgene's expression efficiency, we inserted another cHS4 insulator into the upstream of the transgene cassette, and the two insulators were in the same orientation. One of the transgenes was introduced into the mouse genomic DNA through microinjection, and the transgenic mice developed venous malformation after the induction of transgene expression.

## 2. Plasmid construction

### 2.1 The construction of regulatory units
### 2.1.1 Cloning of cHS4 insulator, NK10 and teTR gene
The first step of the study was the cloning of cHS4 insulator from chicken genomic DNA. The primers used were: forward primer, 5' AAAACGCCTCCAGGAAACCACTCT3'; and reverse primer, 5'CAGCAATATTCCCCCATCCTCACT3'. To ensure the sequence be in frame with the targeting sequence, pfu enzyme was used for the PCR experiments. PCR conditions were 95℃ for 5 minutes and then 34 cycles of 94℃ for 30s, 60.1℃ for 40s, and 72℃ for 2m40s, followed by 72℃ for 10minutes.

NK10 gene was cloned from brain tissues cDNA. The primers used were: forward primer, 5'GAATTCGGAATGGCCCCGAGACCT3'; and reverse primer, 5'CTGCAGTCAATGCCCC TGGATAGCGACATT 3'. EcoR1 and Pst1 restriction site sequences were incorporated into the 5' end of the PCR primers, respectively. PCR conditions were 95℃ for 5 minutes and then 34 cycles of 94℃ for 30s, 61℃ for 40s, and 72℃ for 1minutes,followed by 72℃ for 10minutes.

TetR gene was cloned from the plasmid of ptet-off. The primers used were: forward primer, 5'AAACTCGAGCCCGGGATGGCTAGATTAGATAAAAG 3'; and reverse primer, 5'AAA GAATTCCTTTCTCTTCTTTTTTGGCGACCCACTTTCACATTT 3'. The Xho1 and EcoR1 restriction sites sequences were introduced into the 5' end of the PCR primers, respectively. PCR conditions were 95°C for 5 minutes and then 34 cycles of 94°C for 30s, 61.5°C for 40s,and 72°C for 1minutes,followed by 72℃ for 10minutes.

### 2.1.2 Cloning the PCR products in pSK-
The 1.36Kb cHS4 insulator was digested by Sac II and Ssp II and the Sac II Ssp I fragment(1.2Kb)  was subcloned into the Sac II-EcoRV fragment of plasmid pSK-,constructing the plasmid of pBShs4.The NK10 and tetR PCR products were subcloned into T-vector, respectively. The pMD-NK10 plasmid was digested by Pst I and EcoR1, and the pMD-tetR was digested by Xho I and EcoR I .The Pst I-EcoR1 fragment containing NK10 gene and the Xho I Eco I fragment containing tetR gene were subcloned into the Pst I-Xho I fragment of the pSK- plasmid, forming the plasmid of pBSNK10-tetR,in which the NK10-tetR gene will be translated into a fusion protein. The plasmid of pBSNK10-tetR was digested by Xho I. After the end blunted and purified, the fragment was then digested by

Not I. The plasmid of pIRES-EGFP was first digested by Bstx I and after the end blunted and purified the fragment was also digested by Not I, cutting off the EGFP gene. The Xho I-Not I insert fragment containing NK10-tetR gene and the Bstx I-Not I vector fragment were ligated together to generate the plasmid pIRES-NK10-tetR.To build the plasmid of pVP16-NK10-tetR, a EcoR I-Pst I digestion was carried out on the plasmid of pIRES- NK10-tetR, generating the 4Kb EcoR I-Pst I vector fragment, and a BamH1- Pst1 double digestion was also performed on the same plasmid to obtain the 1.6Kb BamH1- Pst1 insert fragment. The ptet-on plasmid was digested by EcoR1 and BamH I, isolating the 1.6Kb EcoR I- BamH I insert fragment rtTA. The three fragments, EcoR I-Pst I vector fragment, BamH I- Pst I insert fragment and EcoR I- BamH I insert fragment, which were all sticky ends were ligated in one ligation reaction, and the pVP16-NK10-tetR plasmid was constructed. pVP16-NK10-tetR plasmid was digested with BamH1 and Not1 cutting off the IRES- NK10-tetR fragment ,and then ligated itself after the end blunted, generating the plasmid of pVsp-teton.

