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Development of a doxycycline-inducible lentiviral plasmid with an instant regulatory feature

Tian Yang [a,b], Christopher Burrows [a], Jeong Hyeon Park [a,*]

[a] Institute of Fundamental Sciences, Massey University, Palmerston North, New Zealand
[b] Institute of Somatology, Nanjing Medical University, 140 Hanzhong Road, Nanjing, Jiangsu 210029, PR China

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ABSTRACT

Lentiviruses provide highly efficient gene delivery vehicles in both dividing and non-dividing cells. Inducible gene expression systems often employ a specific cell line that constitutively expresses a regulatory protein for transgene expression. As one of such inducible expression systems the Tet-On system uses a cell line expressing reverse tetracycline-responsive transcriptional activator (rtTA). The rtTA protein binds to the tetracycline-responsive element (TRE) in the promoter and activates transcription of a transgene in a doxycycline-dependent manner. To establish a universal and instant regulatory system without generating Tet-On cell lines, the cDNAs of *rtTA* and a testing target gene (*PPM1B*) were cloned in the bi-directional TRE-containing promoters. Here, we examined whether a basal leaky expression of rtTA allows instantly inducible expression of both *rtTA* itself and the target gene, *PPM1B* in a single plasmid using the two mini-CMV promoters. Transient transfection of the lentiviral plasmids into human embryonic kidney HEK293T cells showed a significant induction of *PPM1B* expression in response to doxycycline, suggesting that these lentiviral plasmids can be used as an instantly inducible mammalian expression vector. However, the expression of *rtTA* by lentiviral transduction shows a minimal expression without a consistent response to doxycycline, suggesting that the utility of these lentiviral vectors is limited. A potential solution to overcome lentiviral transgene inactivation is proposed.

1. Introduction

Lentiviral delivery system is known to be one of the most effective methods to deliver a gene of interest into target cells, tissues and organs, by integrating a target gene into the host cell genome for a long-term stable expression (Zufferey et al., 1998). This system has been widely used as a research tool for targeted gene expression and could potentially serve as a gene delivery method for gene therapy. An ideal gene expression system for gene therapy should be stable, bio-safe, non-toxic, non-immunogenic and most importantly, tightly regulated by a regulatory protein to facilitate precise control of therapeutic gene expression (Goverdhana et al., 2005). Lentiviruses are a subtype of human immunodeficiency virus (HIV). They are prepared as pseudoviral particles by co-introducing a lentiviral plasmid together with the packaging plasmids co-expressing foreign viral envelope proteins and the vesicular stomatitis virus G glycoprotein (Mitta et al., 2002). Lentiviral delivery systems have been continuously

Abbreviations: PPM1B, protein phosphatase, Mg^{2+}/Mn^{2+} dependent, 1B; GUSB, β-glucuronidase; RT-qPCR, reverse transcription-coupled quantitative real time PCR; HEK, human embryonic kidney; HIV, human immunodeficiency virus; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; rtTA, reverse tetracycline-responsive transcriptional activator; TRE, tetracycline-responsive element; Dox, doxycycline; CFU, colony forming unit.

* Corresponding author. Address: Institute of Fundamental Sciences, Massey University, Private Bag 11222, Palmerston North 4442, New Zealand. Fax: +64 6 350 5688.
E-mail address: J.Park@massey.ac.nz (J.H. Park).

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improved over the years, from the first generation of lentivirus that comprised of all HIV proteins with a high risk of wild-type virus re-construction; to the second generation that contains only key viral proteins (*gag*, *pol*, *tat* and *rev*); to the third generation that further stripped down to three core HIV-1 genes (*gag*, *pol* and *rev*), and the use of a split viral genome to reduce the replication competent virus production (Gasmi et al., 1999; Mitta et al., 2002; Zufferey et al., 1998).

To establish a lentiviral expression system with an inducible feature, tetracycline responsive element (TRE) and reverse tetracycline-controlled transactivator (rtTA) have been commonly incorporated in the expression system (Centlivre et al., 2010). The tetracycline derivative, Doxycycline (Dox) is used as an inducer to regulate transgene expression by allowing rtTA binding to the TRE in the promoter. Expression of the regulatory protein rtTA should be achieved by a separate plasmid such as pTet-On regulator plasmid (Clontech) or by generating a stable cell line that constitutively expresses rtTA. This binary system consisting of a TRE-regulated transgene and a separate rtTA expression makes lentiviral system impossible for gene therapies or *in vivo* use in primary cells. Moreover, overexpressed rtTA has been implicated in cytotoxicity and unstable transgene expression (Shockett et al., 1995; Strathdee et al., 1999). To overcome these problems, autoregulatory expression of rtTA has been attempted in which constitutively expressed rtTA is maintained at low levels in the absence of Dox but induced to the higher levels in response to Dox by incorporating TRE in the rtTA expression cassette (Gould et al., 2000; Markusic et al., 2005; Strathdee et al., 1999). However, previous autoregulatory rtTA expression systems depend on relatively high basal level of rtTA expression through constitutive promoters. Additionally, most of autoregulatory rtTA expression studies have not been investigated in lentiviral expression system involving a stable integration of expression cassette in the host chromosome.

