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ORIGINAL PAPER

Design of a Lentiviral Vector for the Inducible Expression of *MYC*: A New Strategy for Construction Approach

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Abstract Lentiviral vectors are powerful tools for gene expression studies. Here we report the construction of pTIJ, a vector for inducible gene expression. pTIJ was generated from pTRIPZ backbone, which is designed for the inducible expression of shRNA sequences, by the introducing of a multiple cloning site upstream of the Tet promoter and the removal of miR30 flanking sequences. To evaluate pTIJ as a tool for the inducible expression of genes of interest, we introduced *MYC* cDNA into pTIJ and infected two small cell lung cancer cell lines, H209 and H345. Induction of *MYC* expression by doxycycline was detectable in both cell lines by real-time PCR and western blot analysis. This study highlights the relevance of pTIJ vector to allow the inducible expression of any gene of interest. In our belief, pTIJ will be an extremely useful tool to simplify the generation of genetically engineered cell lines for the inducible expression of cDNA sequences in biological studies. Furthermore, we report the generation of a pTIJ-MYC vector for the inducible expression of the oncogene *MYC*.

Keywords Lentivirus · Myc · MCS · SCLC · Dox · Inducible vector

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Introduction

Gene transfer is a simple and an efficient method to stably introduce foreign genes into the target cells [1, 2]. The efficacy of gene transfer and expression is dependent on the methodology employed and the characteristics of the vector used. While choosing the vector, it should be considered that different tissues/cells require vectors with different properties and based on the application, vector choice can also be different [3]. Tools for gene delivery can be classified into two major groups as viral and non-viral vectors [4–6]. For what concerns viral vectors, parts of viral genome were deleted and replaced by genetic elements [7]. Lentiviral vectors, in particular, contain accessory genes from the viral genome which enable regulation of gene expression and offer long-term expression of the gene of interest [8].

As mentioned in recent studies, inducible expression vectors are powerful tools for gene expression of many transcription factors [9, 10]. Since expression of genes involved with cell cycle regulation or progression causes indirect effects to cell viability such as mitotic stress in the cells, it is difficult to identify their direct targets when overexpressed by constitutive systems [11–14]. Therefore, to identify the direct targets of this category of genes, it is fundamental to control their expression in a refined and time-dependent manner [15]. Hence, inducible systems could be helpful for this purpose.

Several systems that are capable of regulating gene expression in eukaryotic cells are available [16]. Reverse tetracycline-inducible expression rtTA, alias Tet-On system, that will authorize graduated gene expression response is quite accepted so far [17]. Lentiviral vectors have the advantage to integrate with the host genome, offering long-term expression of gene of interest [18–20]. It would be

important also to choose a vector which contains a selectable marker such as puromycin to confer antibiotic resistance and enrich cells with stably integrated vector.

In this study we developed a lentiviral inducible expression vector modifying the pTRIPZ plasmid (Dharmacon) by replacing the shRNA insertion site with a multicloning site (MCS). We also inserted *MYC* gene in the MCS, downstream the Tet CMV promoter (six copies of Tet operon placed upstream of the CMV). We infected H209 and H345 small cell lung cancer cell lines, and stably infected cells were afterward selected with puromycin selection.

Materials and Methods

Cell Culture

HEK293T, H345 and H69 cells were obtained from Dr. Jun Yokota. HEK293T cells were maintained in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% fetal bovine serum. H345 and H69 cells were cultured in RPMI medium (Life Technologies) supplemented with 10% tetracycline-free FBS (Clontech) in a humidified atmosphere with 5% CO_2 at 37 °C.

Construction of pTIJ Inducible Expression Vector

To construct inducible expression vector, we used pTRIPZ empty vector (Dharmacon) as backbone. tRFP and shRNA flanking sequences were removed from the pTRIPZ vector by restriction digestion using AgeI and MluI enzymes. To generate a multiple cloning site (MCS), two overlapping oligos containing AgeI, EcoRI, HpaI, ClaI, BstBI, XhoI and MluI restriction sites were designed and annealed (Table 1). The ligation reaction was performed for 3 h at room temperature using T4 ligase (NEB). Transformation of each ligation products into DH5α competent cells was performed by heat shock. Plasmid isolation was performed with Nucleospin Plasmid EasyPure kit. Plasmids were validated by restriction digestion using AgeI, MluI, HpaI and BamHI restriction enzymes. We named our Tet-inducible expression vector pTIJ.