## 2.2 The construction of responsive element
### 2.2.1 The cloning of MT gene
The plasmid pPyMT1 containing MT gene was digested by Bstx1 restriction enzyme. A 0.8% agarose gel electrophoresis that was loaded with all samples was used to isolate the 1.53Kb fragments. After the ends blunted and purified, the fragments were then digested by HindIII, and the Bstx I（blunting）- HindIII fragment(1.48Kb)containing ORF of MT gene was subcloned into the Sma I -HindⅢ fragment of pSK- ,forming the plasmid of PbsMT. A BamH I-HindIII digestion reaction was done on the plasmid of PbsMT, and the BamH I-HindIII fragment containing MT gene was cloned to the BamH I-HindIII vector fragment of the pTRE2 plasmid, forming the vector of pTREMT.

The pBSHS4 plasmid was first digested with Cla I, and then Sac I digestion was done on the same fragment after the Cla I ends were blunted. The 1.2Kb Cla I (blunt)-Sac I insert fragment containing Hs4 insulator was ligated with the Sac I -EcoR I  vector fragment of pN2-EGFP plasmid that the EcoR I ends were also blunted, and this plasmid was labeled pHs4-N2EGFP. The Xho I-BamH I fragment of pTRE2 plasmid containing TRE gene was cloned to the Sal1-BamH1 vector fragment of pHs4-N2EGFP plasmid. As the Xho I is an isoschizomer of Sal I,this ligation reaction was sticky-ends, and the plasmid was named pHs4TRE-N2EGFP. The next step was to replace the EGFP gene of pHs4TRE-N2EGFP vector with MT. The experiments were as follows: pHs4TRE-N2EGFP was digested with BamH I and Not I, and the Not I ends were blunted. The vector fragment ligated with the BamH I-EcoRV insert fragment of pTRE-MT plasmid containing the MT gene, and this plasmid was designated as pN2-Hs4TRE-MT.

## 2.3 Ligation of the regulatory unit and the responsive element
The Ssp I -Vsp I  insert fragment of pVP16-NK10-tetR plasmid(2.5Kb) and pVsp-teton(1.9Kb) were ligated with the Nhe I - Vsp I  vector fragment of pN2-Hs4TRE-MT, respectively, whose Nhe I  ends were blunted. Thus the plasmid of pVP16NK10-Hs4-TREMT and pVP16-Hs4-TREMT were constructed.

In order to insert another Hs4 insulator upstream of the transgene cassette, a plasmid was constructed. First, a pair of primers were designed: forward primer, 5' AAAATCG ATCATATGGCGGAGCGAGGAACTGAG3'  having Cla I  and Nde I  restriction site sequences; and reverse primer, 5' AAACTCGAGGATATCCATATGCCGCCCTGGGAA3' having Xho I , EcoRV  and Nde I  restriction site sequences, and the template DNA was

1.5Kb MT gene originated from pBSMT plasmid. PCR was carried out to amplify a 500bp fragment. The product as a spacer was digested with Cla1 and Xho1 and cloned to the Cla1-Xho Ⅰ vector fragment of pBS Hs4.Thus the pBSHs4-Nde plasmid was constructed, in the upstream of the spacer there was Nde Ⅰ restriction site and the downstream were Nde Ⅰ and EcoⅤ. Therefore, the Vsp Ⅰ-Ssp Ⅰ insert fragments of pVP16NK10-hs4-TREMT(6.9Kb) and pVP16-Hs4-TREMT(5.4Kb) could be cloned to the pBSHs4-Nde's Nde Ⅰ-EcoRⅤ vector fragment(4.2Kb).However, we tried many times and failed. So we changed the strategies: pBSHs4-Nde plasmid was first digested with Vsp1 and Nde1. The 1.4Kb fragment containing two kinds of DNAs was extracted and the Ssp1 digestion was done on it. Thus two kinds of DNAs were divided, and the 1.4Kb fragment which contained 1.2Kb insulator flanking Vsp Ⅰ, Nde Ⅰ sticky ends was cloned to the Vsp Ⅰ site of plasmid of pVP16NK10-hs4-TREMT and pVP16-Hs4-TREMT, respectively, constructing the final plasmids, pHs4-VP16NK10-hs4-TREMT and pHs4-VP16-Hs4-TREMT, and the Bstx1 digestion was performed to determine the insert directions.

