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Development of a tetracycline-inducible lentiviral vector with an instant regulatory system

A thesis presented in partial fulfillment of the requirements of the degree of
Master of Science (MSc)
in
Biochemistry

at Massey University, Manawatu,
New Zealand

Tian Yang

2013
Abstract

Lentiviral vectors, originally derived from human immunodeficiency virus, provide highly efficient viral gene delivery vehicles. Lentiviral vectors often use a constitutive promoter to drive the expression of a therapeutic gene. To regulate the expression of a therapeutic gene, a regulatory system such as Tet-On needs to be established in the target cell lines to produce a regulatory protein, reverse Tet-responsive transcriptional activator (rtTA). The expressed rtTA binds to the tetracycline responsive element (TRE) in the promoter in response to doxycycline and activates transcription of gene of interest. A hypothesis in this study is based on the speculation that a basal leaky expression of rtTA in the bi-directional TRE vectors allows instantly inducible expression of a gene of interest and thereby avoids the time-consuming procedures for generating Tet-On cell lines. Based on this hypothesis, a novel lentiviral vector has been developed to examine an instant induction of PP2Cβ as a target gene. Three instantly inducible bicistronic lentiviral vectors [pLenti-Bi-TRE-Tet-on (V), pLenti-Bi-TRE-Tet-on-PP2Cβ WT (WT), pLenti-Bi-TRE-Tet-on-PP2Cβ MUT (MUT)] were constructed and characterised to assess the usefulness of these vectors. Transient transfection of both WT and MUT vectors into HEK293T cells showed a great induction of PP2Cβ expression upon 24 h of 1 μM doxycycline treatment. The result promises the use of these vectors as a mammalian expression plasmid with a feature of inducible target gene expression. However, viral infection studies involving lentiviral packaging and infection procedures did not show a reproducible expression of rtTA or PP2Cβ in HEK293T cells. Therefore, the inducibility of viral transduction needs to be improved for the future studies of PP2Cβ in primary cells.
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Abbreviations

A absorbance
AD activation domain
APS ammonium persulfate
ATP adenosine triphosphate
attL Left integration attachment site
attR Right integration attachment site
b path length
bp base pair
BAD Bcl-2-associated death promoter
BSA bovine serum albumin
CA capsid
CAK CKD-activating kinase
cDNA complementary deoxyribonucleic acid
CDKs cyclin-depend protein kinase
CFU colony-formation unit
CIP calf intestinal alkaline phosphatase
CMV cytomegalovirus
Cp/Ct crossing point
CFU colony-formation unit
DBD DNA binding domain
DMEM Dulbecco’s Modified Eagle medium
DMSO dimethyl sulphoxide
DNase deoxyribonuclease
dNTP deoxyribonucleotide triphosphate
Dox doxycycline
DTT dithiothreitol
ε extinction coefficient
EDTA ethylene diamine tetra-acetic acid
FBS fetal bovine serum
FCS fetal calf serum
GFP green fluorescent protein
GPCR G protein-coupled receptor
GUSB beta glucuronidase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>HEK 293T</td>
<td>human embryonic kidney 293T cell line</td>
</tr>
<tr>
<td>hES</td>
<td>human embryonic stem cell</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
</tr>
<tr>
<td>IN</td>
<td>integrase</td>
</tr>
<tr>
<td>IkB</td>
<td>NFKB inhibitory binding partner</td>
</tr>
<tr>
<td>IKK</td>
<td>IkB kinase</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>JUK</td>
<td>Jun N-terminal kinase</td>
</tr>
<tr>
<td>kb</td>
<td>kilo base</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>LV</td>
<td>Lentiviral vector</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MA</td>
<td>membrane associated matrix</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCS</td>
<td>multiple cloning site</td>
</tr>
<tr>
<td>MEKK</td>
<td>MAP kinase kinase kinase</td>
</tr>
<tr>
<td>MLK</td>
<td>mixed lineage kinase</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MKK</td>
<td>MAPK kinase</td>
</tr>
<tr>
<td>MKKK</td>
<td>MKK kinase</td>
</tr>
<tr>
<td>MTK1</td>
<td>MAP three kinase 1</td>
</tr>
<tr>
<td>MUT</td>
<td>vector containing mutant PP2Cβ</td>
</tr>
<tr>
<td>NC</td>
<td>nuclear capsid</td>
</tr>
<tr>
<td>NCBI</td>
<td>national centre for biotechnology information</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>nM</td>
<td>nanomolar</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>penicillin-streptomycin</td>
</tr>
<tr>
<td>PIC</td>
<td>pre-integration complex</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>PP2Cβ</td>
<td>protein phosphatase type 2C isoform beta</td>
</tr>
<tr>
<td>PPMs</td>
<td>protein phosphatase magnesium-dependent enzymes</td>
</tr>
<tr>
<td>PPM1B</td>
<td>PP2Cβ</td>
</tr>
<tr>
<td>PPPs</td>
<td>phospho-protein phosphatases</td>
</tr>
<tr>
<td>PR</td>
<td>protease</td>
</tr>
<tr>
<td>PTPs</td>
<td>protein tyrosine phosphatases</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride transfer membrane</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative real-time PCR</td>
</tr>
<tr>
<td>RCR</td>
<td>replication competent recombinant</td>
</tr>
<tr>
<td>RCV</td>
<td>replication competent virus</td>
</tr>
<tr>
<td>RE</td>
<td>restriction endonuclease</td>
</tr>
<tr>
<td>RHD</td>
<td>Rel homology domain</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RIN</td>
<td>RNA integrity number</td>
</tr>
<tr>
<td>RRE</td>
<td>Rev-responsive element</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription/reverse transcriptase</td>
</tr>
<tr>
<td>RT-</td>
<td>reverse transcriptase free reaction</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>reverse transcription quantitative real-time PCR</td>
</tr>
<tr>
<td>rTetR</td>
<td>Reverse TetR</td>
</tr>
<tr>
<td>rtTA</td>
<td>tetracycline-responsive transcriptional activator</td>
</tr>
<tr>
<td>SAPKs</td>
<td>stress signaling pathways</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SIN</td>
<td>self-inactivating vector</td>
</tr>
<tr>
<td>SU</td>
<td>Surface/envelop glycoprotein</td>
</tr>
<tr>
<td>TAK1</td>
<td>TGFβ-activated kinase 1</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween 20</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N’,N’-tetramethylethylenediamine</td>
</tr>
<tr>
<td>tetO</td>
<td>tet operator</td>
</tr>
<tr>
<td>TetR</td>
<td>Tet repressor protein</td>
</tr>
<tr>
<td>TGFα</td>
<td>Transfomring growth factor α</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TM</td>
<td>Trans membrane protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Tm</td>
<td>melting temperature</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TRE</td>
<td>tetracycline-response element</td>
</tr>
<tr>
<td>TREmod</td>
<td>modified TRE-response element</td>
</tr>
<tr>
<td>tTA</td>
<td>tetracycline-controlled transactivator</td>
</tr>
<tr>
<td>U</td>
<td>units</td>
</tr>
<tr>
<td>μM</td>
<td>micromolar</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>vector only/no PP2Cβ control vector</td>
</tr>
<tr>
<td>VSV-G</td>
<td>vesicular stomatitis virus G glycoprotein</td>
</tr>
<tr>
<td>WT</td>
<td>vector containing wild type PP2Cβ</td>
</tr>
<tr>
<td>ψ</td>
<td>packaging signal</td>
</tr>
</tbody>
</table>
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Chapter 1: Introduction

1.1 Generation of a new vector system

Vector delivery systems are commonly used as gene transfer vehicles in gene therapy, which provide promising therapeutic treatment to revert disease phenotypes. An ideal vector system for gene therapy should be bio-safe, non-toxic, non-immunogenic, tightly regulated, efficient and stable in gene expression [2]. In addition, an efficient delivery system must easily penetrate into target cells, tissues or organs, and have strict regulatory systems in place to allow precise control of gene expression [2].

A number of vector systems including viral and non-viral vectors have been used to deliver genes of interest into target cells while providing efficient and robust expression of the transgenes. Viral vectors are considered superior to non-viral vectors because they offer broader tropism, higher transduction efficacy, greater cloning capacity, and more efficient penetration into target cells and their nuclei [3]. Most importantly, viral vectors have the ability to integrate into the host genome rather than remaining episomal, allowing long-term stable expression [4, 5]. Due to safety concerns, however, viral vectors without most of the virus-derived coding sequences have been used in gene therapy. This helps to minimize any adverse immune responses by preventing the recognition and destruction of transduced cells by cytotoxic T lymphocytes [5].

1.1.1 Lentiviral systems

Lentiviral vectors are retroviruses that are derived from human immunodeficiency virus (HIV). Pseudotyping, a process that produces viral vectors in combination with foreign viral envelope proteins, with the vesicular stomatitis virus G glycoprotein (VSV-G) allowed a broad transduction tropism of lentiviruses [6]. A unique characteristic of LV vectors is their ability to replicate in non-dividing cells via a nucleoprotein complex, called pre-integration complex (PIC), allowing the association of viral genetic material to their host genome [3, 6-10]. This feature made lentiviral vectors more appealing to researchers.
1.1.1.1 Basic biology and evolution of lentiviral vector

Generally, lentiviruses consist of two copies of RNA, a capsid (CA), a nuclear capsid (NC), a membrane associated matrix (MA), and various envelope proteins and enzymes (Table 1.1 and Figure 1.1). The major envelope proteins include surface/envelope glycoproteins and trans-membrane proteins (TM); while the major envelope enzymes include integrase (IN), protease (PR) and reverse transcriptase (RT). The coding region of gag, encodes the CA, NC and MA proteins while the pol and env genes are critical for pro-virus integration and polyadenylation, respectively. The gag, pol and env regions are further flanked by long terminal repeats (LTRs) and cis-acting sequences [3]. The two regulatory genes, tat and rev, regulate HIV gene expression transcriptionally and post-transcriptionally and are critical for viral replication. Lentiviruses also carry four accessory genes nef, vif, vpu, vpr which are dispensable for viral growth but have been shown to be required for in vivo replication and pathogenesis [3, 7].

<table>
<thead>
<tr>
<th>Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capsid (CA)</td>
<td>gag gene; protect the core</td>
</tr>
<tr>
<td>Nuclear Capsid (NC)</td>
<td>gag gene; protect the genome and formation of the core</td>
</tr>
<tr>
<td>Membrane associated matrix (MA)</td>
<td>gag gene; lines envelope</td>
</tr>
<tr>
<td>Surface/envelope glycoprotein (SU)</td>
<td>env gene; Polyadenylation</td>
</tr>
<tr>
<td>Tans membrane protein (TM)</td>
<td>The inner component of the mature envelope</td>
</tr>
<tr>
<td>Integrase (IN)</td>
<td>pol gene; integration of the provirus</td>
</tr>
<tr>
<td>Protease (PR)</td>
<td>Essential for gag protein cleavage during maturation</td>
</tr>
<tr>
<td>Reverse transcriptase (RT)</td>
<td>Reverse transcribes the RNA genome</td>
</tr>
<tr>
<td>Accessory protein for HIV</td>
<td>nef, vif, vpu, vpr; in vivo replication and pathogenesis</td>
</tr>
</tbody>
</table>

*Table is obtained from Virginia Commonwealth University (VCU) website [11].
Figure 1.1: A schematic representation of HIV-1-derived vector structure. Nine of HIV genes: gag, pol, tat, rev, two regulatory genes tat and rev, and four accessory genes nef, vif, vpu, vpr. Both long terminal repeat (LTR) includes U3, R and U5 regions. att, integrase attachment site; SD, splice donor; ψ, packaging signal; ppt, polypurine tract (Figure adapted and modified from [12], permission for publishing obtained through RightsLink®)

The complexity of the lentiviral genome has been exploited by scientists for years to build a novel vector with appropriate biosafety to minimize reconstitution of the wild-type virus by recombination. To avoid the emergence of replication competent recombinants (RCR), many precautions were taken, for example, the genes required for virus packaging and replication are now expressed by two separate plasmids [5, 6].

1.1.1.2 Packaging process

The construction of a lentiviral gene delivery system requires two major components: (i) a vector genome containing the gene of interest for transfer (Figure 1.2a), and (ii) a packaging helper genome, also called a packaging plasmid, which provides necessary enzymes and protein structures to generate infectious viral particles (Figure 1.2b) [5, 7]. For biosafety concerns, the helper virus genome is devoid of the packaging signal (ψ) preventing repackaging of the virus. Upon co-transfection, the vector and packaging plasmid are introduced into a packaging cell line, commonly human embryonic kidney (HEK) 293T cells. Here, the packaging plasmid undergoes transcription and translation to produce viral structural and replication proteins (Figure 1.2c) while the vector genome is transcribed into a single-stranded RNA genome that is recognized by the packaging signal (ψ) (Figure 1.2d). Once virions are packaged and assembled inside of the packaging cell line (Figure 1.2e), they are released as viral particles containing viral vector (Figure 1.2f).
**Figure 1.2: Virus packing system.** Harboured within the helper viral DNA (b) are viral genes encoding for proteins necessary for viral genome replication (a) The therapeutic gene expression cassette, flanked by the inverted terminal repeats (ITR) and cis-elements which is required for genome encapsidation is contained within the vector genome. a: vector genome; b: helper virus genome; c: transcription and translation of virus structural and replication proteins; d: vector genome replicates into single stranded RNA genome; e: virions package and assembly; f: viral vectors release out of packaging cells (Figure adapted from [2], permission for publishing obtained through RightsLink®).

### 1.1.1.3 Development of lentiviral vectors

The first generation of HIV-derived vectors comprised of all HIV proteins (Table 1.1) apart from the envelope protein [5, 7]. Although the viral particles were produced in a separate packaging plasmid, the high content of HIV proteins could risk re-constitution of the wild-type virus. To alleviate this, second generation vectors contained only the key components gag, pol, tat and rev. Later studies suggested that tat is also dispensable in the packaging construct, by replacing the tat-dependent U3 sequence from the 5' LTR with strong heterologous promoter sequences to hinder the transcription of vector genomic RNA in producer cells, therefore, promotes a tat-independent primary transcription without affecting the efficiency of the vector generation [5, 7]. Optimal
production of viral vector requires the presence of rev which encodes a post-transcriptional regulator, Rev-responsive element (RRE), that strictly regulates the expression of gag and pol [7, 13]. It has been shown that vectors containing Rev/RRE systems exhibit the highest yield in production [6]. The third generation of HIV-derived vectors was further stripped down to only three core HIV-1 genes, gag, pol and rev. In addition, the original viral genome has been further split to express rev from a separate construct [14]. It has been demonstrated that a split genome dramatically reduces the emergence of RCR, eliminates targeted infection of CD4+ T-lymphocytes and extends cell tropism allowing safer and broader application of therapeutic interventions [3, 6]. This third-generation vector system significantly prevents the risk of parental virus regeneration, since 60% of the genome has been completely eliminated [13].

To further improve these vector systems, the two terminal repeat (LTR) sequences have been removed to form self-inactivating (SIN) vectors. Removal of these sequences abolishes the generation of replication competent viruses (RCV). The LTR sequences are transcriptional elements containing enhancer and promoter sequences, the left integration attachment site (attL) in U3 at 3’ LTR, the right integration attachment site (attR) in U5 at 5’ LTR, polyadenylation signal in R, part of the polyadenylation signal, and Tat-interacting TAR sequence overlapping the R region (Figure 1.1) [12, 15]. SIN vectors are achieved by further deletion of viral enhancer and promoter sequences, in order to eliminate the transcriptional capacity of the viral LTR in integrated target cells, which prevents the mobilization of RCV [3, 12, 14, 16, 17].

In addition, it has been shown that Tat trans activation requires an intact TATA box; mutations in the TATA box dramatically reduce viral titers [15, 18]. In contrast, Tat-independent transcription, achieved by replacing the U3 region of 5’ LTR with the cytomegalovirus (CMV) promoter, did not affect viral titer production [12, 16]. Other modifications, for example, U3 deletion and U5 substitution of the 3’ LTR also exhibited a competitive transduction efficiency compared to the wild type construct [15, 18]. The cis-acting element guides polyadenylation of viral genomic RNA distal to the TATA box, which resides upstream of the R region in LTR (Figure 1.1); however, polyadenylation on the cis-acting element may lead to a decrease in efficiency [5]. Together this evidence suggests that HIV-1 derived vectors may tolerate a large or complete U3 deletion without functional loss; however, the efficiency may be reduced.
to some extent. These modifications of the LV vector design provided additional safety features to the lentiviral vector system.

### 1.1.1.4 Advantages, limitations and concerns of lentiviral vectors

Lentiviral vectors are known to facilitate high-level transduction, broad tropism and long-term expression in a variety of cells and tissues. As one of the gene delivery systems in gene therapy, the superior advantage of lentiviral vectors is their ability to infect non-dividing cells without delivering lethal viral coding sequences. This prevents the recognition and destruction of transduced cells by cytotoxic T-lymphocytes \[5\]. In addition, several advancements have effectively made general safety concerns redundant. These include incorporating pseudotyping with envelope glycoproteins, elimination and reassembly in multiple attenuated split genome packaging systems as well as the development of the third generation and SIN lentiviral vectors \[3, 6, 9, 19\].