Subcloning of Myc Insert into pTIJ Vector

The coding sequence (CDS) of c-MYC cDNA (1364 bp) was amplified from pCMV-Sport6-Myc, a modified PCMV-Sport6 vector by PCR reaction. The reaction conditions were as follows: denaturation phase of 95 °C for 5 min, followed by 35 cycles of 95 °C for 40 s, 64 °C for 40 s, 72 °C 2 min and 72 °C for 5 min. 1364 bp PCR product was visualized by 2% agarose gel electrophoresis (Fig. 1). Both PCR product and plasmid DNA were digested with EcoRI and XhoI restriction enzymes (New England Biolabs) according to manufacturer's instructions and verified by agarose gel electrophoresis (Fig. 2a). Purified digested PCR insert and vector were ligated to form the new recombinant plasmid pTIJ containing the MCS. DH5 alpha competent cells were used for transformation. The transformation reactions were plated on LB agar plates containing the appropriate antibiotic. Selected colonies were sequenced with pTIJ sequencing primers.

Transfection

H69 cells (3×10^5 cells) were plated in a 35-mm tissue-culture dish with 3.0 ml of medium which achieved >70% confluency at the time of transfection. After 24 h in culture, the culture medium was replaced with fresh medium. DNA was diluted with 100 µl of OptiMEM (Life Technologies). Then FuGENE® HD Transfection Reagent (8 µl) was added directly into the OptiMEM/DNA mixture and mixed well by vigorously tapping. Fifteen minutes after incubation at room temperature (RT) for complex formation, the mixture was added drop wise to the cells. Cells were

Table 1 Primer/oligonucleotide sequences

Primer/oligonucleotide sequence 5′ → 3′	Primer name
CCGGTGAATTCGTTAACATCGATTTCGAACTCGAGA	MCS oligonucleotide 1
CGCGTCTCGAGTTCGAAATCGATGTTAACGAATTCA	MCS oligonucleotide 2
TAAGCAGAATTCCTGGATTTTTTTCGGGTA	MYC PCR EcoRI primer F
ATCGCACTCGAGTTACGCACAAGAGTTCCG	MYC PCR XhoI primer R
CGTCCTCGGATTCTCTGCTC	MYC sequence primer 1
CAGCGACTCTGAGGAGGAAC	MYC sequence primer 2
GGAAAGAATCAAGGAGG	pTIJ sequence primer
CTTCTCTCCGTCCTCGGATTCT	MYC forward qRt-PCR primer
GAAGGTGATCCAGACTCTGACCTT	MYC reverse qRT-PCR primer
CGCGAGAAGATGACCCAGAT	Beta-actin forward qRT-PCR primer
GATAGCACAGCCTGGATAGCAAC	Beta-actin reverse qRT-PCR primer

incubated for 6 h, and then medium was changed. Doxycycline (2 μg/ml) was added 24 h after transfection. Cells were analyzed 72 h after medium renewal.

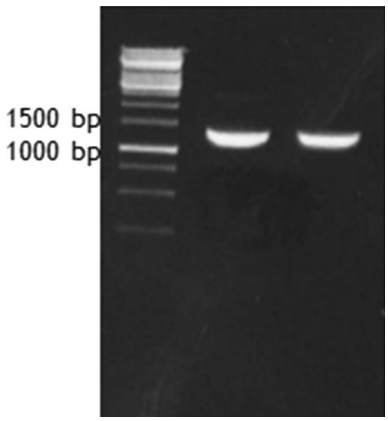

Fig. 1 Product of PCR synthesis and identification of the recombinant lentiviral vector. The agarose gel band at 1364 bp was derived from PCR amplification

Lentivirus Preparation, Infection and Selection of Infected Cells

Lentiviral vectors expressing the *MYC* gene were produced as follows. Twenty-four hours prior to transfection, 3×10^6 293T cells were plated onto 75 cm^2 flasks. Two hours prior to transfection, medium was replaced with medium containing 25 μM chloroquine. All solutions were sterilized by filtration through 0.22 μm filters. Solutions were stored in aliquots at −20 °C and thawed prior to use.