## 3. Experiments of the two plasmids *in vitro*

### 3.1 Transient transfection

To testify the function of the two plasmids, transient transfection was carried out. COS7 cells were cultivated in DMEM for 72 h and transfected by electroporation with the same amount of substance of the two plasmids. The electroporation conditions were: voltage, 220V; electrical capacity, 975uF; No. of pulse,1. After the electroporation and adding 1.5ml DMEM to the cuvette, the cells were carefully transfered into two culture dishes equally, respectively. Dox(15µg/ml DMEM) was added to one dish three hours after the electroporation, and the other was used as control.

### 3.2 Semi-quantitative RT-PCR experiment

A semi-quantitative RT-PCR( reverse transcription-polymerase chain reaction) method was applied to measure the marker genes expression of the two plasmids. Total RNA was isolated from the cells by TRIzol method, according to manufacturer's instructions (Invitrogen, Cergy-Pontoise, France), after 72 hours of incubation. In order to resolve the problem of genomic DNA contamination ,2µg RNA was digested with RNase Free Dnase I (New England BioLab, England). RT-PCR was carried out to 1µg DNase-digested RNA. The cDNA was generated using First-Strand Synthesis System for RT-PCR (TaKaRa, Dalian city, China), followed which was the PCR amplification of marker gene, and the expression of β-actin, a housekeeping gene which is assumed to be expressed at roughly equal levels in different cell types and under different conditions, was examined as an internal control. The primers and templates were the same amount of substances in different reactions. The primers used were: MT forward primer, 5′ AAAGAATTCCCATTTTTTGGCTGG GCAGATATCGCGGAGCGAGGAACTGAG3′; MT reverse primer, 5′ AAAAAGCTT CCATTTTTTGGATGGCGAGATATCCGCCCTGGGAATGATAG 3′. β-actin PCR primers were kindly provided by Dr Fei (IBCB, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China), which was designed spaning an intron of the genomic sequence , and this will result in a PCR product from genomic contamination that will be larger in size than the product generated from the cDNA. The PCR conditions were 95°C for 5 minutes and then 27 cycles of 94°C for 30s, 60°C for 1m30s, and 72°C for 1m, followed by 72°C for 10minutes. 0.8% agarose gel loaded with the same volume of PCR products was run to test the differences of the marker genes expression levels.

## 4. Transgenic mice

The 8.5 kb microinjection construction of the plasmid pHs4-VP16NK10-hs4-TREMT was excised from the vector by digestion with SSP I and VSP I, and separated by agarose gel electrophoresis, followed by purification. The purified fragment was microinjected at a concentration of 10 $\mu$ g/ml in phosphate-buffered saline into fertilized C57/BL6 mouse eggs. Transgenic animals were identified by PCR of tail-tip genomic DNA. The sense and antisense primers were 5'-GCGGAGCGAGGAACTGAG- 3' and 5'-CCGCCCTGGGA ATGATAG-3', respectively. Founder mouse mate with wild type C57/BL6 mouse was used to obtain the F1 generation. Dox (0.5 mg/ml) and Sucrose (1 %) were added to drinking water to induce the expression of MT in 6-week-old F1 mice. Skin and tumor samples were fixed in formaldehyde (10% in phosphate-buffered saline) and embedded in paraffin. Sections were cut at 5 $\mu$ m and stained with hematoxylin-eosin.