Despite a lentiviral vector gene delivery system being an effective tool in gene therapy, there are still some limitations. For instance, lentiviral vectors often lack convenient multiple cloning sites (MCS), increasing the difficulty of introducing transgenes into the system \[3\]. Although transgene expression can be readily controlled by administration of ligand of transcriptional regulators such as the antibiotic, doxycycline (Dox) in Tet-On/Off systems, regular or prolonged exposure may induce unwanted side effects such as drug resistance in clinical trials \[2\]. It has also been shown that the relatively safe SIN vectors which contain deletions in the TATA box, displayed a lower viral titer and were impaired in induced penetration of the target tissue and organ resulting in reduced transduction efficiency \[5, 16\]. Another disadvantage of these systems is that the absence of the accessory protein vpr and vip abolishes in vivo infection in target tissues such as liver and muscle \[13\]. Thus, vectors used for targeting these tissues could potentially increase the cytotoxicity and the generation of RCRs. In addition, untoward immune effects of the transcriptional activator proteins are also a major concern in lentiviral systems \[2, 20, 21\].

A more recent lentiviral system has been improved by incorporating regulatory switches to tightly regulate transgene expression (see Section 1.1.2). These switches are able to induce transgene expression to increase the transduction efficiency, and therefore, the
requirement of the viral vector dose is reduced for enhanced utility and biosafety of gene transfer vectors [2]. A concern still remains however, that these regulatory systems can be leaky resulting in toxicity and inefficient induction.

1.1.2 Tetracycline regulatory system

Regulatory systems of lentiviral vectors have been developed to control the expression of transgenes in vivo and in vitro. Successful gene therapy not only requires an efficient delivery vehicle, but also needs a stringent and reliable regulatory system. An ideal pharmacologically-regulated system in human gene therapy should: (1) contain an On/Off switch to allow tight regulation, (2) be activated by a highly specific orally administered small molecule that does not interfere with endogenous metabolic pathways, (3) have low basal expression and high inducibility, (4) have low immunogenicity and high bio-safety, and (5) be dose-dependent and orally bio-available [17, 22]. There are a number of regulatory systems available, including the most widely used tetracycline-controlled system.

The tetracycline-controlled system can be activated by the antibiotic tetracycline and its derivatives such as doxycycline (Dox), which is a second generation semisynthetic analog of tetracycline. Dox was developed in 1967 and has since been successfully used in clinical treatments for more than 40 years [23-26]. Dox offers several advantages over tetracycline including (1) a much easier dose schedule compared to tetracycline; (2) a reasonably long half-life; (3) being more readily absorbed when taken with food avoiding gastrointestinal upset, and (4) is generally well tolerated in the body with fewer side effects [27]. Additionally, the high lipid solubility of Dox promotes better distribution and tissue penetration compared to tetracycline, allowing potentially improved penetration of the blood-brain barrier [23, 25, 27].

Besides these clinical applications, Dox is also widely used in research applications as an inducer in tetracycline-inducible gene regulation systems, especially in the context of viral vectors. In Escherichia coli, the Tet repressor protein (TetR) negatively regulates the genes of the tetracycline-resistance operon on the Tn 10 transposon. In the absence of tetracycline/Dox, TetR dimerises and binds to tet operator (tetO) DNA sequences consequently blocking the transcription of this operon [24, 28, 29]. However, in the
presence of tetracycline/Dox, a conformational change in TetR leads to disassociation from tetO rescuing the transcriptional blockade [28]. The sequence-specific and high-affinity binding between TetR and tetO also provides the basis of regulation and induction for mammalian experimental systems.

The natural Tet-controlled DNA binding domain (DBD) of E.coli TetR is fused to the Herpes simplex virus VP16 heterologous transcriptional activation domain (AD) to form a hybrid protein, tetracycline-controlled transactivator (tTA). In the absence of Dox, the fusion of VP16 AD converts the TetR from a transcriptional repressor to a transcriptional activator activating expression of the transgene (Figure 1.3A). The reverse TetR (rTetR) was developed by four amino acid changes in TetR. In the presence of Dox, rTetR binds to tetO to form the reverse tetracycline-responsive transcriptional activator (rtTA) which activates transcription of the gene of interest (Figure 1.3B) [28-31]. Regulatory proteins tTA and rtTA are encoded by pTet-Off and pTet-On regulator plasmid (Clontech), respectively, both of which contain a neomycin-resistance gene for selection.

The second critical component of the Tet systems is the response plasmid, which expresses a gene of interest under the control of the tetracycline-response element (TRE). The latest version of the pTRE-tight plasmid contains a modified TRE response element (TREmod) that consists of seven direct repeats containing tetO [31, 32]. In the pTRE-tight plasmid, TREmod resides upstream of an altered minimal cytomegalovirus (CMV) promoter ($P_{\text{minCMV}}$) (Figure 1.3). In contrast to the complete CMV promoter, this minimal CMV promoter lacks the enhancer sequence allowing a reduction of the basal expression of the gene of interest. A Dox-dependent gene expression system with these two components, tTA and rtTA, is expected to display tightly controlled expression of the gene of interest. In the Tet-Off system, Dox represses expression (Figure 1.3A) while in the Tet-On system, Dox activates expression of the target gene (Figure 1.3B).
Figure 1.3: Schematic diagram of the gene regulation in the Tet-Off and Tet-On systems.

The tetracycline response element (TRE) is located upstream of the minimal immediate early promoter of CMV (P_{minCMV}). **A. Tet-Off:** transcription of gene of interest remains active in the absence of Dox through tetracycline-concilled transactivator (tTA) binding to the TRE. Addition of Dox results in conformational changes in tTA and, therefore, disassociates tTA from the promoter to block gene transcription. **B. Tet-On:** transcriptional activator tetracycline-responsive transcriptional activator (rtTA) has a reverse response to Dox due to four amino acid changes compared to rTA. Transcription remains inactive without Dox. In the presence of Dox, rtTA undergoes conformational change and binds to TRE to activate gene transcription (Figure adapted from Clontech Laboratories user manual [33]).
Both Tet systems allow stringent regulation of the target gene in a precise and dose-dependent manner. Some minor drawbacks still remain however, for instance, since these systems are derived from bacteria, they may illicit undesirable immunogenic responses when utilized in humans [20, 21]. In addition, Dox has been demonstrated to accumulate in the bone and teeth [34], which may delay the silencing of the Tet-On and activation of the Tet-Off systems [17, 21]. Conversely, a major drawback of the Tet-Off system is that patients would face long-term exposure to Dox for inactivation of the system which may lead to drug resistance and insensitivity. The re-induction of this system relies on the complete clearance of the drug and, therefore, the efficiency and effectiveness of this system could also be a problem. On the other hand, the Tet-On system requires a reasonably large dose of Dox to achieve activation of the target gene expression. In addition, the residual affinity of rtTA to tetO in this system may have an elevated leaky background activity [29, 30, 35]. This also poses a problem in stringent regulation especially in humans. To alleviate this issue, a mutant rtTA2S-M2s has been developed which activates the Tet-On system at a 10-fold lower Dox concentration than that of wild type rtTA. The mutant rtTA2S-M2s has been shown to offer greater stability and zero background expression in the absence of Dox [30, 35].

1.1.3 Applications of lentiviral systems in primary and stem cells

Lentiviral systems are widely used in gene therapies and as a research tool, especially in primary cells and stem cells mostly using a constitutive gene expression system. One of the most common uses of lentiviral vectors is to silence genes using RNA interference (RNAi) [36, 37]. Lentiviral vectors can establish a stable genetic modification of human embryonic stem (hES) cells [38], retain sustained transgene expression in hES cells [39], and induce the generation of pluripotent stem cells [40]. It has been reported that a lentiviral vector governs the stable transduction of cells to induce the reversible immortalization of human primary cells regardless of their cycling status [41]. Furthermore, inducible Tet-On lentiviral systems have been demonstrated to be able to avoid down-regulation of the target gene in ex vivo gene transfer to the central nervous system [42]. An adverse effect also exists however, in the in vitro application of lentiviral vectors [43]. Therefore, development of a novel Tet-On lentiviral system with an instant induction feature is critically required to overcome several drawbacks that the
current lentiviral gene delivery system has been suffering in primary and stem cell applications.

1.2 Molecular ageing in association with Protein phosphatase type 2C isoform beta (PP2Cβ)

Decline in the functional abilities of organisms, organs, tissues and cells occur during the ageing phase of their life span. Cellular senescence is a terminally differentiated cellular state, characterised by exhaustion of proliferative potential and permanent cell cycle arrest in human diploid primary cells. This process is also known as replicative senescence or the Hayflick phenomenon [44]. Since ageing accompanies diverse and multiple levels of changes in gene expression, investigating the mechanisms controlling age-dependent gene expression is likely to enhance understanding of the ageing phenomena at the molecular level. Replicative senescence is known to be induced by multiple signaling pathways and their resultant gene expression, which are governed by senescence-associated transcriptional regulators. This current study aims to provide a tool to study the role of protein phosphatase PP2Cβ in relation to molecular ageing.

1.2.1 Protein phosphatase type 2C isoform beta (PP2Cβ)

In cellular signal transduction, protein phosphorylation is one of the most important mechanisms mediated by a balance between protein kinases and cognate protein phosphatases [45]. Based on their structural and functional diversity, protein phosphatases can be classified into three major families: (i) protein tyrosine phosphatases (PTPs) that dephosphorylate phosphotyrosine residues; (ii) phosphoprotein phosphatases (PPPs) containing a highly conserved catalytic domain and are further classified by distinct amino acid sequences, that dephosphorylate serine/threonine residues and are further divided into three distinct subtypes of phosphatases including type PP1, PP2A and PP2B in eukaryotes, and finally (iii) protein phosphatase magnesium-dependent (PPMs) enzymes that are characterised by their requirement of Mg$^{2+}$ or Mn$^{2+}$ ions for activity. PPMs mainly consist of pyruvate dehydrogenase phosphatase and PP2C, and dephosphorylate phosphoserine and phosphothreonine residues like PPPs [46-50].
Chapter 1: Introduction

PP2C belongs to the second family, PPPs, in eukaryotes, but shares little homology with other phosphatases PP1, PP2A and PP2B [51]. These three phosphatases share ~40% amino acid identity in their primary structure. In addition, they are sensitive to phosphatase inhibitors and are associated with their cognate regulatory subunits by forming heteroligomeric complexes [1, 52]. Unlike these three phosphatases, monomeric PP2C is a free catalytic protein that requires metal ions for activity. So far, no specific inhibitor of PP2C has been identified [1, 46, 48, 50]. In contrast, unsaturated fatty acids with special structural features like oleic acid are known to activate PP2C activity [48]. PP2C is able to dephosphorylate a broad spectrum of substrates involved in diverse cellular events and signaling pathways. There are at least 16 different PP2C genes encoding more than 20 distinct PP2C isozymes [45, 47, 53, 54]. Among those, the paralogs α and β were the first identified and have been studied in the greatest detail.

PP2Cβ has six distinct isoforms, β-1,-2,-3,-4,-5 and -X, which are produced from alternative splicing of a single pre-mRNA [46, 47, 55]. The amino acid sequences of these isoforms differ only at the C-terminal ends, suggesting that substrate specificity is determined by the C-terminus [47, 49, 56]. PP2Cβ is mostly localised in the cytoplasm however, isoforms β-1 and β-2 are also known to be present in the nucleus [45]. Expression in mice revealed that isoforms β-1, β-2 and β-X are expressed ubiquitously, and β-3,-4, and -5 are exclusively expressed in intestine, adult testis and liver cells [45-47].

The primary sequence of PP2Cβ shares 77% identity and 87% similarity to its paralog PP2Cα in mammalian cells. These proteins, PP2Cα and β share similar roles, for instance, they are involved in suppressing the stress activated protein kinase pathway. Their functions however, appear to be dependent on tissue type and stage of cell development [46, 49].

1.2.2 Known functions of PP2Cβ

Members of the PP2C family are known to lack regulatory subunits due to their monomeric structure [1, 46, 48, 50]. In living systems, the physiological concentration of divalent cations, such as Mg\textsuperscript{2+} and Mn\textsuperscript{2+}, which are required for PP2C function, remain constant and are difficult to manipulate hence the cation-dependent functionality
of PP2C proteins has been challenging to assess [1, 46, 47, 50]. PP2C phosphatases are known to play a role in cell proliferation, differentiation, and apoptosis and stress signaling pathways [1, 48, 52, 55, 57]. Overexpression of PP2Cβ in HEK 293T cells leads to cell-growth arrest or cell death [49]. Early reports suggested that the PP2Cβ mRNA level is increased during the first wave of spermatogenesis in pachytene spermatocytes and more highly differentiated germ cells, implying differentiation dependent regulation of PP2Cβ [58]. Later on, the same group confirmed that tissue-specific expression of PP2Cβ uses alternative promoters within the PP2Cβ gene to direct its expression [46]. Findings of later knock down studies, suggest that PP2Cβ may play a role in gametogenesis, fertilization and the early stages of embryonic development [47]. Although PP2Cβ is also believed to have critical roles in a number of pathways, very little is known about the molecular mechanisms behind PP2Cβ regulation.

1.2.2.1 Regulation of stress signaling pathways (SAPKs)

PP2Cβ is known to be involved in the regulation of the stress-activated protein kinases (SAPKs) pathways. SAPKs are a subfamily of the mitogen-activated protein kinase (MAPK) superfamily which is highly conserved from yeast to humans and respond to SAPKs stimuli including extracellular stress and inflammatory cytokines. There are two prominent classes of SAPKs in mammalian cells, C-Jun amino-terminal kinases (JNKs) and p38 kinases, which are activated by extracellular stresses through phosphorylation of conserved tyrosine and threonine residues in the catalytic domain by protein kinases belonging to the family of MAPK kinases (MKK) (Figure 1.4) [51, 54, 55]. JNK is predominantly phosphorylated by the MKK4 and MKK7 protein kinases while p38 is specifically phosphorylated by the protein kinases MKK3 and MKK6 [51, 55]. Interestingly, MKK4 can also phosphorylate p38. The MKKs are further activated by phosphorylation by MKK kinases (MKKK) which can directly sense extracellular stress signals. There are numerous MKK kinases (MKKK) such as apoptosis signal-regulating kinase 1 (ASK1), MAPK kinase kinase (MEKK) group (MEKK1, 2, 3, 4), MAP three kinase 1 (MTK1), mixed lineage kinase (MLK) family, transforming growth factor β (TGFβ)-activated kinase 1 (TAK1) [45, 51, 55].
Upon a stress signal, MKK kinases (MKKK) activate MKKs which subsequently activate JNK or p38, followed by activation of transcriptional factors c-Jun or ATF2, thus, gene expression is changed to allow survival under stress conditions [45, 51, 54, 55]. PP2Cβ1 expression has been shown to suppress anisomycin- and NaCl- enhanced phosphorylation of p38, as well as the phosphorylation of MKK3b and MKK6b [55]. Overexpression of PP2Cβ1 reduces the basal and stress-enhanced phosphorylation of MKK4 and MKK7 in the JNK pathway [47, 55, 59, 60], suggesting that PP2Cβ is a negative regulator in SAPK pathways.

Figure 1.4: Regulation of stress-activated protein kinase (SAPK) signal pathways by PP2Cβ1. The protein kinase cascade of SAPK signaling pathways is shown, PP2Cβ1 interferes with MKKKs to regulate SAPK pathway. ASK1, apoptosis signal-regulating kinase1; ATF2, activating transcription factor; JUK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase kinase; MKK, motigen-activated protein kinase kinase; MKKK, mitogen-activated protein kinase kinase kinase; MLK, mixed lineage kinase; MTK1, MAP three kinase 1; PP2Cβ, protein serine/threonine phosphatase 2C isoform beta; TAK1, transforming growth factor β (TGFβ)-activated kinase 1 (Figure is modified from Tamura et al., 2006 [54], permission for publishing obtained through Wiley).

Transforming growth factor β (TGFβ)-activated kinase 1 (TAK1) was originally identified as a MKKK in the TGFβ signaling pathway. Previously, TAK1 was found to activate JNK and p38 pathways by phosphorylating MKK. More recent studies demonstrated that a phosphatase-negative mutant PP2Cβ1 acts as a dominant negative mutant to inhibit dephosphorylation of TAK1 by wild type PP2Cβ1, suggesting that the
expression of wild type intact PP2Cβ1 dephosphorylates and inactivates the TAK1 signal, preventing an efficient stress response [44, 47, 51]. These studies strongly support the concept that PP2Cβ functions as a negative regulator in SAPKs pathways.

1.2.2.2 Implication of PP2Cβ in regulation of cell division

In eukaryotes, cell cycle progression relies on the periodic activation and inactivation of cyclin-dependent protein kinases (CDKs) [52]. Activation of CDK is controlled by extracellular and intracellular signals, such as associated regulatory proteins (cyclins, inhibitors, and assembly factors), reversible protein phosphorylation, transcriptional regulation, selective proteolysis, and subcellular localization [61]. Full activation of CDK for normal cell cycle progression requires the recruitment of a cyclin, followed by the removal of inhibitory phosphate groups by Wee1-like protein kinases, and addition of activating phosphates in a region of the CDK called the “T-loop” by CDK-activating kinases (CAKs) [52, 62]. PP2Cβ2 and recombinant PP2Cβ2 have been shown to remove T-loop phosphates on CDK2 and CDK6 [45, 52]. Furthermore, it has been demonstrated that PP2C-like enzymes predominantly dephosphorylate human CDK2 in HeLa cell extracts suggesting that PP2Cβ is likely to be an important inhibitor in cell cycle progression [62].