pTIJ-MYC colony 2 (15 μg), together with pPAX2 (6.5 μg) packaging and pMD2.G (3.5 μg) envelope plasmid DNA, was transfected using calcium phosphate method. Sixteen hours post-transfection, the medium was replaced with RPMI supplemented with 10% Tetracycline-free FBS and incubated at 5% CO_2 for 24 h. After 24 and 48 h, first and second aliquots of media were collected. Media containing viruses were filtered through a 0.45-μm PVDF filter and stocked at −80 °C. H209 and H345 cells were infected with polybrene (2 μg/ml) for 24 h. After

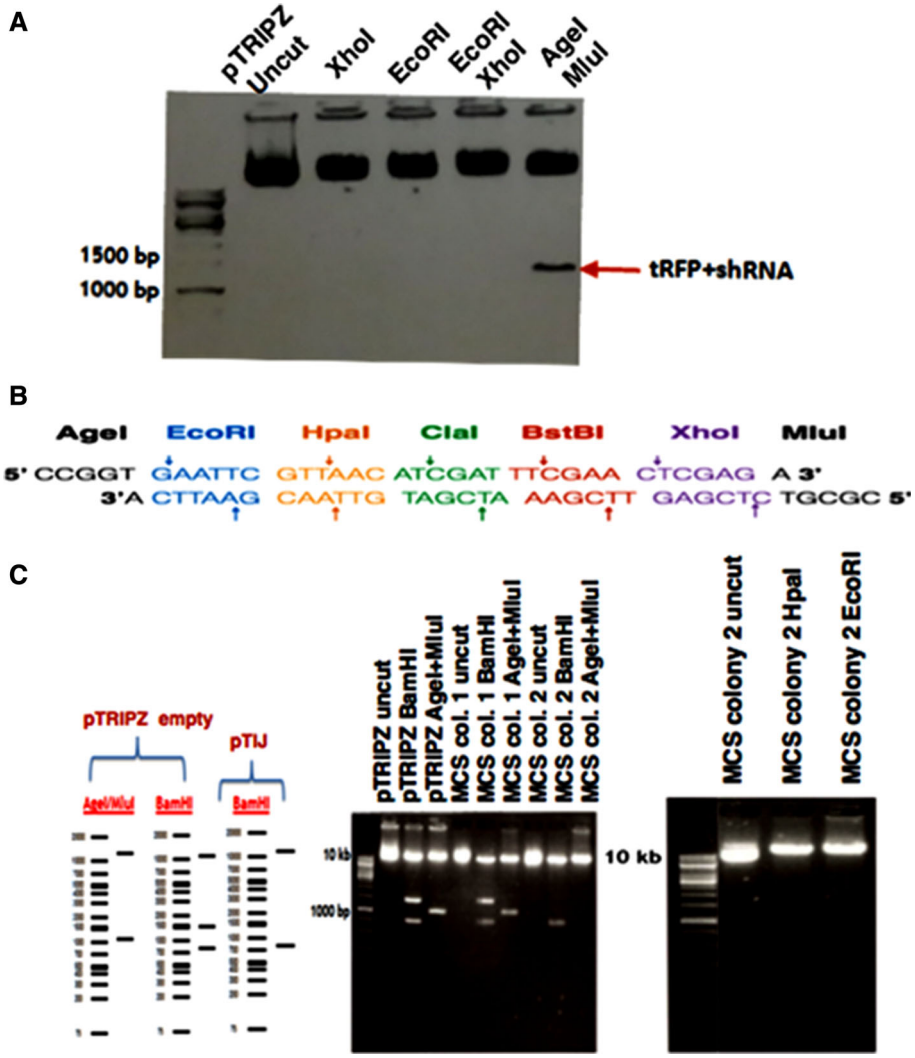

Fig. 2 Restriction digestion of pTRIPZ vector. **a** Removal of tRFP and shRNA sequences by using MluI and AgeI restriction enzymes. **b** Sequences of complementary oligonucleotides including 7 restriction sites. **c** Validation of selected colonies by digestion reactions: *left figure* represents the predicted sizes of AgeI + MluI and BamHI digested products for pTRIPZ and pTIJ vectors. *Middle* and *right figures* correspond to the restriction digestion of selected colonies

1 week of expansion, puromycin was added to kill non-infected cells. Optimal dose for puromycin selection was evaluated as 2 μg/mL in H209 and H345 cells. Puromycin concentration (2 μg/ml) was then adjusted to maintain optimal plasmid integration. H345 cells were induced with various Dox concentrations (0.5, 2.0 and 4.0 μg/ml) in order to obtain the suitable Dox dose.