## 5. Results

In construction of the vectors the following plasmids were used: pTtet-On , pTet-Off , pTRE2, PBSsk-, pIRES2-EGFP and pEGFP-N2, and the vector backbone of the final plasmid was pEGFP-N2.The tet-off and tet-on systems have been developed to regulate transgene expression which provided negative and positive control of transgene expression, respectively. Although the tet-on system produces high basal expression in the absence of an inducer, as it is positive regulation, it is still applied widely. In order to eliminate the basal expression, in this study, we cloned the 'kruppel-associated box' (KRAB) silenceing domain of the zinc finger protein NK10 gene, a powerful transcriptional repressor, and ligated it with tet repressor to translate a fusion protein .The fusion protein binds to the tet operator sequences in the response element only in the absence of Dox and reduce leaked gene transcription. As dox added, the silencer dissociates from the tet operator, relieving the transcriptional inhibition, while the rtTA binds to the TRE and activate the gene's expression. To compare the efficiency and the effectiveness of diverse operating systems, two different Tet-On based inducible expression systems carried in a single cassette were constructed (Fig.1) .
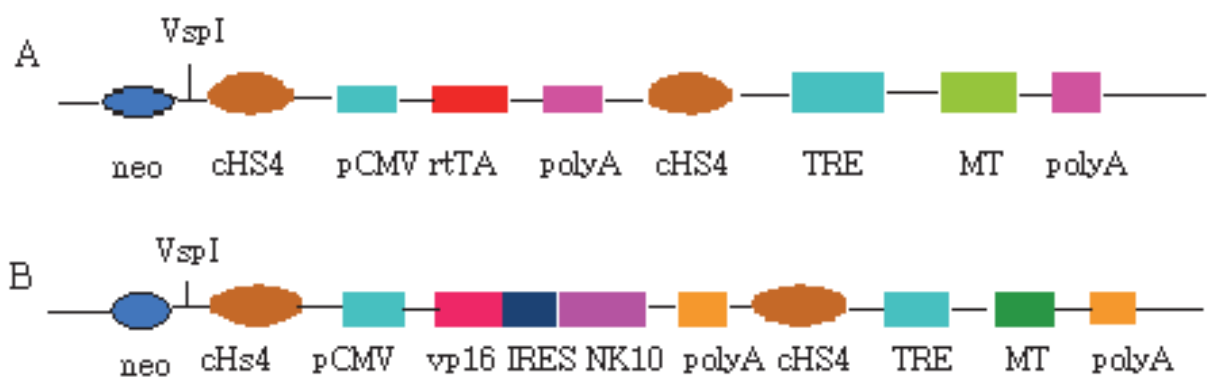


Fig. 1. The maps of the plasmids pHs4-VP16-Hs4-TREMT(A) and pHs4-VP16NK10-hs4-TREMT(B) after the SspI digestion.

In this study, three genes were successfully cloned by PCR method, and the sequences were right through the identification of sequencing and restriction enzyme digestion (Fig.2).
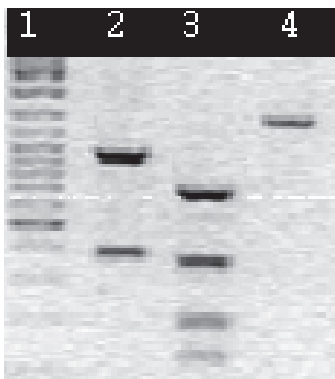
Fig. 2. CHS4 insulator restriction enzyme digestion. Lane 1: Marker, lane 2: Hind III, lane 3: Pst I, lane 4: cHS4 PCR product.

Restriction digestions were carried out to identify the final two transgene vectors, and analysis of the gel showed the two plasmids were constructed successfully (Fig.3).
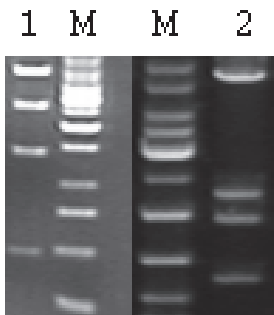


Fig. 3. The EcoRI digestion results of the two plasmids, pHs4-VP16-Hs4-TREMT(1) and pHs4-VP16NK10-hs4-TREMT(2).

The relative quantification of MT transcripts (target) was analyzed by semi-quantitative RT-PCR method in mRNA samples, by comparison with the mRNA abundance of β-actin (control), a housekeeping gene, which is assumed to be expressed at roughly equal levels in different cell types and under different conditions. The results showed that the two plasmids, pHs4-VP16NK10-hs4-TREMT and pHs4-VP16-Hs4-TREMT, had certain levels of transgene expression in the absence of tetracycline, and the inducibility of the latter was higher than that of the former (Fig.4).
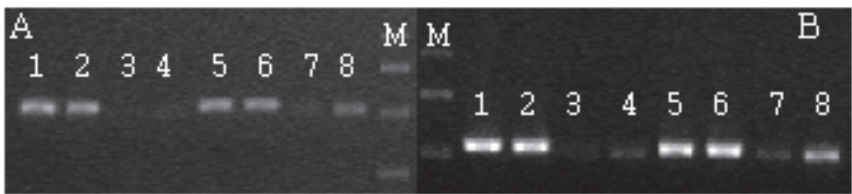


Fig. 4. The results of semi-quantitative RT-PCR of the two plasmids, pHs4-VP16NK10-hs4-TREMT(lane 1,2,3,4) and pHs4-VP16-Hs4-TREMT(lane 5,6,7,8) . The cycles were 22(A) and 24(B).There was no DOX added in the lanes 1,3,5 and 7, while dox added in lanes 2,4,6, and 8. There was no difference in the beta-actin expression before and after the DOX added (lane 1,2,5,6).