1.2.2.3 Involvement in the regulation of apoptosis

Phosphorylation and dephosphorylation of proteins are essential to ensure proper cellular development and function, and has important implications in controlling cell apoptosis. For instance, phosphorylation of Bcl-2-associated death promoter (BAD) by 14-3-3 proteins keeps BAD in the cytosol and abolishes BAD-mediated pro-apoptotic responses. In contrast, dephosphorylation of BAD by PP2Cα and PP2Cβ induces cell death by heterodimerization with the anti-apoptotic oncogenes Bcl-2 and Bcl-XL at the mitochondrial membrane [53, 63]. BAD belongs to the pro-apoptotic protein family of Bcl-2 gene and when de-phosphorylated interacts with anti-apoptotic molecules Bcl-2 and Bcl-XL to promote apoptosis [53, 63, 64]. PP2Cβ dephosphorylates serine 155 in BAD which is critical for its interaction with Bcl-XL in the mitochondrial matrix, suggesting that PP2Cβ has an important role in the regulation of mitochondrial apoptosis [63, 64]. It has been demonstrated that unsaturated fatty acids with special
structural features, such as oleic acid, activate PP2Cβ-dependent apoptosis in neuronal and endothelial cells [48, 53].

1.2.2.4 Involvement of PP2Cβ in molecular ageing through p53 pathway and NFκB signaling pathway

The pivotal tumour suppressor p53 is mainly regulated by its negative regulator Mdm2 through ubiquitination and subsequent proteasomal degradation [57, 65, 66]. In response to DNA damage, the interaction between p53 and Mdm2 is disrupted and the level of p53 is dramatically increased. Overexpression of PP2Cα, a close paralog to PP2Cβ, has been shown to enhance the stability of p53 resulting in higher expression of the p53 downstream target gene, p21, which is a key player in G2/M cell cycle arrest and apoptosis [57, 67]. PP2Cβ has also been proposed to increase the stability and activity of p53 by down-regulating Mdm2 [45, 57, 66], however, the mechanism of this is unclear. Recently, Park et al. have demonstrated that PP2Cβ associated with the brain cancer-amplified gene GAS41, which is a negative regulator of p53 tumour suppressor [65, 66]. The GAS41-PP2Cβ complex was shown to be required for dephosphorylation of p53 at serine 366 [66]. These studies suggest that PP2Cβ appears to be involved in both positive and negative regulation of the p53 pathway.

Nuclear factor kappa B (NFκB) belongs to a family of dimeric transcription factors containing a Rel homology domain (RHD) which are involved in numerous cellular pathways from cellular stress response to apoptosis and immunity [68-71]. NFκB is activated by a variety of stimuli including cytokines, toll-like receptors (TLRs), antigen receptors, G protein-coupled receptors (GPCRs), and growth factors [26, 72, 73]. In the absence of stimuli, NFκB is sequestered in the cytoplasm in an inactive form by its inhibitory binding partner, IκB. Upon stimulation, IκB is phosphorylated, ubiquitinated and eventually degraded by the proteasome, freeing NFκB and allowing it to translocate into the nucleus to activate its target genes [1, 70]. Cytokines such as tumor necrosis factor (TNFα), interleukin-1 (IL-1β) and transforming growth factors (TGFα) activate the phosphorylation of IκB by IκB kinases (IKKs) [1, 70]. The IKKs form complexes consisting of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IK γ /NEMO [70]. PP2Cβ is known to associate with the IKK complex upon cytokine stimulation. It has also been shown that PP2Cβ association with IKKβ, decreases IKKβ
kinase activity therefore terminating IKKβ-induced NFκB activation pathway [1]. This suggests that PP2Cβ down regulates cytokine-induced NFκB activation by altering IKK activity. A recent study confirmed that overexpression of PP2Cβ resulted in dephosphorylation of IKKβ while knocking down PP2Cβ expression promotes cytokine-induced NFκB activation by enhancing IKKβ phosphorylation [70]. In context with the multi-function of NFκB in cell survival, apoptosis and cellular stress signaling, PP2Cβ seems to play a role as an inhibitor of cellular progression and cellular stress signaling pathways.

NFκB is also known as a master regulator of gene expression programs associated with mammalian ageing [74, 75]. It is an ultimate effector that senses multiple age-associated signals from oxidative stress, DNA damage, cell survival and inflammation [71]. Constitutive activation of NFκB induces ageing phenotypes among various cell lines and animal models in which NFκB-dependent gene expression contributes deleterious effects of age-associated diseases such as induced insulin resistance and neurotoxicity in Alzheimer's disease [74, 76-78]. Blocking NFκB activity in aged cells has been shown to reverse many of these phenotypes by inhibiting age-associated gene expression [74]. PP2Cβ is known to play a role as a negative regulator of IKKβ terminating IKKβ-induced NFκB activation [1, 70]. PP2Cβ is a common regulator of NFκB and the p53 pathways which are likely to exhibit “cross-talk” with each other to coordinate precise control of gene expression in aged cells. Given a common negative regulatory role of PP2Cβ on p53 and NFκB transcription factors, it is conceivable that PP2Cβ would be significantly down-regulated in ageing process.

1.3 Background to this project

The original design of the tetracycline regulatory system consists of two plasmids. One plasmid expresses rtTA constitutively, and the other contains the gene of interest. This binary system has been combined into an autoregulatory plasmid which contains rtTA and the transgene [28, 31, 32]. This current study employed a bi-directional pTRE-Tight-BI lentiviral plasmid, coupled with the Tet-On system (Figure 1.5). This promoter consists of two mini CMV promoters flanking the TREmod element, in which rtTA and PP2Cβ, are under the control of their respective mini CMV promoters (Figure 1.5 a). In theory, almost no background levels of rtTA can be detected in this system without Dox
induction. To activate the mini CMV promoters, Dox is added to the system which forms a complex with rtTA (Figure 1.5 b). This Dox-rtTA complex binds to the TRE element activating the mini CMV promoters and the expression of the downstream genes, rtTA and PP2Cβ (Figure 1.5 c). As more rtTA is produced in the presence of Dox, more Dox-rtTA complexes form which bind the TRE element, further inducing the expression of PP2Cβ. To switch off this system, Dox is removed to silence both mini CMV promoters and terminate the expression of PP2Cβ (Figure 1.5 d).

Figure 1.5: Activation of bi-directional lentiviral vector of PP2Cβ. a. Tetracycline response element (TRE) flanked with two mini CMV promoter, reverse tetracycline response transcriptional activator (rtTA) and the gene of interest PP2Cβ reside downstream of each mini CMV promoter. b. Addition of Dox to the system allows its binding to rtTA in a basal expression level to form an activator complex, Dox-rtTA. c. Binding of Dox-rtTA complex to TRE activates both mini CMV promoters and, therefore enhances the transcription of its downstream gene, PP2Cβ and rtTA, respectively. d. Removal of Dox from the system leads to disassociation of Dox-rtTA complex, thus, blocks the transcription activation.

This positive feedback regulatory promoter in the lentiviral system is thought to be superior to a normal constitutive promoter in a number of aspects, including higher viral titer production, undetectable levels of rtTA and the transgene in the Off state, improved induction kinetics, and repeatable induction of the target gene expression with multiple
cycles of Dox exposure [28, 32]. Most importantly, the induction of the transgene can be achieved within a single cassette using a bi-directional autoregulatory vector. Compared to the binary system in general Tet-On systems, this autoregulatory system has been shown to significantly reduce the process of establishing homogeneously transduced populations to prevent unwanted cytotoxicity and immunogenicity and thus preventing its utilization in vivo [31, 32]. An early study reported that the autoregulatory Tet-Off system could produce a possible adverse effect at cellular levels due to higher VP16 levels. After 48 h of rTA induction, cells have been observed to increase in size with a significant (over 50%) reduction in growth rate [31, 32]. Therefore, the expression of transcriptional regulators should be tightly regulated as well to minimize cellular toxicity.

1.4 Project outline

This study was undertaken to investigate the involvement of PP2Cβ in NFκB pathway-mediated cellular ageing by utilizing a Tet-on induced lentiviral system. PP2Cβ is implicated in a number of cell processes including, cell proliferation, differentiation, senescence and apoptosis. Importantly, recent studies revealed a chronological down-regulation of PP2Cβ during cellular senescence of human diploid fibroblast, suggesting that PP2Cβ plays a key role in cellular ageing process (unpublished result, Dr. Jeong Park).

This research aims to construct and verify the Dox-inducible lentiviral vectors for the stable expression of PP2Cβ [pLenti-Bi-TRE-Tet-On (V), pLenti-Bi-TRE-Tet-On-PP2Cβ WT (WT) and pLenti-Bi-TRE-Tet-On-PP2Cβ MUT (MUT)] in immortalized cancer cells. These viral vectors will be used to determine the role of PP2Cβ in cellular senescence and regulation of NFκB activity in primary human diploid cells in the future.

1.4.1 Hypothesis

A basal expression of rtTA in the bi-directional TRE vectors allows instantly inducible expression of PP2Cβ without establishing a Tet-On cell line.
1.4.2 Research aim

The first aim of this study was to construct viral vectors for tetracycline inducible PP2Cβ (WT, MUT).

Objectives:
- Clone a T7-tag reverse tetracycline responsive transactivator (rtTA) into an empty lentiviral vector (V) or lentiviral vectors expressing PP2Cβ (WT) and PP2Cβ (MUT), respectively.
- Confirm instant inducibility of lentiviral vectors in HEK 293T cells by transient transfection upon Dox treatment.

The second aim was to examine PP2Cβ expression levels in lentiviral infected HEK 293T cells.

Objectives:
- Produce lentiviral stocks for vector only (V), PP2Cβ (WT) and PP2Cβ (MUT) in co-packaging cells.
- Examine the protein level of PP2Cβ in infected HEK 293T cells in the presence and absence of Dox.
- Compare PP2Cβ expression levels of WT and MUT using western blot and real-time quantitative PCR analyses.


## Chapter 2: Materials and Methods

### 2.1 Materials

#### 2.1.1 General

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#### 2.1.2 Cloning

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Clal, EcoRI, XmaI
### Chapter 2: Materials and Methods

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#### 2.1.3 Cell culture and lentiviral preparation

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<td>Caffeine, Crystal violet dye, Doxycycline hyclate, Puromycin, Polybrene</td>
<td>Sigma-Aldrich, Missouri, USA</td>
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<tr>
<td>Dulbecco’s Modified Eagel Medium (DMEM), OPTIMEM, Penicillin/streptomycin (Pen/Strep), Trypsin/EDTA</td>
<td>Invitrogen, CA, USA</td>
</tr>
<tr>
<td>Fetal Bovine Serum (FBS), Fetal Calf Serum (FCS)</td>
<td>SAFC Biosciences, Hampshire, UK</td>
</tr>
<tr>
<td>FuGENE HD transfection reagent, X-tremeGENE HP transfection reagent</td>
<td>Roche, Auckland, NZ</td>
</tr>
<tr>
<td>Microtube 2 mL (Cryovials)</td>
<td>SARSTEDT, Nümbrecht, Germany</td>
</tr>
<tr>
<td>Minisart Syringe filter, hydrophilic, 0.45 µm</td>
<td>Sartorius stedim Biotech, Gottingen, Germany</td>
</tr>
<tr>
<td>Plasmocin</td>
<td>InvivoGen, CA, USA</td>
</tr>
</tbody>
</table>

#### 2.1.4 RNA extraction

<table>
<thead>
<tr>
<th>Materials</th>
<th>Manufacturer/ Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>Merck, Darmstadt, Germany</td>
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<tr>
<td>DNase, RNase free water</td>
<td>Roche, Auckland, NZ</td>
</tr>
<tr>
<td>Trizol® LS reagent</td>
<td>Invitrogen, California, USA</td>
</tr>
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### 2.1.5 DNA extraction

<table>
<thead>
<tr>
<th>Materials</th>
<th>Manufacturer/ Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phusion DNA polymerase and Phusion HF reaction buffer</td>
<td>Thermo Scientific, Waltham, USA</td>
</tr>
<tr>
<td>dNTP</td>
<td>Takara Bio, Shiga, Japan</td>
</tr>
<tr>
<td>Veriti™ 96 well Thermal Cycler</td>
<td>Applied Biosystems</td>
</tr>
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</table>

### 2.1.6 Protein extraction and western blotting

<table>
<thead>
<tr>
<th>Materials</th>
<th>Manufacturer/ Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% Acrylamide/bis-acrylamide solution 19:1, Bradford reagent, Mini PROTEAN® 3 gel casting system, Minigel apparatus, Mini Trans-Blot® filter paper</td>
<td>BioRad Laboratories, California, USA</td>
</tr>
<tr>
<td>Ammonium persulfate (APS), Dithiothreitol (DTT), N,N,N',N'-Tetramethylethylenediamine (TEMED), NP-40, Phenylmethanesulfonyl fluoride (PMSF), Sodium azide, Sodium dodecyl sulphate (SDS), Triton X-100, Tween-20</td>
<td>Sigma-Aldrich, Missouri, USA</td>
</tr>
<tr>
<td>Biomas general blue film, Biomas maximum sensitivity film, Biomas MS intensifying screen cassette 8” × 10”</td>
<td>Kodak, NY, USA</td>
</tr>
<tr>
<td>Microplate reader</td>
<td>BioTek, Vermont, USA</td>
</tr>
<tr>
<td>Goat Anti-Mouse IgG-HRP</td>
<td>Invitrogen, California, USA</td>
</tr>
<tr>
<td>PageRuler™ prestained protein ladder</td>
<td>Fermentas, Burlington, Canada</td>
</tr>
<tr>
<td>Pierce® ECL Western Blotting Substrate, SuperSignal® West Femto Maximum Sensitivity Substrate</td>
<td>Thermo Scientific, Waltham, USA</td>
</tr>
<tr>
<td>Polyvinylidene fluoride (PVDF) membrane</td>
<td>GE Healthcare, Wisconsin, USA</td>
</tr>
</tbody>
</table>

### 2.1.7 Reverse transcriptase quantitative real-time PCR

<table>
<thead>
<tr>
<th>Materials</th>
<th>Manufacturer/ Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>LightCycler® 480 SYBR Green I Master, LightCycler® 480 instrument</td>
<td>Roche, Auckland, NZ</td>
</tr>
<tr>
<td>SuperScript® Platinum® Taq Mix</td>
<td>Invitrogen, California, USA</td>
</tr>
<tr>
<td>Frame Star 480 Q-PCR plate (96-well)</td>
<td>4titude®, Ockley, UK</td>
</tr>
</tbody>
</table>
2.2 Methods

2.2.1 Cloning

The cDNA of reverse tet-responsive transcriptional activator (rtTA) was first amplified from the pTet-On plasmid (Clontech) using conventional PCR amplification (Section 2.2.5.1). In the Tet-on system [28-31], rtTA binds to the tet-responsive element (TRE) and activates the transcription in the presence of doxycycline. The rtTA PCR product was sub-cloned into three bi-directional TRE-inducible lentiviral vectors (pLenti-Bi-TRE, pLenti-Bi-TRE-PP2Cβ WT and pLenti-Bi-TRE-PP2Cβ MUT; previously constructed by Dr. Jeong Park) to control the expression of PP2Cβ in response to Dox.

2.2.1.1 Restriction endonuclease digestion

Three bi-directional plasmids and the rtTA PCR product were digested with two restriction endonucleases. Most digestions were carried out overnight at 37°C. Reaction mixtures consisted of 2 µg of DNA, 0.5-1 µL of each restriction endonuclease (10 U/µL), 2 µL of 10 × bovine serum albumin (BSA, 1mg/mL) and 2 µL 10 × recommended buffer, in a total volume of 20 µL. Reaction mixtures were analysed by agarose gel electrophoresis (Section 2.2.5.2) and the desired DNA fragments were excised from the gel, followed by gel purification and PCR clean-up for subsequent cloning.

2.2.1.2 Calf intestinal alkaline phosphatase (CIP) treatment

In order to reduce self-ligation of the vector, plasmids were treated by incubation with 0.5 µL CIP (10 U/µL) at 37°C for 1 h after restriction endonuclease digestion.

2.2.1.3 Ligation

Ligation was performed using T4 DNA ligase (Invitrogen). A vector:insert molar ratio of 1:3 was used to set up each ligation. Reactions were performed in a total volume of 10 µL containing 0.5 µL ligase (400 U/µL) and 1 µL of 10 × ligase reaction buffer (500 mM Tris-HCl, 100 mM MgCl2, 100 mM dithiothreitol, 10 mM ATP, pH 7.5/25°C). The ligation mixture was incubated at 16°C or room temperature overnight.
2.2.1.4 Transformation

Transformation was carried out using *E. coli* DH5α competent cells (in 80 mM CaCl₂, 10% glycerol). Two µL of ligation reaction mixture was added into 50 µL of competent cells and incubated for 30 minutes on ice, then heat-shocked at 42°C for 1 minute followed by 2-3 minutes incubation on ice. Two hundred µL of sterile LB broth was added to the transformation mixture, and incubated at 37°C for 1 h with constant shaking. The transformation mixture was transferred onto LB agar plate supplemented with ampicillin (50 µg/mL). Plates was incubated at 37°C overnight. These procedures were carried out using the aseptic technique.