Real-Time Quantitative PCR (qRT-PCR)

Total RNA was extracted 2 days after Dox induction by using TRIzol reagent from H345 and H209 pTIJ-MYC colony 2 infected cells. Reverse transcription of RNA was performed with high-capacity cDNA reverse transcription kit. qRT-PCR was performed by using Kilogreen MasterMix (ABM). qRT-PCR primers and oligonucleotide sequences are listed in Table 1. mRNA expression of Myc between Dox+ versus Dox− was measured using the Eq. $2^{-\Delta\Delta Ct}$. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control.

Western Blot Analysis

Forty-eight hours after Dox induction, cells were lysed in lysis buffer (50 mM TRIS, 0.5% sodium deoxycholate, 1.0% NP-40, 0.1% SDS, 150 mM NaCl, 2 mM EDTA) supplemented with protease inhibitors (Roche). Lysates (15–30 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membranes and probed with the following antibodies: c-Myc (sc-40, Santa Cruz) and α-tubulin (CP06, CalBiochemicals). Membranes were then incubated with a peroxidase-conjugated antibody. Enhanced chemiluminescence was performed according to manufacturer's instructions (Western Lightning Plus, Perkin Elmer).

Results

Construction of Tet-Regulated Lentiviral Vector

To generate an inducible gene expression system, we modified pTRIPZ empty vector. pTRIPZ is 13.32 kb size plasmid containing tRFP and shRNA flanking sequences. To add a multicloning site for later insertion of cDNA inside the vector, tRFP and shRNA flanking sequences were removed by using MluI and AgeI restriction enzymes (Fig. 2a), in order to avoid excessive increase in the size of vector that cause a decrease in the efficacy of packaging. Besides that removal of those sequences was important to avoid interfering with MYC activity. To generate a new multiple cloning site (MCS), two overlapping oligos were designed and generated by restriction digestion of existing sites in the original vector (Fig. 2b). For the validation of selected colonies which contain new multiple cloning sites (MCS), restriction digestion by using BamHI, AgeI and MluI were performed (Fig. 2c). The map of pTIJ is shown in Fig. 3.

Expression of Myc in pTIJ-MYC Vectors Transfected in H69 Cells

To validate if selected colonies contain Myc cDNA EcoRI and XhoI restriction digestion was performed (Fig. 4a). According to the restriction digestion we obtained that 7 out of 10 colonies were found to have Myc insert. To check if vectors induce Myc overexpression, lipofection was performed in H69, MYC non-amplified cell line, by using Fugene HD transfection agent. Colony 2, 4 and 6 were selected for transfection. Two days after Dox induction c-Myc expression was observed in H69 cells (Fig. 4b). Highest MYC expression was observed in colony 2. For further experiments we have chosen to continue with colony 2.

Infection of SCLC Cells with pTIJ-MYC Lentiviral Vector

pTIJ-MYC vector contains a tetracycline response element and a CMV minimal promoter upstream the *MYC* sequence. The expression of MYC was augmented depending on Dox dosage (Fig. 5a). Induction of Dox effectively induced expression of MYC within 48 h of culture. The effect of pTIJ-MYC lentivirus was evaluated at mRNA and protein levels in H345 and H209 cells by real-time quantitative PCR and western blot analysis (Fig. 5b,c). The results revealed that the expression levels of MYC in pTIJ-MYC colony 2 infected H345 cells were markedly increased. Both qRT-PCR and western blot analysis demonstrated that in Dox-treated H345 cells, MYC expression was detected on the contrary to in cells cultured without Dox.

Discussion

Several methods are described to integrate the tetracycline-inducible components into a single vector [21, 22] that will give us the hope that the development of tetracycline-regulated promoters allowing robust expression of gene of interest upon induction [23, 24].