Five founders were identified by PCR analysis of DNA from tail biopsies. As expected from the inducible nature of transgene expression, no apparent phenotype was observed. After hybridization，three F1 mice were obtained, Dox (0.5mg/ml) and Sucrose（1%）were added to drinking water to induce the expression of MT in 6-week-old F1 mice .Two months later 2 mice developed vascular anomalies in brain and skin. The lesion was more venous malformation than hemangioma in histopathology (Fig.5., Fig.6.) .



Fig. 5. Cystic tumor developed in the chest and skin of the mouse after 2 months of induction (arrow shows).
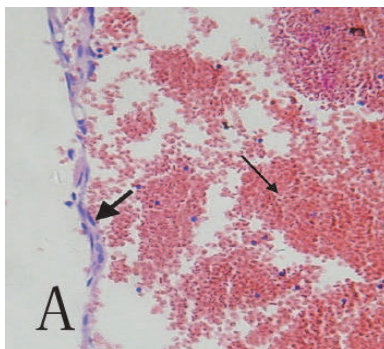


Fig. 6. Histological analysis of vascular anomalies induced in F1mice. The tumor was cystic and lined by endothelial cells(thick arrow shows). The cyst was full of red blood cells (thin arrow shows)

## 6. Discussion

The concept of gene therapy was originally introduced to treat inherited genetic disorders with the goal of replacing the defective gene with a normal one in diseased cells. This early concept has rapidly switched to a broader vision of gene therapy that uses genetic materials to alter the disease process. Currently, clinical trials are ongoing to examine the use of gene therapy in life-threatening diseases, such as malignant tumors and inherited genetic disorders. In gene therapy, the choice of gene delivery systems is most important.

Transgenic models have provided a wealth of information about the function of specific genes. However, the limitations of promoter-controlled transgene expression have become clear. The constitutive system has no control over the time of expression, which depends entirely on the properties of the promoters used. The promoters in this setting are active constitutively, many staring early in embryonic stages. The target gene is activated as soon as the promoter becomes active. If the transgene product happens to be toxic to the organism, especially during gestation, it can be detrimental to the developing embryo. This will results in a failure to generate live progeny carrying the transgene and that of the transgene cannot be studied past embryogenesis. If the gene product is not toxic to the

embryos, the animals are viable and many demonstrated an interesting phenotype. However, there will still be questions remaining such as whether the phenotype is the results of transgene expression or is that of the mutation of endogenous genes. To deal with the limitations a number of investigators have established transgenic systems in which the expression of the transgene can be externally regulated. Although a variety of approaches have been utilized, tetracycline-controlled expression systems have been employed most frequently (10).

In the present study, we constructed a single plasmid containing both a tet-responsive transactivator together with the transgene responder successfully. This strategy circumvents the need to generate two independent transgenetic lines in the first place and greatly facilitates the mouse breeding strategy as effector and responder will not segregate.

Recent development of gene transfer approaches in plants and animals has revealed that transgene can undergo silencing after integration in the genome (11.12.13). Gene expression is influenced not only by the presence or absence of nearby transcription factors but also by the structure of surrounding chromatin. Variable repression of transcription often is associated with heterochromatin and frequently manifests itself as position effect variegation. In an extensive study of this phenomenon, Cerutti et al. (12) arrived at the conclusion that in C.reinhardtii the local heterochromatin structure rather than DNA methylation has a role in transgene inactivation. Insulators are DNA sequence elements that function as blocking the action of an enhancer and shielding a transgene from chromosomal position effect in transgenic animals and cell lines (14). Flanking a transgene with two copies of an insulator can shield it from chromosomal position effect following stable integration. In our preliminary study we attempted to insert two copies of 1.2Kb insulators in the flank of the transgene cassette, if that there will be three copies of direct repeat sequences in one plasmid and the plasmid will be unstable. We tried many times to construct it , but failed, though the STBL4 competent cells that are capable of stabilizing direct  repeat sequences was used. As transgenes are usually found to be inserted into a single chromosomal location as multiple copies forming head-to-tail concatermers (10), so insulators can still be effective when placed on only one side of transgenes when in multi-copy arrays. This strategy has been employed successfully in this study and others (15.16.17).Theoretically only the end copy of the transgene would be susceptible to position effect after the concatemers integrated with germ lines genomic DNA. This not only can help save some cloning time and lower the plasmid size, but also can improve the transgene's expression efficiency.