2.2.1.5 Inoculation

Single colonies were picked into LB supplemented with ampicillin (50 µg/mL), 3 mL of LB for small scale or 200 mL of LB for medium scale plasmid extraction, and incubated at 37°C overnight with constant shaking for plasmid preparation.

2.2.1.6 Manual small scale plasmid preparation

Manual small scale plasmid preparation [79] is an economic and efficient way to check successful sub-cloning immediately after inoculation. In general, 1.5 mL of *E.coli* culture was pelleted by centrifugation at 12,000 rpm for 1 minute. The cell pellet was resuspended in 350 µL of STET buffer (0.1M NaCl, 10 mM Tris-Cl, 1 mM EDTA, pH 8) and 25 µL of freshly prepared lysozyme (10 mg/mL in sterile water), then boiled for 40 seconds followed by immediate centrifugation at 12,000 rpm for 10 minutes. The pellet was removed using a toothpick and discarded. Four hundred µL of isopropanol was added to the supernatant and incubated at -20°C for 20-30 minutes to precipitate the DNA. Centrifugation at 12,000 rpm for 5 minutes was performed and the supernatant was removed. Another 500 µL of cold 95% ethanol was added to wash the pellet followed by centrifugation at 12,000 rpm for 1 minute, and the supernatant was removed. The pellet was air dried at room temperature and resuspended in 50 µL of TE (10 mM Tris, pH 8.0, 1 mM EDTA) containing 0.1 mg/mL of RNase A. Purified plasmids were then digested with restriction endonucleases and electrophoresed to identify recombinant plasmids.
2.2.1.7 Medium scale plasmid preparation

Once a *E. coli* colony was confirmed to have the plasmid of interest by manual plasmid preparation, LB supplemented with ampicillin (50 µg/mL) was inoculated with the appropriate clone overnight at 37°C with constant shaking. Overnight cultures were centrifuged at 2,600 g at 4°C for 20 minutes to pellet the plasmid DNA. Medium scale plasmid preparation was carried out using PureLink™ HiPure Plasmid Midiprep Kit according to manufacturer’s instructions. Plasmid DNA was eluted with 800 µL of sterile TE.

2.2.1.8 Polyethylene glycol (PEG) purification

PEG purification was performed after medium scale plasmid preparation to increase DNA purity. NaCl (5M) and 300 µL of PEG solution (30% PEG 8000) were added to the plasmid DNA, and incubated on ice for 30 minutes followed by centrifugation at 16,000 × g at 4°C for 15 minutes. The supernatant was discarded and the pellet was spun with additional 1 minute at 16,000 × g followed by removal of residual liquid. Five hundred µL of 70% ethanol was added to the pellet and centrifuged at 16,000 × g for 5 minute. The supernatant was aspirated in aseptic hood, the pellet was air dried for 10 minutes then resuspended in 200 µL sterile TE buffer. Concentration of purified plasmid DNA was determined using Nanodrop® ND-1000 spectrophotometer (Section 2.2.5.4).

2.2.2 Cell culture

The human embryonic kidney (HEK) 293T cell line was cultured in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% v/v fetal calf serum (FCS) or fetal bovine serum (FBS), 0.5% v/v penicillin (5000 U/mL) and streptomycin (500 µg/mL) (Pen/Strep). Virally transduced HEK 293T cells were cultured in supplemented DMEM containing 1 µg/mL of puromycin. Addition of plasmocin (5-10 µg/mL) and modification of the medium contents were also applied to some procedures. All cells were cultured at 37°C with 5% CO₂ in a humidified atmosphere. All tissue culture procedures were carried out in an ESCO Class II Biohazard safety cabinet.
2.2.2.1 Starting cell line

Frozen stocks (1 mL) were thawed and transferred into 5 mL of supplemented DMEM pre-warmed to 37°C. The mixture was centrifuged at 200 × g for 5 minutes to pellet the cells. The cell pellet was gently resuspended in 2 mL DMEM and seeded into a 6 cm plate in a total volume of 4 mL.

2.2.2.2 Cells passaging

After growth medium was removed from the plate, the monolayer was washed once with 1×PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.46 mM KH₂PO₄, pH 7.4, 10 mL/10 cm plate), and 0.5×Trypsin/EDTA (1 mL/10 cm plate) was applied to the monolayer, swirled and immediately removed. The plate was left to stand for 2-5 minutes at room temperature to allow sufficient trypsinisation. The side of the plate was tapped to ensure complete dislodgement. Cells were resuspended in supplemented DMEM (10 mL/10 cm plate) and an aliquot of 1 mL cell suspension were transferred to a fresh 10 cm plate or 6 cm plate containing ~9 mL or ~3 mL of growth medium, respectively.

2.2.2.3 Medium change

In cases where a cell culture had not reached ~60% confluency over 3 days of passage, medium was removed by aspiration and replaced with fresh growth medium.

2.2.2.4 Freezing cells

Cells at approximately 80% confluence after the first passage were used for freezing stocks. Cells were dislodged as described in Section 2.2.2.2, resuspended in 5 mL of growth medium and transferred into 15 mL centrifuge tube for 5 minutes centrifugation at 1000 rpm. The cell pellet was then resuspended in ~2 mL/3 mL freezing solution (10% DMSO, 20% FBS and 70% DMEM) for one 6 cm/10 cm plate, then dispensed 1 mL aliquots into two sterile cryovials. Cryovials were well wrapped with tissue paper to allow slow freezing in the -80 °C freezer.
2.2.2.5 Transient transfection

X-tremeGENE HP DNA Transfection Reagent (Roche) was used to carry out transient transfection reactions. HEK 293T cells were seeded onto 6 cm plate and incubated until they reached ~60% confluency. In a sterile microcentrifuge tube, pre-warmed OPTI-MEM (200 µL/6 cm plate) was dispensed with 1 µg of DNA and 3 µL of transfection reagent, briefly tapped and incubated for 15 minutes at room temperature to allow formation of a transfection complex. Transfection mixture was then transferred onto designated 6 cm plate.

2.2.2.6 Viral transduction and puromycin selection

Lentivirus stocks were prepared as described in Section 2.2.3. HEK 293T cells were seeded onto 24 well plate (approximately $1 \times 10^4$ cells per well with 0.4-0.5 mL growth medium) and allowed to grow for 24 h. Growth medium was replaced with 400 µL of lentivirus transduction mixture (Table 2.1) and 0.1% v/v of polybrene (8mg/mL) was added to increase the efficiency of viral transduction. After 24 h of transduction, mixture was replaced with puromycin-containing medium to allow selection for successfully transduced cells. Once ~80% confluency was reached under puromycin selection, cells were ready to be harvested or treated for downstream experiments.

Table 2.1: Component of lentivirus transduction mixture for each vector.

<table>
<thead>
<tr>
<th>Lentivirus</th>
<th>Virus Titer* (µL)</th>
<th>Supplemented DMEM (µL)</th>
<th>Polybrene (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLenti-Bi-TRE-Tet-on</td>
<td>100</td>
<td>300</td>
<td>0.4</td>
</tr>
<tr>
<td>pLenti-Bi-TRE-Tet-on-PP2Cβ WT</td>
<td>100</td>
<td>300</td>
<td>0.4</td>
</tr>
<tr>
<td>pLenti-Bi-TRE-Tet-on-PP2Cβ MUT</td>
<td>100</td>
<td>300</td>
<td>0.4</td>
</tr>
</tbody>
</table>

* Viral titer is expressed as a volume of a viral titer, eg. 100 µL of XX cfu/mL.

2.2.2.7 Dox induction of TRE-controlled constructs

Doxycycline induction was performed on > 80% confluent monolayers by adding 1 µM Dox (1 mg/mL) to the medium. In general, Dox treatment was performed 24 h post-
transfection, 24-72 h post-transduction or upon reaching ~ 80% confluency for stable cell lines derived from lentiviral infection. Dox was, unless otherwise stated, removed from the medium 24 h post-transfection.

### 2.2.3 Lentiviral preparation and optimisation

Lentiviral preparation and optimisation was carried out in an ESCO Class II Biohazard safety cabinet using sterile solutions and reagents. HEK 293T cells were used as a producer packaging cell line.

#### 2.2.3.1 Packaging

**Preparation:** One day before co-transfection, a 10 cm plate of HEK 293T cells was prepared for each lentiviral vector using 10 mL of DMEM medium supplemented with 10% FBS per plate. Cells were seeded to a final density of $1.5 \times 10^6$ cells per plate (approximately 1:8 split), and incubated at 37°C with 5% CO₂ in a humidified atmosphere.

**Co-transfection:** After 24 h incubation, cells at ~80% confluency were co-transfected with the desired plasmid DNA and packaging mix (provided by Dr. Jeong Park) using FuGENE® HD Transfection Reagent (Roche). In a sterile microfuge tube, pre-warmed OPTI-MEM (500 µL/10 cm plate) was dispensed with 5 µg of desired plasmid DNA, 3 µg of packaging plasmid mix and 24 µL of transfection reagent, briefly tapped and incubated for 15 minutes at room temperature to allow formation of transfection complex. Transfection mixture was then transferred onto designated 10 cm plate, and incubated for 17 h at 37°C with 5% CO₂ in a humidified atmosphere.

**Medium change:** The initial medium was replaced with 10 mL viral harvest medium containing DMEM supplemented with 10% FBS, 1% Pen/Strep and 10 µg/mL of plasmocin (25 mg/mL). Caffeine was added to the medium to a final concentration of 1 mM to increase viral titer [80]. Viral harvest medium supplemented with caffeine was incubated for 24 h at 37°C with 5% CO₂ in a humidified atmosphere for collection.
Collection: Viral harvest medium containing viral particles was collected into a 50 mL falcon tube every 24 h for three days. Each plate was refilled with 10 mL of fresh viral harvest medium after each collection for further harvest.

2.2.3.2 Concentration

The harvested viral soup was centrifuged at 3000 × g for 5 minutes, then its supernatant was removed and filtered through a 0.45 µm filter. Six mL of 50% PEG 6000 and 3 mL of 4 M NaCl (final 0.3 M) were added to the sterile viral solution. The mixture was thoroughly mixed and stored at 4°C for 1.5 h followed by centrifugation at 7000 × g for 10 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 1.1 mL of DMEM, followed by incubation at 4°C overnight. This overnight stock was gently mixed by a pipette, and aliquots of 200 µL were placed into sterile cryovials for storage at -80°C freezer. The residual stock was stored at 4°C for titration.

2.2.3.3 Titration

Two 6-well plates of HEK 293T cells were prepared at a density of 1 × 10^5 cells per well. Ten-fold serial dilutions with 3 mL of growth medium and 8 µg/mL of polybrene according to Table 2.2 were prepared for three viral stocks. Cells were incubated at 37°C with 5% CO₂ in a humidified atmosphere. After 24 h of incubation, initial growth medium was replaced with 2 mL virus containing medium in each well according to Table 2.2, and incubated for another 24 h. Lentivirus containing medium was replaced with 2 µg/mL puromycin selection medium to assess successful viral transduction. For selection, 2 µg/mL of puromycin selection medium was replaced every two days for the first week and reduced to 1 µg/mL of puromycin to replace every two days for another two weeks. After a total of three week growth, cells were stained with 1 mL 0.5% crystal violet solution in 20% ethanol and incubated for 30 minutes at room temperature. Each well was washed twice with distilled water. The number of blue-stained colonies was counted in each well. The functional viral titer was calculated according to the formula: (#colonies/2mL medium) × dilution factor = cfu/mL viral stock.
### Table 2.2: Ten-fold serial dilutions of three viral stocks.

<table>
<thead>
<tr>
<th>Loading Positions</th>
<th>10-fold serial dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plate 1, well 1-3</td>
<td>Tet-on 1:1,000</td>
</tr>
<tr>
<td></td>
<td>WT 1:100</td>
</tr>
<tr>
<td></td>
<td>MUT 1:100</td>
</tr>
<tr>
<td>Plate 1, well 4-6</td>
<td>Tet-on 1:10,000</td>
</tr>
<tr>
<td></td>
<td>WT 1:1,000</td>
</tr>
<tr>
<td></td>
<td>MUT 1:1000</td>
</tr>
<tr>
<td>Plate 2, well 1-3</td>
<td>Tet-on 1:100,000</td>
</tr>
<tr>
<td></td>
<td>WT 1:10,000</td>
</tr>
<tr>
<td></td>
<td>MUT 1:10,000</td>
</tr>
<tr>
<td>Plate 2, well 4-6</td>
<td>Tet-on 1:1,100,000</td>
</tr>
<tr>
<td></td>
<td>WT 1:100,000</td>
</tr>
<tr>
<td></td>
<td>MUT 1:100,000</td>
</tr>
</tbody>
</table>

#### 2.2.4 RNA

**2.2.4.1 Phenol/chloroform extraction**

Human HEK 293T cells were grown in 6 cm or 10 cm plates. Growth medium was aspirated from each plate, and 1 mL of PBS was added for harvest. Cells were harvested in PBS with a rubber scraper. Trizol® LS reagent (Invitrogen) was used to extract RNA from a liquid source. A 250 μL aliquot of cell suspension was transferred to a sterile microcentrifuge tube with 10 μg of glycogen, and 750 μL of Trizol® LS reagent was added to the tube, mixed and incubated at room temperature for 5 minutes. After incubation, 200 μL of chloroform (Merck) was added into the tube followed by vigorous vortex for 15 seconds and incubated at room temperature for another 5 minutes. The tube was centrifuged at 12,000 \( \times \) g for 15 minutes at 4°C. The colourless upper aqueous layer was transferred into a new RNase-free microcentrifuge tubes and an equal volume of isopropyl alcohol was added for subsequent purification.

**2.2.4.2 RNA purification**

The tube containing RNA was centrifuged at 12,000 \( \times \) g for 10 minutes at 4°C. Supernatant was removed and the RNA pellet was washed once with 1 mL of 75% ethanol for 5 minutes followed by centrifugation at 12,000 \( \times \) g for 10 minutes at 4°C. Pellet was air-dried for 30 minute and dissolved in 50 μL of RNase free water.

**2.2.4.3 RNA quantification and quality assessment**

The amount of RNA was quantified from the absorbance at 260 nm measured using the Nanodrop® ND-1000 spectrophotometer. RNA purity was assessed by the OD\(_{260}\)/OD\(_{280}\)
ratio and the OD$_{260}$/OD$_{230}$ ratio generated by the Nanodrop® ND-1000 spectrophotometer. The RNA OD$_{260/280}$ ratio less than 2 indicates possible contamination by DNA, protein or phenol, because these molecules absorb electromagnetic radiation at a wavelength of around 280 nm. The OD$_{260/230}$ is a secondary measure of nucleic acid purity with uncontaminated RNA having an OD$_{260/230}$ greater than 1.8. A lower OD$_{260/230}$ ratio may indicate the presence of co-purified contaminants.

2.2.4.4 DNase treatment

Potential contamination by genomic DNA in the RNA sample was eliminated by DNase treatment. RNA samples were normalised to the lowest RNA concentration, and 45 μL of normalized RNA sample was transferred to 0.6 mL PCR tubes. One μL of DNase and 5 μL of 10 × DNase buffer were added, followed by incubation at 37°C for 30 minutes and then at 75°C for 5 minutes for DNase inactivation.

2.2.5 DNA

2.2.5.1 PCR

The cDNA of the rtTA insert was initially amplified from the pTet-On plasmid (Clontech) using conventional PCR. PCR amplification was performed using the Veriti™ 96 well Thermal Cycler (Applied Biosystems). A total of 50 μL of reaction cocktail contained 0.5 μL of Phusion DNA polymerase (2U/μL), 10 μL of 5 × Phusion HF reaction buffer (contains 7.5 mM MgCl$_2$), 5 μL of 10 × dNTP (2.5 mM), 2.5 μL of each forward (10 μM) and reverse (10 μM) primer, 10 ng/μL of pTet-On plasmid DNA template and distilled water. A negative control containing no DNA template was included.

The standard PCR amplification cycling steps were as follows:

- Enzyme activation: 95°C for 1 minutes
- Denaturation: 95°C for 15 seconds
- Annealing: 54°C for 30 seconds
- Extension: 72°C for 1 minutes

35 × cycles

- Final Extension: 72°C for 5 minutes
2.2.5.2 Agarose gel electrophoresis

PCR and digestion products were analysed by horizontal electrophoresis using 0.8% to 2% w/v agarose gels in 0.5 × TBE buffer (44.5 mM Tris-HCl, 44.5 mM boric acid, 1 mM EDTA). Samples were loaded into designated wells with the addition of 1 µL of 6 x DNA loading dye (0.25% Bromophenol blue, 50% w/v glycerol). Electrophoresis was carried out at ~120 V for 1 h, and the gel was thereafter immersed in ethidium bromide (0.5 μg/mL) staining solution for 12 minutes. After destaining in water for 2 minutes, bands were visualised under ultraviolet light on the Gel Doc™ system (BioRad) and the sizes of the PCR products were compared to the DirectLoad™ Wide Range DNA Marker (Sigma).

2.2.5.3 Gel extraction and PCR purification

Desired bands were excised from agarose gel, and purified using E.Z.N.A.® Gel Extraction Kit (Omega) according to manufacturer’s instructions. PCR and some digestion products were also purified using this kit. For vector size over 5000 bp, elution buffer were heated to 65°C prior to elution. A final volume of 30 µL of DNA was eluted.