Tet operator segments are fused with a CMV core promoter, and expression is constitutive in the presence of tetracycline or its analog doxycycline. In the Tet-On system, reverse tTA (rtTA), tetracycline induces rtTA binding to the operator and subsequent transcriptional activation. Additional variants have been developed that greatly

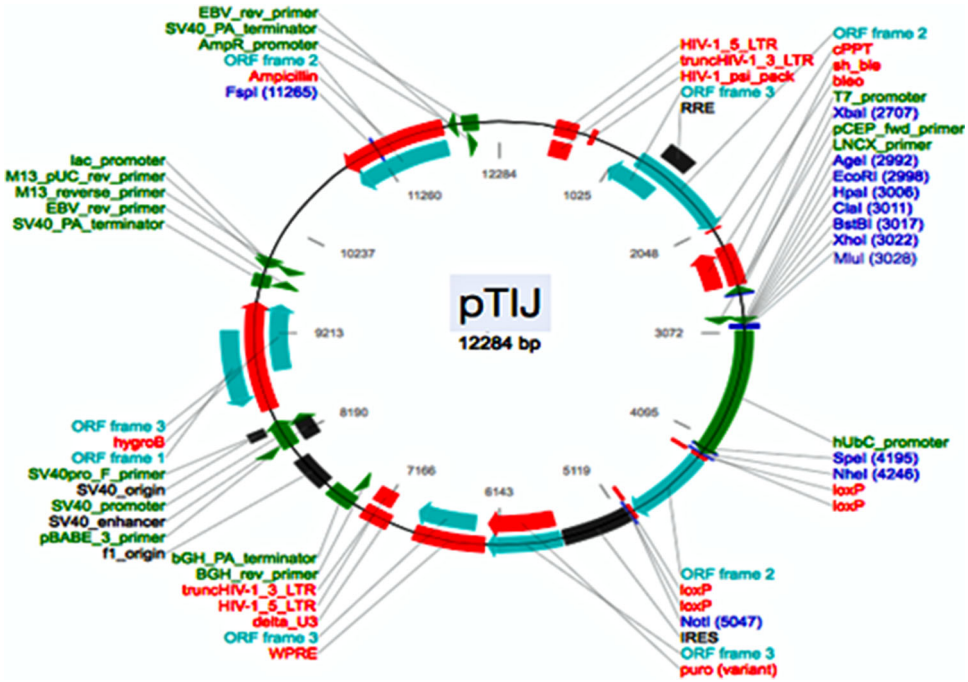

Fig. 3 Schematic process of generating lentiviral vector expressing human MYC gene (pTIJ)

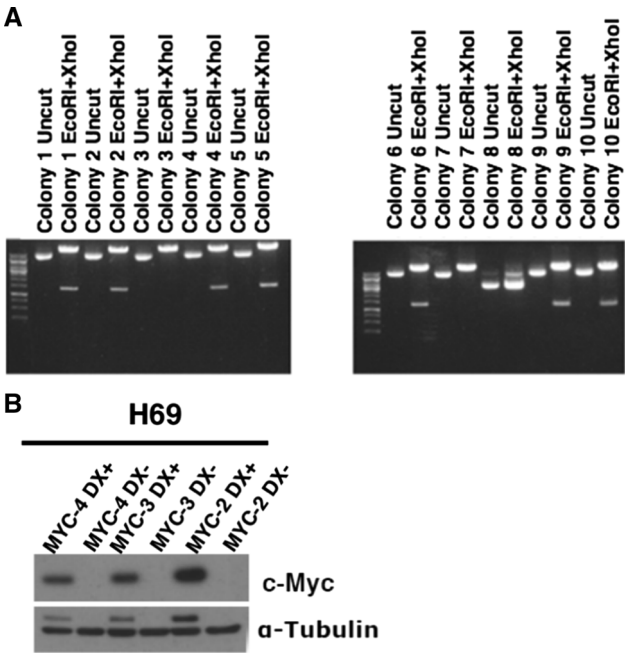

Fig. 4 Validation of subcloned Myc cDNA into selected colonies by restriction digestion. **a** MYC cDNA is subcloned to pTIJ vector by using EcoRI and XhoI restriction enzymes. **b** 2 days after Dox induction MYC expression was validated with western blot analysis

enhance induction levels, such as rtTA3 [25]. Since lentiviruses tend to integrate into active chromatin, this design would theoretically be expected to minimize silencing by adjacent heterochromatin, a problem that limited utility of the original system [26].