Theoretically tetracycline inducible transgene expression systems should have negligible basal expression levels and high inducibility. Unfortunately, these goals are not achievable in our project. The results of this study showed that background expressions still existed in the two vectors, pVP16NK10-hs4-TREMT and pVP16-Hs4-TREMT, but the former's was much lower than that of the latter, and the inducibility of the later was much higher than that of the former. The reasons were as follows : first, in the two plasmids there was a enhancer/promoter of neo gene which could also act on the CMV minipromoter of TRE at a distance promoting the expression of the target gene. In *in vivo* experiment this factor will be excluded after the linearization of the plasmid with Vsp I and Ssp I digestions.  Second, in the plasmid of pVP16NK10-hs4-TREMT the products of rtTA and tetR-NK10 can form heterodimers that do not function appropriately and compromise the overall inducibility of this system. On the other hands, competition existed between rtTA and tetR-NK10 for the DNA binding sites of TRE which could also lower the Dox-inducibility. This plasmid would be modified in our next project. Basal expression was also associated with the site(s) of

integration of the transgenic constructs, and enhancer sequences near the target gene have been repeatedly demonstrated to increase basal target gene expression, thereby compromising the tight regulation that is desired (18.19). To eliminate the basal expression, insulator was used to block the effect of enhancer near the transgene integration sites on TRE in our study. Although the results in the test of transient transfections showed a degree of leakage expression, in some circumstances, the leak is acceptable and does not negate the ability of the experimental system to appropriately address the hypothesis that is being investigated. In the upstream of our transgene cassettes there was a neo gene, respectively. If the vectors were only digested with Ssp I this gene being a selective marker will be included in the transgene cassettes, and this will benefit stable transfection of the two vectors.

Vascular anomalies are classified into hemangiomas and vascular malformations. Hemangiomas are the most common cutaneous tumors of infancy, which demonstrated by rapid growth after birth followed by a slow spontaneous involution or regression within five to seven years. Unlike hemangiomas, vascular malformations enlarge proportionately with the growth of the child and do not undergo spontaneous involution (Mulliken JB and Glowacki J. 1982). Vascular malformations can be subdivided based on blood flow rate: "slow flow" (capillary, venous, lymphatic or mixed) vs "fast flow" (arterial, arteriovenous, fistulae or shunt) subtypes. Vascular anomalies occur mostly in the head and neck regions, and there is no useful animal model with this disease until now. In our study, we established conditional transgenic mice model with cavernous malformations base on tet-on system, and this animal model could be a useful tool to investigate the pathogenesis and intervention of venous malformation, as well as the function of PyMT gene.

In summary, in the present study we combined the two expression units of Tet-On system on a single plasmid constructing two conditional transgenic vectors, one of which contained Nk10 gene fused to TetR. CHS4 insulator elements were utilized in the construction of the vectors to eliminate the basal expression and transgene silencing. Our study *in vitro* demonstrated that the two "two-in-one" systems were functional. This would minimize the breeding and genotyping required for phenotypic characterization. Above all, If the vectors were only digested with Ssp I a neo gene as a selection marker will be included in the transgene cassettes. Therefore, the Tet-On system presented here may be easy to use and widely applicable to the study of gene function in transfected cells and gene therapy, as well as for the creation of conditional transgenic animal models to study disease and development *in vivo*. As the Dox-inducibility of pHs4-VP16NK10-hs4-TREMT was lower, modifications should be made on this plasmid in the future.

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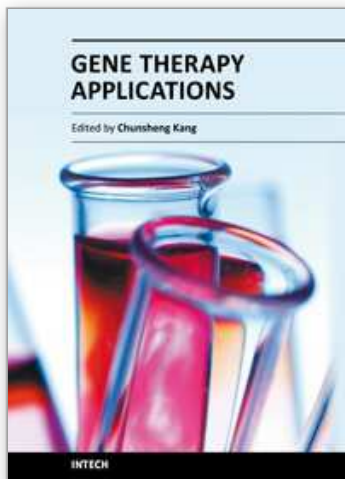
**Gene Therapy Applications**

Edited by Prof. Chunsheng Kang

The aim of our book is to provide a detailed discussion of gene therapy application in human diseases. The book brings together major approaches: (1) Gene therapy in blood and vascular system, (2) Gene therapy in orthopedics, (3) Gene therapy in genitourinary system, (4) Gene therapy in other diseases. This source will make clinicians and researchers comfortable with the potential and problems of gene therapy application.

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