2.2.5.4 DNA quantification

DNA was quantified by the absorbance at 260 nm measured with the Nanodrop® ND-1000 spectrophotometer. DNA purity was assessed by the OD$_{260}$/OD$_{280}$ and OD$_{260}$/OD$_{230}$ ratios.

2.2.6 Protein analysis

2.2.6.1 Protein extraction

Growth medium was aspirated from each plate (usually 6 cm plate), and 1 mL of PBS was added for harvest. Cells were harvested with a rubber scraper. The dislodged cells were transferred to a sterile microcentrifuge tube, and centrifuged at 3000 rpm for 5 minutes at 4°C. All traces of supernatant were aspirated after centrifugation. The pellet in each tube was resuspended in 100 µL of NP-40 lysis buffer (500 mM NaCl, 50 mM Tris pH 7.5, 0.2% NP-40, 10% glycerol) containing 1 mM of serine protease inhibitor.
phenylmethanesulfonyl fluoride (PMSF, 100 mM), and incubated on ice for 10 minutes followed by a briefly vortex. Protein lysate was centrifuged at full speed for 5 minutes at 4ºC. The supernatant representing the protein extract was transferred into a new sterile microcentrifuge tube for storage at -20ºC.

### 2.2.6.2 Cell fractionation for protein manipulation

Cells were harvested, as described in Section 2.2.6.1, and centrifuged at 3000 rpm for 5 minutes at 4ºC to pellet the cell. Cell pellet was gently resuspended in 0.5 mL buffer A (10 mM HEPES, pH7.5, 10 mM KCl, 1.5 mM MgCl₂) supplemented with 0.5 mM dithiothreitol (DTT) and 0.5 mM PMSF, and were immediately spun at 3000 rpm for 5 minutes at 4ºC. The swollen pellet was resuspended in 50 μL of buffer A. Another 50 μL of buffer A containing 1% NP-40 was added to the cell suspension, gently mixed by tapping and incubated on ice for 10 minutes. After incubation, the mixture was spun at 6,500 rpm for 10 minutes, and the supernatant was collected as a crude cytoplasmic fraction. The pellet was further submerged in 50 μL of F buffer (25 mM Tris, pH 7.5, 50 mM NaCl, 30mM Na₃P₂O₇, 50 mM NaF, 10% glycerol, 0.5% Triton X-100), vortexed for 1 minute and incubated on ice for 30 minutes. Another 1 minute vortex was performed followed by centrifugation at 16,000 for 10 × minutes. The supernatant was collected as nuclear fraction.

### 2.2.6.3 Bradford protein quantification

A protein standard curve from 0-1 mg/mL was prepared from Bovine Serum Albumin (BSA) stock (10 mg/mL) using Bradford reagent in 96-well plates. Each well contained 10 μL of pre-determined BSA standard or target protein sample and 200 μL of 1:5 diluted protein assay dye reagent concentrate (BioRad). All BSA samples and target protein samples were prepared in triplicate and loaded onto 96-well flat-bottom microplates. Samples were incubated at room temperature for 5 minutes to allow colour development, and their absorbance was measured at 595 nm using a spectrophotometer microplate reader (BioTek). The Gene5™ software was used for analysis and a standard curve was generated in Excel (Microsoft office 2010).
2.2.6.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Target protein samples were assessed using 8% or 10% SDS-PAGE gel electrophoresis. Mini PROTEAN® 3 gel casting system (BioRad, 1.5 mm-spaced) was filled with ~7.5 mL of 8% resolving gel, followed by ~2.5 mL of 4% stacking gel on top of the polymerised resolving gel with an appropriate comb. The components of resolving and stacking gel are listed in Table 2.3. Protein samples were prepared with 1 × SDS loading buffer (33 mM Tris pH6.8, 1.7% SDS, 5% glycerol, 0.05% w/v bromophenol blue, 0.7 M β-mercaptoethanol) and denatured for 3 minutes at 100ºC. Denatured samples and PageRuler™ prestained protein ladder (Thermo Scientific) were loaded into designated wells in a minigel apparatus (BioRad), and electrophoresed at a constant voltage of ~130 V for 1.5 h in protein running buffer (25 mM Tris, 200 mM glycine, 0.1% w/v SDS).

Table 2.3: Components of 8% and 10% SDS-PAGE gel.

<table>
<thead>
<tr>
<th>Components</th>
<th>Resolving Gel (10 mL)</th>
<th>Stacking Gel (4 mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8%</td>
<td>10%</td>
</tr>
<tr>
<td>4×Tris/SDS, pH 8.8</td>
<td>2.5 mL</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>4×Tris/SDS, pH 6.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>40% Acrylamide</td>
<td>2 mL</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>10% APS</td>
<td>100 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td>TEMED</td>
<td>20 µL</td>
<td>20 µL</td>
</tr>
</tbody>
</table>

2.2.6.5 Western blotting

SDS-PAGE gel was soaked in transfer buffer (2 M Tris, 192 mM glycine, 20 % v/v methanol) for at least 5 minutes and electro-transferred onto a positively charged PVDF membrane (pre-soaked in methanol) at a constant current of 150 mA for 2.5 h. The membrane was incubated in blocking buffer (5% w/v skim milk powder, 0.02% sodium azide, TBST: 10mM Tris pH7.6, 150 mM NaCl, 0.2% v/v Tween-20) for 1 h at room
temperature on an orbital shaker (Labnet). Membrane was briefly rinsed with TBST, and incubated with the primary antibodies overnight at 4°C on a shaker. Primary antibodies of target proteins were diluted 1:500 in TBST containing 0.04% blocking solution and 0.001% sodium azide. β-actin was used as a loading control with 1:1000 dilution containing 0.004% blocking solution and 0.001% sodium azide. Membrane was washed 3 × 15 minutes in TBST and incubated with HRP-conjugated secondary antibody (1:10,000 dilution) for 30 minutes at room temperature on a shaker, followed by 4 × 10 minutes wash steps in TBST. Membrane was developed using Pierce® ECL substrate (Thermo Scientific) according to the manufacturer’s instructions and immunoreactive bands were detected on medical X-ray films (Kodak) using an automated film processor according to the manufacturer’s instructions.

2.2.6.6 Membrane stripping and reprobing

Membranes were left wet in TBST prior to stripping. For membrane stripping, TBST buffer was removed and the membrane was incubated with stripping buffer (0.2 M Glycine, pH2.5-3, 0.05% Tween 20) in a 75°C water bath for 20 minutes. Membranes were washed 2-3 times for 10 minutes with TBST and blocked with blocking buffer as described in Section 2.2.6.5 for 30 minutes prior to reprobing.

2.2.7 Reverse transcriptase-coupled quantitative real-time PCR (RTqPCR)

Reverse transcriptase-coupled quantitative real-time PCR amplifications were performed on the Lightcycler® 480 real time PCR instrument (Roche) according to the manufacturer's instructions. The synthesis of first strand cDNA of rtTA and subsequent quantitative real-time PCR was conducted using one-step RT-qPCR analysis (Roche LightCycler® 480 SYBR Green Master I). Three oligonucleotide primer pairs were designed using the Roche LightCycler® Probe Design 2.0 software (Table 3.2). Oligonucleotide primers were received as dry powder and resuspended in distilled water to make a 100 μM stock and then diluted further to 10 μM working concentration for the assays. The specificity of the designed oligonucleotide primer pairs were tested by conventional PCR (Section 2.2.5.1) and visualized on agarose gel electrophoresis (Section 2.2.5.2).
### 2.2.7.1 Quantitative real time PCR

Quantitative real time PCR cocktails were made in a volume of 18 μL, containing 10 μL of 2 × LightCycler® 480 SYBR Green Master I, 0.2 μL of reverse transcriptase mix, 2 μL of 10 × forward and reverse primer mix (10 μM) and 5.8 μL of RNase free water. Two μL of RNA template was added to the SYBR Green master cocktail for PCR amplification. LightCycler® 480 SYBR Green Master I specific cycling conditions on the Lightcycler® 480 real time PCR system were performed according to the manufacturer's instructions (Table 2.4). First strand cDNA synthesis was performed prior to PCR amplification and forty cycles were used for amplification.

<table>
<thead>
<tr>
<th>Mode</th>
<th>Cycles</th>
<th>Target (°C)</th>
<th>Acquisition Mode</th>
<th>Hold (hh:mm:ss)</th>
<th>Ramp Rate (°C/s)</th>
<th>Acquisition (per °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA synthesis</td>
<td>1</td>
<td>50</td>
<td>None</td>
<td>0:10:00</td>
<td>4.4</td>
<td>-</td>
</tr>
<tr>
<td>Pre-incubation</td>
<td>1</td>
<td>95</td>
<td>None</td>
<td>0:05:00</td>
<td>4.4</td>
<td>-</td>
</tr>
<tr>
<td>Amplification</td>
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<td>95</td>
<td>None</td>
<td>0:00:10</td>
<td>4.4</td>
<td>-</td>
</tr>
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<td></td>
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<td>60</td>
<td>Single</td>
<td>0:00:30</td>
<td>2.2</td>
<td>-</td>
</tr>
<tr>
<td>Melting Curve</td>
<td>1</td>
<td>65</td>
<td>None</td>
<td>0:00:01</td>
<td>4.4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95</td>
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<td>-</td>
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</tr>
<tr>
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<td>40</td>
<td>None</td>
<td>0:00:30</td>
<td>2.2</td>
<td>-</td>
</tr>
</tbody>
</table>

### 2.2.7.2 Relative quantification and statistical analysis

Three reverse transcription repeats were carried out for each sample, and each sample was tested in triplicate. A standard curve with crossing point (X axis) versus Log concentration (Y axis, Log 2000, Log 200, Log 20) was constructed for both rtTA and GUSB (Appendix VI). Relative Log concentration of each sample was calculated from their standard curves and the formula shown in Appendix VII, which were constructed using Ct values generated from RT-qPCR reactions. Averages of relative Log concentration were calculated for each sample from their triplicate. Relative expression of each sample was calculated according to the formula \[ \text{Relative expression} = 10^{\text{Relative Log Conc.}}. \] A normalization ratio based on a reference gene, GUSB expression between
induced (Dox) and uninduced (No Dox) cells (Normalization ratio = Uninduced/Induced GUSB expression) was calculated, where the normalization ratio of uninduced cells was set to be 1. This normalization ratio for each sample was used to calculate relative target gene expression of rtTA. A relative fold difference was calculated from normalized expression levels of rtTA. Student’s t test was used to determine whether gene expression in induced cells was statistically different to that in uninduced cells.
Chapter 3: Results

3.1 Construction of inducible lentiviral vectors

3.1.1 Introduction

An efficient gene delivery vector should include two components: (1) a delivery plasmid containing a gene of interest, and (2) a regulatory system that manipulates expression of the gene of interest in a drug inducible manner. Lentiviral vectors are superior to other retroviral vectors in their ability to deliver a gene of interest into non-dividing cells as well as dividing cells. The main aim of this study was to construct three inducible lentiviral plasmids [pLenti-Bi-TRE-Tet-On (V), pLenti-Bi-TRE-Tet-On-PP2Cβ WT (WT) and pLenti-Bi-TRE-Tet-On-PP2Cβ MUT (MUT)] which place the gene of interest under the control of the tetracycline dependent system, Tet-On.

3.1.2 Cloning strategies

A two-step strategy was implemented to construct these plasmids. The cDNA of reverse tetracycline-responsive transcriptional activator (rtTA) was PCR amplified from pTet-On plasmid (Appendix II and Figure 3.1). In the Tet-On regulatory system, rtTA is sensitive to the presence of doxycycline and binds to tetracycline response element (TRE) to activate the transcription of adjacent genes [28-31]. Oligonucleotide primer sequences were designed and synthesized (Table 3.1), which incorporate the restriction endonuclease (RE) sites, XmaI and ClaI, located upstream and downstream of the cDNA of rtTA respectively, to enable insertion into bi-directional lentiviral vectors (Appendix III). A T7 tag was appended in the forward primer sequence to facilitate easy detection of rtTA protein expression. Once the rtTA fragment was amplified from the pTet-On plasmid, the next step was to sub-clone this T7-tag containing rtTA fragment individually into each bi-directional lentiviral vector [pLenti-Bi-TRE, pLenti-Bi-TRE-PP2Cβ WT and pLenti-Bi-TRE-PP2Cβ MUT] through XmaI and ClaI sites (Figure 3.1). PP2Cβ MUT construct differs from WT construct by a single base pair substitution at R179G resulting in mutation of arginine to glycine (Appendix V) [1]. Three pLenti-Bi plasmids [pLenti-Bi-TRE, pLenti-Bi-TRE-PP2Cβ WT and pLenti-Bi-TRE-PP2Cβ MUT] were linearized by double-digestion with XmaI and ClaI at their multiple cloning sites (MSC) (Left, Figure 3.1). The amplified rtTA fragment, containing XmaI and ClaI site,
was digested with these enzymes to allow insertion into pLenti-Bi plasmids (Right, Figure 3.1). Construction of V, WT and MUT was attempted by ligation of *rtTA* with the pLenti-Bi plasmid at *Xmal* and *ClaI* sites.

**Figure 3.1: Cloning strategy.** Bi-directional pLenti-Bi-TRE-PP2Cβ plasmid were linearized using *Xmal* and *ClaI* (Left). The cDNA of the tetracycline-responsive transcriptional activator (*rtTA*) fragment was amplified with a T7-tag-containing primer pair (Table 3.1) using PCR from pTet-on plasmid (Right). This *rtTA* fragment was then inserted into each pLenti-Bi plasmid by ligating at *Xmal* and *ClaI* sites.
Table 3.1: Primers used for rtTA fragment amplification.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5' → 3')*</th>
<th>Tm (°C)</th>
<th>Appended Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>TetOn_Xma_T7_F</td>
<td>GAC CCC GGG CAC CAT GGC TAG CAT GAC TGG TGG ACA GCA AAT GGG TAT GTC TAG ATT AGA TAA AGG T</td>
<td>71.0</td>
<td>XmaI site, T7 tag</td>
</tr>
<tr>
<td>TetOn_Cla_R</td>
<td>TCC TCG ATC GAT CTA CCC ACC GTA CTC GTC AAT</td>
<td>64.9</td>
<td>ClaI site</td>
</tr>
</tbody>
</table>

*T7-tag: blue, restriction sites: red, random end sequences: underlined.

Figure 3.2: Screening of rtTA in V and MUT constructs using 1% agarose gel electrophoresis. Electrophoresis was carried out for 1 h at 120 V in 0.5 × TBE buffer and stained with 0.5 µg/mL ethidium bromide. M: DNA molecular weight marker. Lane 1-5: XmaI and ClaI digestion of candidate plasmid DNA isolated from colonies transformed with ligation reaction for the construction of V plasmid. Lane 6-18: XmaI and ClaI digestion of candidate plasmid DNA isolated from colonies transformed from MUT construct ligation reaction.

3.1.3 Construction of pLenti-Bi-TRE-Tet-On-PP2Cβ MUT (MUT)

The ligation reactions for pLenti-Bi plasmids [pLenti-Bi-TRE, pLenti-Bi-TRE-PP2Cβ WT and pLenti-Bi-TRE-PP2Cβ MUT] were used for E. coli transformation and successful transformants were selected on LB/agar plates containing ampicillin (50 µg/mL). Five colonies containing V and thirteen colonies containing MUT plasmids
were screened by one-step double digestion with XmaI and Clal. Only one of the MUT colonies appeared to have an insert corresponding to the molecular weight of the rtTA fragment ~1000 bp (Lane 11, Figure 3.2). The size of rtTA in MUT construct (Lane 3, Figure 3.3) was identical to that of PCR amplified rtTA fragment (Compare lane 1 with lane 3, Figure 3.3). The sequence of rtTA, PP2Cβ and the R179G mutation in this MUT construct were confirmed by Sanger sequencing analysis, carried out by the Massey genome service. An additional XmaI restriction site was found at the C-terminal end of rtTA (Appendix IV and V), therefore, the observed size of rtTA fragment on agarose gel was ~900 bp rather than the full length ~1100 bp (Figure 3.3). The plasmid map and the corresponding sequence of rtTA and PP2Cβ are presented in Appendix IV and V. The PP2Cβ primer pair used for sequencing is illustrated in Appendix I.