The size of pTRIPZ vector is 13.32 kb. It consists of tRFP and shRNA flanking sequences in addition to structural genes. We removed tRFP and shRNA flanking sequences with AgeI and MluI restriction enzymes in order to avoid excessive increase in the size of vector that cause a decrease in the efficacy of packaging. Besides that removal of those sequences was important to avoid interfering with MYC activity.

The multiple cloning site (MCS) is a short segment of DNA that is located downstream of an internal promoter allowing convenient cloning of the gene of interest. We chose seven unique restriction enzymes (AgeI, EcoRI, HpaI, ClaI, BstBI, XhoI and MluI) in the light of inserting any gene of interest.

pTIJ vector was generated with a similar structure like Tet-inducible system vectors with puromycin selection marker for mammalian cell and regulation of gene expression in the presence of Tetracycline. Puromycin selection is quite important for positive selection of colonies containing pTIJ vector. Optimal dose for puromycin selection was evaluated as 2 μg/mL in H209 and H345 cells.

Dox, a synthetic derivative of Tet, can be administered to the cells in low concentrations to induce gene expression [27]. Due to its serum half life which is normally 14–20 h, low toxicity and low cost, it is preferred as an effector for tetracycline-inducible expression systems [28]. In the present study, dose response of the pTIJ vector system for Dox was studied in H209 and H345 cells and optimal dose of Dox is determined as 2 μg/mL.

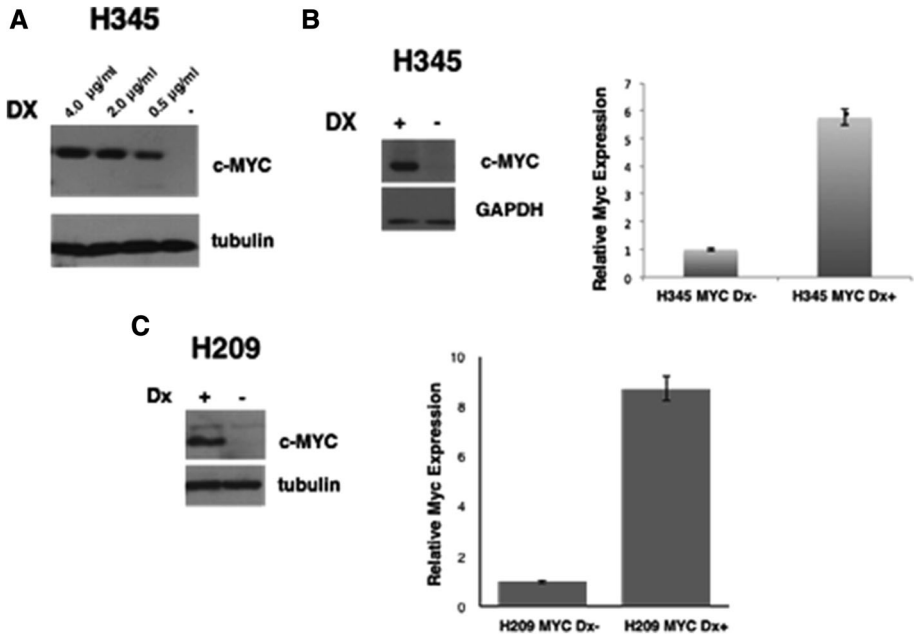

Fig. 5 Evaluation of the pTIJ-Myc vector by a transient and lentiviral vector systems. **a** The expression of MYC was augmented depending on Dox dosage. **b**, **c** The effect of pTIJ-MYC lentivirus was evaluated at mRNA and protein levels in H345 and H209 cells by real-time quantitative PCR and western blot analysis

In this study we demonstrated a successful transduction of human *MYC* gene with a new lentiviral vector, pTIJ, for potential use to induce MYC expression as well as any gene of interest. The techniques used in this study give a point of new view for designing and construction of inducible lentiviral vectors. We believe that this inducible expression vector system will be of great use to check the effect of different genes in different processes both in vitro and in vivo.

Conclusion

pTIJ expression vector system can serve as a host for the expression of any gene of interest. In our belief, pTIJ vector will be a useful tool for identifying the function of genes that can be therapeutic targets both in vivo and in vitro.

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Compliance with Ethical Standards

Conflict of interest Authors declared that there is no competing interest.

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