Here we investigated (i) whether an extremely low basal level of rtTA from the silenced TRE-containing promoter can induce its own synthesis by a positive feed forward regulation (ii) whether a TRE-flanked target gene can also be induced by rtTA in response to Dox. We hypothesized that a leaky basal rtTA expression will be sufficient for a Dox-dependent autoregulatory induction and therefore avoid a requirement of rtTA expression by a constitutive promoter in previous studies. We constructed lentiviral plasmid vectors containing bi-directional mini-CMV-driven expression cassettes to promote expression of both rtTA and the transgene under the control of their respective TRE-flanked mini-CMV promoters. In the absence of Dox, extremely low levels of rtTA will be present by a leaky expression of silenced mini-CMV promoter, but can trigger strong induction of both *rtTA* and target gene in response to Dox. To switch off the system, Dox will be simply removed from the system to silence mini-CMV promoters, leading to terminate the expression of both rtTA and the transgene.

PPM1B is a member of protein phosphatase 2C (PP2C) family and has been implicated in regulating stress signaling pathways, cell proliferation, embryonic development and differentiation (Lammers and Lavi, 2007; Park et al., 2011). Since we are interested in the physiological functions of PPM1B in cellular senescence pathway, lentiviral plasmids in this study were examined to see the inducible expression of *PPM1B* is achieved in response to Dox. Here, we describe the bi-directional lentiviral expression plasmids that display a great functionality in short-term transient gene expressions. However, lentiviral vector gene transfer using pseudoviral particles show a minimal, inconsistent expression of target gene, suggesting that rtTA cannot properly access the chromatin-embedded expression cassette in the host chromosome.

2. Material and methods

2.1. Cell culture

Human embryonic kidney 293T (HEK293T) cells were maintained in a 5% CO_2 incubator with growth medium composed of Dulbecco's Modified Eagel's Medium (DMEM), 10% fetal calf serum (FCS), 0.5% of penicillin (5000 U/ml) and streptomycin (500 μg/ml). Cultures were passaged in a subcultivation ratio of 1:8 – 1:12 every four days. Lentivirus packaging plasmid mix was purchased from System Biosciences and used to prepare lentivirus in HEK293T packaging cells. Virally transduced HEK293T cells were selected for at least 7 days against puromycin (1 μg/ml at final).

2.2. Construction of lentiviral vectors

To construct a bi-directional, bi-cistronic lentiviral vector, a gene expression cassette from the commercially available pTRE-Tight-BI (Clontech) was used to replace a CMV-driven expression cassette in the lentiviral plasmid, pCDH-CMV-MCS-EF1-puro (System Biosciences) (Fig. 1A). pCDH-CMV-MCS-EF1-puro was digested with ClaI and NotI to get rid of the original CMV promoter, followed by T4 DNA polymerase fill-in reaction to make blunt-ended DNA. A bi-directional expression cassette was amplified from pTRE-Tight-BI (Clontech) by primers containing restriction enzyme sites for BstBI and SwaI (Table 1). In the Tet-On regulatory system, rtTA responds to the presence of Dox and binds to tetracycline response element (TRE) to activate the transcription of flanked genes (Centlivre et al., 2010). The new lentiviral vector, pLent-TRE-Bi plasmid (Fig. 1B) carries puromycin resistance marker and is expected to show the same transgene expression features of pTRE-Tight-BI, including two mini-CMV promoters-driven bi-directional expressions in a Dox and rtTA dependent manner. Three inducible lentiviral vectors were constructed containing either *rtTA* alone or *rtTA* together with the *PPM1B* gene (pV-TRE-T7-rtTA, pPPM1Bwt-TRE-T7-rtTA and pPPM1Bmut-TRE-T7-rtTA). *PPM1B* wild type or mutant was first cloned downstream of one of the mini-CMV promoters using XbaI and HindIII (Fig. 1C). Subsequently, the cDNA of rtTA was PCR amplified from pTet-On plasmid (Clontech) with primers containing T7 tag sequence and restriction sites for XmaI and ClaI (Table 1). PCR-amplified T7 tagged rtTA (T7-rtTA) was cloned into