Figure 3.3: Confirmation of rtTA containing constructs using 1% agarose gel electrophoresis. Electrophoresis was carried out for 1 h at 120 V in 0.5 × TBE buffer and stained with 0.5 µg/mL ethidium bromide. M: DNA molecular weight marker. Lane 1: positive control, rtTA fragment amplified from PCR and digested with XmaI. Lane 2, 4, 6: uncut V, WT, MUT constructs. Lane 3, 5, 7: V, WT, MUT constructs digested with XmaI. The size of rtTA from a restriction enzyme digestion of the candidate plasmid was consistent to that of amplified rtTA size ~1000 bp. The construction of V and WT was later performed by different strategy as described in Section 3.1.4.
3.1.4 Construction of pLenti-Bi-TRE-Tet-On (V) and pLenti-Bi-TRE-Tet-On-PP2Cβ WT (WT)

The second XmaI site within the cDNA of rtTA caused a major problem in the construction of V and WT plasmids. A unique restriction enzyme site other than XmaI site at the N-terminal was thought to solve this problem. However, no suitable endonuclease site was found to give a single cleavage in either the N-terminal end of rtTA fragment or pLenti-Bi plasmids. Therefore an alternative cloning strategy was developed to accomplish V and WT plasmid construction. A unique XhoI restriction site was found on the pLenti-Bi plasmids, which is located upstream of the PP2Cβ’s open reading frame (ORF). Most importantly, XhoI does not cut cDNA of rtTA. Therefore, the second cloning strategy employed XhoI and ClaI sites to cleave a larger fragment containing rtTA from MUT plasmid, instead of using PCR amplified rtTA from pTet-On plasmid, and insert this fragment into XhoI and ClaI digested pLenti-Bi V and pLenti-Bi WT plasmids. However, no rtTA-containing DNA fragment was produced from XhoI and ClaI digested MUT plasmid. To see if XhoI site really exists in the MUT plasmid, plasmid DNA was digested with XhoI and ClaI individually. Surprisingly, digestion with ClaI failed to produce a linearized plasmid DNA on agarose gel, possibly due to DNA methylation-mediated inhibition of ClaI endonuclease activity. Therefore, cleavage of rtTA from ClaI site was no longer feasible even though DNA sequencing analysis confirmed the ClaI site on the MUT plasmid. The restriction enzyme, EcoRV, which will cut adjacent to the 3’ end of ClaI site, was then selected to substitute ClaI site.

The second strategy was employed using XmaI, which has been allowed to cleave the 5’ end of rtTA for a limited time frame for a partial digestion. MUT plasmid was first digested with EcoRV for 1 hour, followed by partial digestions with XmaI (1:5, 1:10 and 1:20 dilution) under a restricted time frame. The resultant digestion mixtures were analysed on an agarose gel. Digestion with 1:5 diluted XmaI was found to produce a full length rtTA fragment at a size of ~1100 bp (a, Figure 3.4 and Appendix IV), simultaneously with a fully digested rtTA fragment at a size of ~980 bp (b, Figure 3.4 and Appendix IV). In addition, two plasmid bands (~10,000 bp) were also produced by incomplete digestion (Lane 2, Figure 3.4). Due to the high efficacy of XmaI, majority of the MUT construct underwent complete digestion. Therefore, a brighter band of smaller
size of rtTA band (b, Figure 3.4) was observed compared to the partial digested product (a, Figure 3.4). This upper rtTA band were then excised from a preparative agarose gel and ligated into digested pLenti-Bi V and WT plasmids. The correct size of rtTA in V and WT constructs were observed on an agarose gel after XmaI digestion (Lane 5 and 7, Figure 3.3). The sequences of rtTA and PP2Cβ in V and WT constructs were also confirmed by Sanger sequencing analysis that was performed by the Massey genome service. Full sequence of the WT plasmid is presented in Appendix V.

In summary, three bi-directional lentiviral plasmids (V, WT and MUT) were successfully constructed by inserting cDNA of rtTA derived from the pTet-On plasmid.

Figure 3.4: Partially digested MUT construct with XmaI and EcoRV to produce a full length rtTA fragment. One representative 1% agarose gel electrophoresis was carried for 1 h at 120 V in 0.5 x TBE buffer and stained with 0.5 µg/mL ethidium bromide. MUT construct was digested with EcoRV for an hour to linearize the construct (lane 1), followed by a partial digestion with diluted XmaI (1:5, 1:10 and 1:20) under a restricted time frame to produce: a: full length rtTA with a size of ~1100 bp; b: fully digested rtTA with a size of ~980 bp. Lane 1: MUT construct digested with EcoRV; lane 2-4: EcoRV-digested MUT construct further digested with diluted XmaI with a dilution of 1:5, 1:10 and 1:20, respectively.
3.2 Confirmation of inducibility of rtTA and PP2Cβ expression in V, WT and MUT vectors.

3.2.1 Introduction

Lentiviral infection requires the packaging from lentiviral vectors into lentiviral particles, followed by a selection process for successful infections. These processes were expected to take several weeks to complete. Therefore, before attempting infection in HEK 293T cells or aged cells, it was deemed necessary to verify that the rtTA and PP2Cβ are expressed in V, WT and MUT vector constructs, and the expression of these two genes only takes place when Dox is presented as an inducer in the cell. When Dox is added into the cell medium, it was hypothesized that minute leaky expression of rtTA in the cell would lead to the formation of an rtTA-Dox complex. This complex is then bound to TRE-element to activate adjacent mini CMV promoters, therefore enhancing the transcription of their downstream genes rtTA itself and PP2Cβ. Eventually, the elevation of rtTA results in a feed forward loop which simultaneously activates the transcription of rtTA as well as PP2Cβ. Expression of rtTA and PP2Cβ is expected to be maintained in the presence of Dox.

3.2.2 Confirmation of inducibility in transient transfected HEK 293T cells

V, WT or MUT vector constructs were independently transfected into HEK 293T cells, and after 24 h post-transfection, 1 µM of Dox was added for 24 h induction. A parental, pLenti-Bi vector was also transfected into HEK 293T cells as a negative control. Whole cell protein extracts of transfected HEK 293T cells were prepared. T7 tag is an 11 amino acid peptide encoded in the leader sequence of T7 bacteriophage gene 10, and serves as a tag for easy detection of rtTA production in immuno blotting. Upon Dox induction, V, WT and MUT constructs showed a clear induced expression of T7 tag (Lane 4, 6 and 8, Figure 3.5) compared to no Dox induction (Lane 3, 5 and 7, Figure 3.5). Consistent with the hypothesis proposed in Section 1.4.1, 24 h Dox induction increased the amount of rtTA which was correlated with increased PP2Cβ expression in the WT and MUT constructs (Lane 6 and 8, Figure 3.5). The size of PP2Cβ is around 60 kDa, however, a minor band below 60 kDa was also observed in Lane 6 and 8 in Figure 3.5. This extra band may be caused by PP2Cβ antibody which could recognize more than one PP2Cβ isoforms, as PP2Cβ has six distinct isoforms being produced by
alternative splicing of a single pre-mRNA [46, 47, 50]. However, HEK 293T cells were shown to produce alternative PP2Cβ isoforms at very low quantities. Alternatively, this extra band could result from degradation of PP2Cβ during sample preparation. In this experiment, the empty pLenti-Bi plasmid (V) which does not contain rtTA or PP2Cβ was used as a negative control. As expected, cells transfected with this vector did not show any expression of rtTA and PP2Cβ regardless of Dox induction (Lane 1 and 2, Figure 3.5). Overall, these results confirmed that V, WT and MUT constructs were able to express either rtTA alone or rtTA and PP2Cβ together. These results also demonstrated that the expression of rtTA and PP2Cβ was dependent on Dox in these vectors.

![Figure 3.5: Confirmation of Dox-dependent rtTA and PP2Cβ expression via transient transfection on the immunoblot.](image)

Western blot analyses were performed on 10% SDS-polyacrylamide gel electrophoresis for 1.5 h at 130 V and transferred for 2 h at 150 mA onto a PVDF membrane. Dox inductions were performed on >80% confluent HEK 293T monolayers for 24 h. Equal amount of HEK 293T protein extracts were prepared, and 100 µg of each sample were loaded on the gel. β-actin, a size of ~42 kDa, was included as a loading controls. Lane 1, 2: plenti-Bi, uninduced and induced; lane 3, 4: plenti-Bi-Tet-On (V), uninduced and induced; lane 5, 6: plenti-Bi-Tet-O-PP2Cβ WT (WT), uninduced and induced; lane 7, 8: plenti-Bi-Tet-O-PP2Cβ MUT (MUT), uninduced and induced.

### 3.2.3 Optimization of Doxycycline concentration

In order to achieve long term stable expression of the transgene under the control of the Tet-On system, the inducer molecule, Dox, must be administered regularly. However, regular administration of an antibiotic can lead to adverse side effects such as loss of
efficacy and drug resistance. Therefore, it was essential to determine the optimal amount of Dox (ideally lowest amount) and the best dose-response time that is required for induction, in order to reduce the possible adverse effects.

Figure 3.6: Determination of the minimum Dox concentration and dose-response time in HEK 293T cells transfected with MUT construct. Western blot analyses were performed on 10% SDS-polyacrylamide gel electrophoresis for 1.5 h at 130 V and transferred for 2 h at 150 mA onto a PVDF membrane. Dox inductions were performed on >80% confluent HEK 293 monolayers in a dose-response (left panel) or a 48 h time course (right panel). Equal amounts of HEK 293T protein extracts were prepared and 100 µg of each sample were loaded. β-actin was included as a loading controls, with a size of ~42 kDa. Lane 1 and 7: Non transfected HEK 293T cell extracts, negative control; lane 2 and 8: HEK 293T transfected with MUT construct, uninduced; lane 3-6: HEK 293T transfected with MUT construct, induced with 1 nM, 10 nM, 100 nM and 1 µM of Dox for 24 h, respectively; lane 9-12: HEK 293T transfected with MUT construct, induced by 1 µm of Dox for 6 h, 12 h, 24 h and 48 h, respectively.

A MUT construct containing both rtTA and PP2Cβ expression was selected for this set of optimization experiments. HEK 293T cells were transiently transfected with the MUT construct. The target gene expressions were examined according to either a dose response manner (Left panel, Figure 3.6) or a 48 h time-course response (Right panel, Figure 3.6), followed by western blot analyses. In the dose response experiment, transfected cells induced by 100 nM and 1 µM of Dox administration for 24 h showed a band in T7 tag western blot (Lane 5 and 6, Figure 3.6) but faint T7-rtTA bands could be detected by Dox lower than 100 nM (Lane 3 and 4, Figure 3.6). Based on signal intensity, a stronger rtTA T7-rtTA expression appeared to be induced by 1 µM of Dox treatment compared to treatment with 100 nM of Dox. Due to the endogenous expression of PP2Cβ, a basal level of PP2Cβ expression was observed in all lanes. However, Dox concentrations at 10 nM, 100 nM and 1 µM clearly induced PP2Cβ.
expression above the basal level (Lane 4, 5 and 6, Figure 3.6). Taken together, these results indicate that the optimal Dox concentration to induce the expression of rtTA and PP2Cβ in MUT was 1 µM. In the time-course experiment (Right panel, Figure 3.6), transfected HEK 293T cells were treated with 1 µM of Dox for a 48 h time course (6 h, 12 h, 24 h and 48 h). Induced T7-rT A expression was observed in all Dox induced cells (Lane 9-12, Figure 3.6). The induced expression of PP2Cβ could only be observed after 12 h of Dox (1 µM) treatment (Lane 10, 11 and 12, Figure 3.6). Cells treated with 1 µM of Dox for 24 h appeared to have the greatest induction in both T7 tag and PP2Cβ. These results indicate that the optimal dose-response time with 1 µM of Dox is 24 h. Thus, to minimise Dox dosage and maximise induction efficiency, 1 µM of Dox was administered for at least 24 h in subsequent experiments. These induction conditions were thought to be the same for V and WT vectors because they were constructed in a similar way as the MUT vector.

### 3.3 Inducibility of lentivirally infected HEK 293T cells

#### 3.3.1 Introduction

Since the inducibilities of rtTA and PP2Cβ in three vector constructs (V, WT and MUT) were confirmed in transient transfected HEK 293T cells using Dox treatment, the inducibilities of rtTA and PP2Cβ in lentivirally infected cells was examined. Given that HEK 293T cells are fast growing and easy to work with, Dox inducibility in lentivirus-infected HEK 293T cells was investigated before attempting primary cell lines. The packaging cell line HEK 293T was used to prepare lentiviral particles from the lentiviral vectors of V, WT and MUT.

#### 3.3.2 Optimisation of lentiviral packaging

Three plates of HEK 293T cells were set up for lentivirus packaging. Individual transfer vectors of V, WT or MUT were independently co-transfected into HEK 293T cells with packaging plasmids. After co-transfection, lentiviral packaging is known to take place inside of HEK 293T cells and be released into the culture medium. After three days of co-transfection, viral particles in the culture medium were harvested, purified and concentrated to produce lentivirus stock (Section 2.2.3).
A density of $1 \times 10^5$ per well of HEK 293T cells were seeded and infected by lentivirus according to 10-fold serial dilutions (1:100, 1:1,000, 1:10,000, 1:100,000). Infected cells were selected with puromycin selection medium, consisted of DMEM supplemented with 10% FBS, 0.5% Pen/Step, 1-2 $\mu$g/mL Puromycin, for 10 days followed by crystal violet staining. Colonies in each well were counted and viral titers were calculated according to the formula, $\frac{\# \text{colonies}}{2 \text{ mL medium} \times \text{dilution factor}} = \text{CFU/mL viral stock}$.

Serial dilutions of these lentivirus stocks were used to determine the optimal viral titer for each lentivirus. HEK 293T cells ($1 \times 10^5$ cells) were seeded onto each well of two 6-well plates and infected by lentivirus according to 10-fold serial dilutions (1:100, 1:1,000, 1:10,000, 1:100,000; Figure 3.7). After infection, all wells were subject to
puromycin selection for approximately 10 days, until colonies were established in each well. Cells were stained with crystal violet for visualization, and colonies were counted and calculated to generate a viral titer for each lentivirus stock. After 10 days of puromycin selection, 1:10,000 and 1:100,000 dilution of each virus displayed almost zero or very low level of HEK 293T survival rate, respectively. Cells infected with V lentivirus appeared to be 5-6 times more efficient, with more colonies observed in each dilution, than that of WT and MUT viruses (Table 3.2). These results together suggest that three lentiviruses were successfully prepared and all of them have a reasonable ability to infect HEK 293T cells, although the infection efficiency is variable between different constructs.

Table 3.2: Viral titer of the three lentviruses.

<table>
<thead>
<tr>
<th>Lentivirus</th>
<th>Viral Titer (CFU/mL*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLenti-Bi-TRE-Tet-on</td>
<td>~8.6 × 10⁴</td>
</tr>
<tr>
<td>pLenti-Bi-TRE-Tet-on-PP2Cβ WT</td>
<td>~1.5 × 10⁴</td>
</tr>
<tr>
<td>pLenti-Bi-TRE-Tet-on-PP2Cβ MUT</td>
<td>~1.6 × 10⁴</td>
</tr>
</tbody>
</table>

*CFU: colony-forming unit.

### 3.3.3 Stability of PP2Cβ in response to Dox induction

The stability of PP2Cβ in response to Dox induction in lentivirally transduced HEK 293T cells was examined to assess the feasibility of this lentiviral Tet-On system. A functional lentiviral Tet-On system requires the ability to induce rtTA and PP2Cβ expression upon Dox induction (On state) and the ability to return to basal level when Dox is removed from the system (Off state). Three plates of lentivirally transduced HEK 293T cells for each lentivirus (V, WT and MUT, vector amount was used according to viral titer in Table 2.1) were cultured and selected until ~80% confluency was obtained. Dox (1 µg/mL) was added into two of these plates (On state) and no Dox was added to the remaining plate as a negative control. The untreated plate and one of the Dox-treated plates were harvested after 24 h of Dox treatment, while the remaining Dox plate was further split into three plates and cultured with no Dox medium for another 24 h, 48 h and 72 h (Off state). Ideally, removal of Dox from the culture medium will result in inactivation of the system (Off state). Western blot analyses were
carried out to examine the stability of rtTA and PP2Cβ in response to the presence and absence of Dox (On vs Off states). However, neither rtTA nor PP2Cβ expression was observed in any of lentivirally infected HEK 293T cells regardless of Dox induction (Data not shown).

This unexpected result could be due to several factors. For instance, the infection efficiency was too low to give a reasonable amount of gene expression. To solve this problem, viral infection was attempted in 24-well plate with the application of more viral particles under puromycin selection until ~80% confluence in a 6 cm tissue culture plate was obtained. Two plates were set up for each lentivirus, one of them were teated with Dox for 24 h before harvest. A negative control, uninfected HEK 293T cells, and a positive control, transfected HEK 293T with V construct were also included in the western blot analyses. Once again, neither rtTA nor PP2Cβ expression was observed upon Dox induction. The increase in viral titer applied to the target cells was expected to increase the infection efficiency, leading to more effective gene induction. However, no induction was observed after three repeats. These negative results showed that virally infected HEK 293T cells were not able to induce rtTA and PP2Cβ regardless of Dox in the system. Given that cells were successfully infected and survived from puromycin selection as demonstrated in Figure 3.7, transduction into a host chromosome seems to affect the Dox inducibility, which was otherwise observed in the transient transfection method (Figure 3.5). Taken these results into consideration, these experiments suggest some difficulties for Dox-dependent instant induction of this lentiviral Tet-On system.

3.3.4 Optimisation of lentiviral induction condition

Since it takes a long time to establish confluent infected cells before Dox induction, an alternative induction strategy was developed. Viral titer experiments showed that HEK 293T cells infected by V virus had 5-6 times higher infection titer than those infected by WT or MUT viruses. Therefore, V virus was used to optimize conditions for lentiviral induction.
Figure 3.8: Time course of rtTA expression in response to Dox induction using V virus infected HEK 293T cells. Western blot analyses were performed on 8% SDS-polyacrylamide gel electrophoresis for 1.5 h at 130 V and transferred for 2 h at 150 mA onto a PVDF membrane. Dox induction was performed in a time course by adding Dox, 24 h, 48 h and 72 h after infection. Equal amounts of infected HEK 293T extracts were prepared after cells are confluent, and 20 µL of each sample were loaded on the gel. β-actin was included as a loading control. Lane 1: Uninfected HEK 293T, negative control; lane 2: infected HEK 293T with no Dox; lane 3-5: infected HEK 293T with Dox induction after 24 h, 48 h and 72 h of infection, respectively; lane 6: positive control, HEK 293T cells transiently transfected with V construct, induced with Dox.

HEK 293T cells were plated and infected in a 24-well plate as described in Section 3.3.3. Dox was added after lentiviral infection in a time course manner, 24 h, 48 h and 72 h post-infection (Lane 3, 4 and 5, respectively Figure 3.8). Dox was maintained in the system until the infected cells reached >80% confluency in a 6 cm plate. Cells were harvested for western blot analysis. Parental HEK 293T cells and transiently transfected HEK 293T cells with V construct were included as a negative and positive control, respectively (Lane 1 and 6, Figure 3.8). Due to a high induction efficiency and the higher cell number (Lane 6 in β-actin panel, Figure 3.8), the positive control sample was developed separately for a shorter time to avoid an intensive signal on a detection film. Consistent with T7-rtTA expression of the positive control (Lane 6, Figure 3.8), virally infected HEK 293T cells with Dox induction showed a band at the same position of T7-rtTA (compare Lane 6 with Lane 3, 4 and 5, Figure 3.8). The addition of Dox 72 h post-infection appeared to have the strongest T7 tag expression (Lane 5, Figure 3.8) compared to the band at 24 h and 48 h (Lane 3 and 4, Figure 3.8) suggesting that lentiviral infection followed by immediate Dox induction improves inducibility of the system. However, this result could not be consistently reproduced using the same cells, suggesting that rtTA was expressed at low levels below the sensitivity threshold of the detection method. In summary, these results showed that V virus infected HEK 293T
cells can be induced by adding Dox within 72 h of post-infection, however, the induction of this system is not efficient enough for a reproducible detection by immunoblot analysis.

![Figure 3.9: Examination of rtTA protein expression in response to Dox induction in stably established V lentivirus-infected HEK 293T cells.](image)

**Figure 3.9**: Examination of rtTA protein expression in response to Dox induction in stably established V lentivirus-infected HEK 293T cells. Western blot analysis was performed on 8% SDS-polyacrylamide gel electrophoresis for 1.5 h at 130 V and transferred for 2 h at 150 mA onto a PVDF membrane. Dox induction was performed on stable cell line over 72 h. Lane 1: positive control, HEK 293T cells transiently transfected with V construct, induced with Dox; lane 2: V stable cell line, induced with Dox for over 72 h.

### 3.3.4.1 Induction in stable cell line

The above studies (Section 3.3.3 and 3.3.4) demonstrated that the inducibility of this Tet-On lentiviral system is not very stable in an infected polyclonal cell population. Individual stable cell lines for all three constructs derived from a single colony were produced and examined for Dox inducibility. HEK 293T cells were plated and infected in 6-well plates as described in Section 3.3.2. Cells were transduced by each virus independently with a 10-fold serial dilution and selected with puromycin until single colonies were developed. Three colonies were picked for each virus and cultured until they became confluent in 6 cm tissue culture plates. Dox treatment was performed for more than 72 h, followed by western blot analysis. One of V lentivirus-infected stable cell lines exhibited weak T7-rtTA expression after Dox induction in which the band matched to the T7-rtTA from transiently transfected positive control cells (compare Lane 2 to Lane 1, Figure 3.9). Cell extract of stable cell line was intentionally loaded more than that of the positive control to visualize a weak expression of T7-rtTA, and therefore β-actin from the stable cell line is more intense than that in positive control. Negative controls from parental HEK 293T cells and V lentivirus-infected stable cell line without Dox, were performed on another gel and did not show any signal of T7-rtTA (Data not shown). Nevertheless, other stable cell lines failed to produce an induced
expression of the T7 tag by Dox. In conclusion, the induced T7 tag expression in V infected stable cell lines was not reproducibly observed every time. This result confirmed that V lentivirus-infected HEK 293T cells could be induced by addition of Dox, but illustrated a instability and inconsistency of this induction system.

3.4 Examine \textit{rtTA} expression using reverse transcriptase-coupled quantitative real-time PCR

3.4.1 Introduction

Reverse transcriptase-coupled quantitative real-time PCR (RT-qPCR) is one of the most sensitive methods for mRNA detection and quantification. This sequence-specific procedure monitors the relative quantities of reverse transcribed mRNA after each PCR cycle in real time which has become a standard method for transcription analysis. HEK 293T cells transiently-transfected with the V construct displayed increased protein expression of rtTA in response to Dox treatment (Figure 3.5). However, lentivirus-mediated infection and subsequent probing by immunoblotting was inconsistent with the Dox induced protein expression of rtTA. This inconsistency could be due to weak protein expression or ineffective Dox induction. Therefore, in order to validate the inducibility in virally infected cells, RT-qPCR was employed to detect the changes in \textit{rtTA} expression in response to Dox treatment at the mRNA level.

3.4.2 Primer design

The first step of RT-qPCR was designing primer pairs for the target gene (\textit{rtTA}) and reference genes. Reference gene Beta Glucuronidase (\textit{GUSB}) primer pair for RT-qPCR analysis was obtained from Qiagen (QuantiTect Primer Assay, catalog number: QT 00046046). Three \textit{rtTA} primer pairs were designed based on the pTet-On plasmid sequence from Clontech, with amplicon sizes of 68 bp, 186 bp and 182 bp (Table 3.2). Intra- and inter-primer complementarities were monitored by the probe design software (Roche) and thus avoided in final primer design.
Table 3.3: Sequences, melting temperatures (Tm °C) and amplicon size of rtTA primer pairs.

<table>
<thead>
<tr>
<th>Primer Pair</th>
<th>Forward Primer (5’ → 3’)</th>
<th>Reverse Primer (5’ → 3’)</th>
<th>Tm (°C)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TAATGAGGTCGGAATCGA</td>
<td>TAGGCTGCTCTACACCAAG</td>
<td>60</td>
<td>68</td>
</tr>
<tr>
<td>2</td>
<td>TAGATGTGCTTTACTAAGTCATC</td>
<td>GATCTTCCAATACGCAACCTA</td>
<td>60</td>
<td>186</td>
</tr>
<tr>
<td>3</td>
<td>CACCTAGACGGCGAGGAC</td>
<td>ATTCCAAGGGCATCGGTA</td>
<td>60</td>
<td>182</td>
</tr>
</tbody>
</table>

The specificity of amplification by each primer pair and amplicon sizes were examined using conventional PCR and visualized on a 2% agarose gel (Figure 3.10). One amplification product was observed for each primer pair (Figure 3.10) with the expected size as indicated in Table 3.3. Negative controls containing no cDNA template were also tested with no visible bands detected (Data not shown). This indicates that each amplicon was specifically produced by the corresponding primer pair rather than by a random non-specific amplification.

Figure 3.10: Specificity check of rtTA amplicon using a conventional PCR. Agarose gel electrophoresis (2%) was carried for 1 h at 120 V in 0.5 × TBE buffer and stained with 0.5 µg/mL ethidium bromide. Ten percent of each 50 µL PCR reactions was loaded into a well and was subject to gel electrophoresis. Lane 1, 2, and 3: PCR reactions using the rtTA primer pairs 1, 2 and 3, respectively. M: molecular weight marker. Negative controls containing no cDNA template were carried out on a separate gel with no detection of any bands (Data not shown).
3.4.3 RNA quality control

High quality RNA preparation is essential in RT-qPCR analysis. Approximately, the same quantity of RNA should be used for each sample for relative quantification by RT-qPCR. Therefore, accuracy of RNA quantification is crucial to generate reliable data. Quantity, purity and integrity are three quality control steps that are normally performed on RNA samples. Spectrophotometry was utilised in this study for RNA quality control using the Nanodrop® ND-1000 spectrophotometer.

DNase treatment was carried out to eliminate genomic DNA contamination using 30 µg of extracted RNA according to the concentration determined by spectrophotometry (Table 3.4, “before” column). RNA concentrations determined using the Nanodrop spectrophotometer, of each sample before and after DNase treatment are listed in Table 3.4. Optical density (OD) of 260/280 and 260/230 ratios were used to assess RNA purity before and after DNase treatment Table 3.4. After DNase treatment, OD_{260/280} ratios for each RNA sample fell into the acceptable range, but most of OD_{260/230} ratios were detected below the 1.8 cut off mark regardless of DNase treatment. These results suggest that all RNA samples were extracted with relatively high purity, however, traces of salt or solvent contamination might be present in RNA samples.

Table 3.4: Comparison of RNA concentration before and after DNase treatment using Nanodrop spectrophotometry.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Before DNase Treatment</th>
<th>After DNase Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK 293T</td>
<td>476.33</td>
<td>0.99</td>
</tr>
<tr>
<td>I-VI-Dox*</td>
<td>514.10</td>
<td>1.76</td>
</tr>
<tr>
<td>I-VII +Dox</td>
<td>861.70</td>
<td>1.90</td>
</tr>
</tbody>
</table>

* I: V lentivirus-infected cell line

3.4.4 Reverse transcriptase negative control

The reverse transcription step introduces substantial errors into RT-qPCR analysis, therefore, it is essential to eliminate the potential interference produced through
contamination by genomic DNA. This study utilized a one-step RT-qPCR analysis that uses RNA samples to produce reverse transcription and PCR amplification together in the same tube. For each sample, a reverse transcription negative control (RT-) was run in parallel whereby the reverse transcriptase was omitted. In general, detection after 35 amplification cycles is considered to be close to background. No detection of amplification (or Crossing point (Cp) value > 40 cycles) was observed in RT- samples, indicating that these samples did not have detectable levels of genomic DNA contamination.

### 3.4.5 PCR efficiency optimization

High PCR efficiency assures robust and accurate RT-qPCR results, especially when relative quantification analysis is involved. Target and reference genes are required to have comparable high amplification efficiencies to allow accurate relative comparison. PCR amplification efficiencies were initially determined by preparing standard curves using the Lightcycler® 480 system. A 10-fold serial dilution series (1:5 to 1:500) of an RNA sample was prepared in triplicate, in order to construct a standard curve for each target and reference gene (Appendix VII-VIII). Crossing points (Cp or Ct) for amplification using each primer pair were collected and plotted against the Log$_{10}$ concentration of each dilution to generate a standard curve. PCR amplification efficiencies were then calculated from the slopes of the standard curve according to

$$\text{Efficiency} = 10^{-\text{slope}} - 1 \ [81]$$

Theoretically, the quantity of PCR product should double with each amplification cycle, therefore, the maximum efficiency of a PCR amplification is 2 [81]. Nevertheless, experimental procedures may introduce errors to PCR amplification, with the range of efficiencies between 1.9 and 2.1 (5% error) being generally accepted. The rtTA primer pair 2 and GUSB primer pair showed reasonably high PCR efficiencies of 2.04 and 1.97 (Appendix VII-VIII), respectively. These efficiencies fall into the acceptable range, and therefore suitable for subsequent analysis. Almost all sample dilutions shared the same melting peak when amplified using the same primer pair, indicating that the primers were highly specific. Together with the acceptable efficiency, rtTA and GUSB are suitable for subsequent analyses to monitor inducibility of rtTA. The melting curve of rtTA at 1:1000 dilution exhibited a shoulder peak around 72°C. This was caused by the formation of primer dimer which was
confirmed by running PCR amplification product on a 2% agarose gel (Data not shown).

3.4.6 Relative quantification

Since the inducibility of rtTA protein level was unstable in V infected HEK 293T cells and hard to be detected in western blotting, real-time quantitative PCR (RT-qPCR) was used to detect induced mRNA level of rtTA. Dox treatment was performed 24 h post-infection, and maintained in the system until the infected cells reached >80% confluency in a 10 cm plate. Cells were harvested for western blot and RT-qPCR analyses. Western blot analysis of V virus-infected HEK 293T cells showed a faint T7-rtTA band when induced with Dox (Lane 5, Figure 3.11A) compared to the un-induced sample (Lane 4, Figure 3.11A). Subsequently, RT-qPCR was carried out to confirm this inducibility. Due to the low reproducibility of this system, only one biological replicate was used to carry out RT-qPCR analysis. Three reverse transcription replicates were carried out and relative induced values (Dox) were calculated compared to the untreated control (no Dox) after normalization against reference gene expression. All samples were assessed in triplicate. Dox induced samples showed 2 Ct cycle difference on average from uninduced control, which correspond to an approximate eight fold increase in rtTA expression (Figure 3.11B). Due to time constraints, untreated control (no Dox) samples were derived from induced cells by removing Dox from the culture medium for over 72 h. This is not an ideal untreated control, which ideally should be derived from infected cells never treated with Dox. Thus, it is possible that traces of Dox residue in the untreated control may result in a higher basal level of expression compared to an ideal untreated control. Parental HEK 293T samples were also analyzed using RT-qPCR as a negative control, which showed an average Ct value of 32, and therefore deemed to be a background level of RT-qPCR assays. Two-tailed Student’s t test was performed with a p-value < 0.001 (asterisk, Figure 3.11B), suggesting that this 8 fold increase in rtTA expression is statistically significant in Dox induced cells than that of un-induced cells. Taken together, these results confirmed that rtTA can be induced in V lentivirus-infected HEK 293T cells. However, primer dimer interference was found in some samples by visualizing RT-qPCR products on a 2% agarose gel (Data not shown). Extra bands around 50 bp and 40 bp for rtTA and GUSB amplicons, respectively, were observed which correspond to the size of their primer dimers.
Therefore, the reliability of these assay results needs to be further confirmed from new primer sets that do not produce primer dimer.

Figure 3.11: Confirmation of induced rTA expression using quantitative real-time PCR analysis. Dox induction was performed 24 h post-infection. (A) Western blot analysis was used to detect rTA protein expression in response to Dox treatment on 8% SDS-polyacrylamide gel electrophoresis for 1.5 h at 135 V and transferred for 2 h at 150 mA onto a PVDF membrane. Positive control (Lane 1) was separately exposed for a shorter time frame to avoid an intensive signal on detection film. Lane 1: HEK 293T cells transiently transfected with V construct, induced with Dox; lane 2-3: HEK 293T cells uninduced and induced with Dox, respectively; lane 4-5: V virus infected HEK 293T, uninduced and induced with Dox, respectively. (B) Relative quantification of gene expression changes in rTA expression in response to Dox induction. Three reverse transcription repeats were carried out for each samples and each sample was tested in triplicate. Dox induced samples exhibited ~8 fold increase in rTA expression compared to uninduced samples. P-values, calculated by Student’s t-test, are presented by asterisk (*: p<0.001).
Chapter 4: Discussion and future directions

Lentiviral vector delivery systems are one of the most efficient ways to transfer a gene of interest into target cells, tissues and organs [4, 82]. These vectors have undergone a number of improvements over many years [3, 5, 7, 16]. A target vector is commonly used in combination with a regulatory component such as tetracycline responsive transcriptional activator or repressor to allow tight regulation of the transgene [29, 82-84]. Almost all inducible lentiviral systems have been constructed using at least two vector components, one harbouring the target transgene and the other carrying the transcriptional regulator inducibly acting on the regulatory promoter element [32, 83].

To improve a conventional lentiviral vector with an inducible gene expression feature, an instantly inducible gene expression system that combines the target gene and transcriptional regulator on a single vector was designed and constructed in this study for investigating gene function in vivo.

PP2Cβ is associated with a number of pathways including cell proliferation, differentiation, senescence and apoptosis [1, 48, 52, 55, 57]. A recent study revealed a down-regulation of PP2Cβ during cellular senescence in human diploid fibroblasts, suggesting its role in cellular ageing process (unpublished result, Dr. Jeong Park). Here, a novel tetracycline inducible lentiviral vector that expresses both PP2Cβ and a regulatory component (rtTA) on a single plasmid was designed, constructed and characterised to provide a novel tool for future investigating the role of PP2Cβ in the NFκB ageing pathway.

4.1 Construction of lentiviral vectors

For many years, effective lentiviral gene transfer with an inducible gene expression feature was undertaken by placing a gene of interest and a regulatory component on separate plasmids [32, 83]. However, such separated systems proved cumbersome due to difficulties in establishing conditions for effective and precise control of gene expression. Markusic et al. was one of the first research groups to incorporate the Tet-On regulatory system into a lentiviral vector as a single cassette and compared the rtTA expression driven by an autoregulatory loop to the one by a constitutive promoter [31]. Since rtTA expression by autoregulatory loop turns out to provide a better lentiviral
vector system [31], this current study made several upgrades to an existing lentiviral vector, allowing more efficient manipulation of the transgenes in vivo. Key features of this improved system include the incorporation of (1) a bi-directional lentiviral vector, allowing transcription of two downstream genes simultaneously; (2) a tetracycline response element (TRE) flanked by two minimal CMV promoters, enabling doxycycline-inducible expression of lentivirally delivered transgene in target cells; (3) a regulatory component, rtTA, facilitating inducible and controlled expression of both the gene of interest and rtTA, and (4) a puromycin marker for selection of successfully infected cells.

In this study, three instant Dox-inducible lentiviral vectors, that inducibly express the protein phosphatase PP2Cβ wild type (WT), mutant (MUT) and no PP2Cβ control (V), were successfully developed using a single cassette (Figure 3.3). The original cloning strategy used to generate these vectors involved independent ligation of the rtTA fragment obtained by PCR into each of the bi-directional pLenti-Bi vectors (Figure 3.1). Ideally, restriction endonucleases used to cut lentiviral vector should not cut the cDNA of interest (rtTA) for cloning. However, the restriction endonuclease XmaI was found to cut inside of the cDNA of rtTA, which caused a time-consuming delay in construction of the V and WT constructs. Only the MUT construct was successfully constructed using this strategy. The success of this MUT construction was a particularly fortunate event, because one of MUT candidate colonies was generated from a partial digest of the cDNA of rtTA, which gave the full length rtTA fragment at a size of ~1100 bp. However, complete digestion of the cDNA fragment of rtTA with XmaI would yield a truncated rtTA fragment (~980 bp) which cannot be used for cloning. Thus, an alternative strategy was attempted, whereby the full size rtTA (~1,100bp) containing fragment was obtained by XhoI and ClaI digestion of the MUT vector. Unfortunately, the ClaI site appeared to be methylated in the MUT vector, which prevented ClaI from cutting at its recognition sequence. Therefore, the ClaI site was substituted by an EcoRV site at the 3’-terminal end of the cDNA of rtTA. The cDNA of rtTA was finally produced from the MUT construct using a time-controlled partial digestion with XmaI and EcoRV (Figure 3.4), and was subsequently sub-cloned into V and WT bi-directional lentivectors.
4.2 Inducibility of lentiviral vector system

Construction of these pLenti-Bi-TRE-Tet-On-PP2Cβ vectors is expected to allow controlled PP2Cβ expression for future studies to investigate its role in molecular ageing. This novel Tet-On inducible lentiviral system provides a number of benefits compared to traditional binary constitutive systems: (1) it enables one step auto-regulation of the transgene and rtTA by Dox; (2) it avoids the cumbersome prerequisite of constructing a cell line for inducibility because this system combines all elements into one single cassette; (3) it does not require co-transduction, selection or screening, unlike previous binary systems, thus allowing it to be used for in vivo applications; and (4) it offers tight regulation of rtTA expression through an auto-regulatory loop, allowing it to minimize the toxic effects associated with traditional systems in which high levels of constitutively expressed rtTA often cause toxicity in vivo.

In this bi-directional lentiviral delivery system, rtTA serves as a transcriptional activator in response to Dox. In the absence of Dox induction, expression of PP2Cβ should remain at low basal levels while in the presence of Dox, leaky expression of free rtTA forms a complex with Dox which associates with the TRE element to enhance the transcription of both PP2Cβ and rtTA (Figure 1.5). The inducibility of this Tet-On lentiviral vector system was confirmed using a transient transfection reaction into HEK 293T cells in which significant induction of rtTA and PP2Cβ protein expression levels were observed in transfected HEK 293T cells upon 1 µM of Dox treatment for 24 h (Figure 3.5). In addition, a mild induction of PP2Cβ and rtTA expression with Dox treatment was observed at a concentration as low as 10 nM, suggesting that the system is highly sensitive to Dox in transiently transfected HEK 293T cells (Figure 3.6). These results also suggest that this novel Tet-On inducible bi-directional lentiviral vector has the ability to manipulate expression of PP2Cβ inducibly, and therefore can be used as an inducible mammalian expression plasmid without viral packaging.

Lentiviral delivery systems have been recognised for their efficient gene transfer into diverse target cell lines [4, 28, 31, 85, 86]. Previously, lentiviral vectors with rtTA expression driven by an autoregulatory loop have been shown to produce higher viral titer and better rtTA inducibility compared to those controlled by a constitutive promoter [31]. Studies by Centilivre et al. further support this observation; they
Chapter 4: Discussion and future directions

demonstrated in mice that more efficient induction of GFP expression occurred when its expression was driven by an autoregulatory loop versus a constitutive CMV promoter [28].

Once a lentivirus delivers a transgene into HEK 293T cells by infection, the transgene is integrated into the cell genome, allowing it to be stably expressed. However, similar Dox sensitivity and the induced expression in transient transfected cells could not be reproduced every time in lentivirally transduced HEK 293T cells (Figure 3.8 and 3.9), probably due to the low viral titers (Figure 4.7 and Table 3.2). One possible explanation is that the number of copies of the transgene integrated into the cell genome was lower than that of transiently transfected cells. Therefore, the minute quantity of free rtTA in the system may not be sufficient to effectively induce strong expression of rtTA and PP2Cβ in the presence of Dox. This is supported by the presence of very faint T7-rtTA bands in V lentivirus-infected cells (Figure 3.8 and 3.11A) and stably established cells (Figure 3.9). RT-qPCR offers greater sensitivity than western blot analysis in gene expression. Due to the difficulties in detecting the gene product via western blot analysis at the protein level, RT-qPCR analysis was employed to identify gene expression at the mRNA level. These set of experiments indicate that Dox treatment of infected HEK 293T cells induced a ~8 fold increase of rtTA expression compared to uninduced cells (Figure 3.11B) These results suggest that gene transcription was induced, however, the protein was not produced at levels detectable by immunoblot analyses.

Tet-On systems tend to have a lower Dox sensitivity than that of Tet-Off systems [31], therefore, Dox at a high concentration is required to activate Tet-On systems. Several studies have reported that the Tet-On system requires nearly toxic levels of Dox treatment for controlled induction of transgene expression in animals [28, 85]. Therefore, it remains to be determined whether the concentrations of Dox used in this study were too low to efficiently induce gene expression in lentivirally transduced cells. Previously, researchers have developed variants for rtTA such as rtTA2S-M2 which display significantly 10-100 fold higher sensitivity to Dox treatment compared to WT rtTA [28, 29, 31, 35]. Thus in future studies incorporating more sensitive variants of rtTA could potentially allow more efficient induction whilst using lower Dox concentrations.
It has also been shown that a loss of gene expression in transduced cells could result from extended Dox induction and repeated induction cycles [28, 31]. This could explain why repeated attempts of Dox induction using the same cell failed to show any induction. This could also be a reason for the low induction levels because cells were shown to be efficiently induced by Dox within 72 h post-infection (Figure 3.8). In addition, experimental error could also lead to lower transduction levels in lentivirally infected cells. Lentiviruses were prepared and stored at -80°C, and an aliquot of lentiviruses stock was thawed prior to infection. This freeze-thaw process could result in a significant reduction of viral titer which could contribute to the loss in transduction efficiency.

### 4.3 Implications in expected PP2Cβ levels in WT and MUT constructs

PP2Cβ is known to associate with IKKβ, and phosphorylation of IKKβ decreases its kinase activity for the activation of NFκB [1]. An arginine to glycine mutant PP2Cβ (R179G) lacking the ability to phosphorylate IKKβ [1], was used to construct MUT vectors to compare the difference in induction level of PP2Cβ by Dox treatment. Unfortunately, the inducibility of PP2Cβ could not be determined in the lentivirus-infected HEK 293T cells by western blot. Due to high viral titer of V lentivirus lacking either PP2Cβ WT or MUT (Figure 3.7 and Table 3.2), V lentivirus-infected HEK 293T cells were used in lentiviral infected experiments. The detectable level of rtTA expression in response to Dox was observed from V lentivirus-infected HEK 293T cells, but not shown in a reproducible and consistent manner (Figure 3.8, 3.9 and 3.11A). Because expressed rtTA protein could be unstable in cells, RT-qPCR was used to confirm the inducibility (Figure 3.11B). An eight fold induction in mRNA level between induced and uninduced samples appeared to be low, which implies the difficulty in induction of this novel lentiviral system.

### 4.4 Summary

Lentiviral vector is commonly used as a gene delivery vehicle that often incorporates a tetracycline inducible system to regulate the transgene expression in target cells, tissues or organs [31, 32, 83]. The transactivator, rtTA, from Tet-On regulatory system was successfully sub-cloned into three lentiviral vectors containing no PP2Cβ control (V),
PP2Cβ wild type (WT) and PP2Cβ mutant (MUT), respectively, which inducibly express rtTA and the transgene in a single plasmid (Figure 3.1 and Figure 3.3). Inducibility of these lentiviral vectors was confirmed by transient transfection in HEK 293T cells. All three vectors revealed responsiveness to Dox in which induced expression of rtTA and PP2Cβ was observed upon 24 h of Dox treatment (Figure 3.5). Lentiviruses were then produced from each lentiviral vector for infection. While induction of rtTA or PP2Cβ expression was observed in lentivirally transduced HEK 293T cells subject to Dox treatment, expression levels were not consistent. Two different strategies were employed to increase the inducibility of this system by: (1) establishing the infected cells at greater cell density followed by Dox induction, and (2) inducing cells within 72 h post-infection. The induction of rtTA was observed in V lentivirus-infected cells, however, this results could not be reproduced consistently (Figure 3.8). Taken together, these results suggest that this novel vector can be used in transient transfection for inducible gene expression; however, its inducibility in transduced cells by infection needs to be improved. In conclusion, this system, which provides inducible gene expression in vivo, could be potentially useful for future studies investigating the role of PP2Cβ in regulating signal transduction pathways and molecular ageing.

4.5 Future directions

4.5.1 Addition of tags in vector construct

This study constructed an instantly inducible tetracycline responsive lentiviral vector to express both rtTA and PP2Cβ upon Dox treatment. Unfortunately, the inducibility of lentivirally infected cells was low and very unstable. The infection efficiency was monitored by a standard colony formation assay (Figure 3.7). This method is time consuming due to colony formation of the infected cells under puromycin selection taking up to 3 to 4 weeks. Additionally, the colony staining could not reveal the functional infection quantitatively due to the possible existence of uninfected but resistant cells. Thus, addition of a fluorescent protein marker on the vector, such as green fluorescent protein (GFP), would allow quicker and intuitive visualization of the infection rate by detecting green fluorescence in the cell.
A T7 tag was fused to the N-terminal end of the regulatory activator, rtTA, to allow easy detection of rtTA expression. However, the absence of a tag on PP2Cβ made it difficult to distinguish between Dox induced and endogenous PP2Cβ expression. Since the level of induction turned out to be extremely low by a lentiviral infection in this study, addition of an epitope tag to PP2Cβ would provide a reliable detection of Dox induced PP2Cβ expression and distinguish this between background levels of this protein.

4.5.2 Improvement of lentiviral induction by priming

This Tet-On lentiviral system is activated by the addition of Dox; however, the inducibility of the Dox-dependent system used in this study was low. Dox treatment within three days of post-infection was shown to improve its inducibility (Figure 3.8); however, this improvement was inconspicuous and difficult to detect using immunoblot analysis. It was initially hypothesized that Dox would form a complex with basal levels of free rtTA produced by leaky expression. This Dox-rtTA complex would then associate with TRE to promote downstream transcription of PP2Cβ. Once HEK 293T cells were infected by lentiviruses, PP2Cβ was integrated into each cell genome at a low copy number. Therefore, the background level of rtTA is insufficient to induce expression in the presence of Dox. To examine if this is the case, rtTA could be transiently expressed to see if this priming of rtTA would increase the lentivirus-mediated expression of target genes.

4.5.3 Applications in cellular senescence

In this study, a novel set of lentiviral vectors were designed and constructed which allow delivery of the gene, PP2Cβ, into a variety of cell lines. Using the transformed cell line HEK 293T, we found optimal induction conditions facilitating controlled PP2Cβ expression. Given that lentiviral systems can deliver a gene of interest into non-dividing senescent cells, the next step would be to optimize conditions in other cell lines such as human diploid fibroblasts IMR-90 cells, which are used for in vitro ageing studies. The vectors constructed in this study could be useful in future studies to investigate the role of PP2Cβ in a number of pathways such as the senescence (i.e. cellular ageing) pathway mediated by NFκB.
To investigate the role of PP2Cβ in NFκB dependent senescence, different stages of senescent IMR-90 cells would be transduced with inducible PP2Cβ WT and MUT, and allowing comparison of the nuclear expression of altered PP2Cβ. This would be followed by analysis of protein and RNA levels of the senescence markers (p21, p16, SA-b-Gal, PAI-1) and NFκB responsive genes (ICAM1, TNF, IL6, CCL2, COX2) would be examined to determine whether PP2Cβ expression is correlated with NFκB activity and senescence-associated gene expression. This would test the hypothesis that elevated PP2Cβ expression can block NFκB activated cellular senescence. It would also shed light on whether administration of PP2Cβ as a therapeutic gene in a Dox dependent manner, could have potential in reversing the ageing phenotype even during later stages of senescence.
Reference


Appendices

Appendix I: Primers used for PP2Cβ sequencing.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5’ → 3’)*</th>
<th>Appended Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP2Cβ F</td>
<td>ACTGAGATCTATGTACCCCATACGATGTTCCTGACTATGGGGCATGGGTGCTTTTTGGATAAAACCCA</td>
<td>BglII site, HA tag</td>
</tr>
<tr>
<td>PP2Cβ R</td>
<td>ACTGTCTAGATCATATTTTTTACACCACACTCATTTTGTCT</td>
<td>XbaI site</td>
</tr>
</tbody>
</table>

*HA tag: blue; restriction sites: red; random end sequences: underlined.

Appendix II: Vector map of pTet-On vector

Source: pTet-On® Vector Information from Clontech Laboratories.
http://www.clontech.com/xxclt_ibcGetAttachment.jsp?clItemId=17935
Appendix III: Vector map of pTRE-Tight-BI


Appendix IV: Vector map of pLenti-Bi-Tet-On-PP2Cβ construct

Vector map determined from Invitrogen Vector NTI software.
Appendix V: Sequence of pLenti-Bi-Tet-On-PP2Cβ construct

PP2Cβ indicated in purple, rtTA indicated in blue, T7 TAG indicated in light green.

1 ACGCGTGTAG TCTTATGCAA TACTCTTGTA GTCTTGCAAC ATGGTAACGA TGCGCACATC AGAATACGTT ATGAGAACAT CAGAACGTTG TACCAT

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351 TTTGGTGACT CTGGAATCTA GAGACCTGCT TTAACCTAAC ATTGCCACCTC TCTAGGGAG ACGACACGAC ATCTCAACAA TGACGTACAA

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1 Refer to Prajapati et al. [1].
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Appendices

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7701 TTTCTCCGGCTC GAAGGGACG TAGGATCTG AAGCATTAGT ACCAGTATCG CACTATTGCAA CACACTAG

7751 CAATCCACA CACATACGA GCCGAAGCA TAAAGTGTAA AGCCTGGGGT GCTATTTAATG

7801 GCCTAATGAG TGAGCTAACT CACATTAATT GCGTTGCGCT CACTGCCCGC CGGATTACTC ACTCGATTGA

7851 TTTCCAGTCG GGAAACCTGT CGTGCCAGCT GCATTAATGA ATCGGCCACCG CAAGGTCGAG TGAGCGGCGG

8001 GCCTAATGAG TGAGCTAACT CACATTAATT GCGTTGCGCT CACTGCCCGC CGGATTACTC ACTCGATTGA

8051 TTTCCAGTCG GGAAACCTGT CGTGCCAGCT GCATTAATGA ATCGGCCACCG CAAGGTCGAG TGAGCGGCGG

86
7901 GCCGCCGGGAG AGGCAGTTTG CGTATGGGCC GCTCTCCTGC TTCCTGCTGC GCAGGACGAG
7951 ACTGACTCAG TGCCTGCTTG CGTTCGCTTG AGCGAGACGG TATCAGCTCA TGACTGAGCC AGCGAGCCAC GCGGCCACGC ATAGTCGAGT
8001 CTCAAAGCGG GTAATACGGT TATCCACAGA ATCAGGGGAT AACGCGAGAA GAGTTTCCGC CATTATGCCA ATAGGCTGCT TAGCTCCTTA TTGCCTCCTT
8051 AGAACATGTT AGCAAAAGGC CAGCAAAGCG CCAGGACAGG TAAAAGGGCC TCTTGGCTGC TCGTATGCTG
8101 GCGTTTGCAGG GCTTTTCCA TACTCGTCTG GCAGGAGCGG TATCAGCTCA TGACTGAGCG ACGCGAGCCA GCAAGCCGAC GCCGCTC GCC ATAGTCGAGT
8151 AAATCGACGC TCAAGTCAGA AATCCAGGGT TAGTGAGCTGA TTTAGCTTGAG AGCTTCGAGTC CCTACCCTTT TAGGCTCTCT CATAGGTGTTT
8201 ACCAGGCCTTT TCCCCCCTGGAGATGAAGGGAGATG AAGTTTCCGC CATTATGCCA ATAGGTGTCT TAGTCCCCTA TTGCGTCCTT
8251 CTTCAAGGGG GTAATACGGT TATCCACAGA ATCAGGGGAT AAGGCCTGTC TATCCACAGA ATCAGGGGAT AAGTTTCCGC CATTATGCCA ATAGGTGTCT
8301 GCTTTCTCAT AGCTCAGCTG TATCAGCTCG TAGTCTAGG TATCAGCTCG TCAAGTCAGA AATCCAGGGT GAAGTTTCCGC CATTATGCCA
8351 GCTCCGCTTA CGCCATACCT GCTCCGGTCT TCCCTTCGAG CAGGAGCTGG GACGGCGAAT GGCCTATGGA CAGGAGCTGG
8401 GCCCTTAAGTG TAAGCTCAGCTG TAGTCTAGG TATCAGCTCG TCAAGTCAGA AATCCAGGGT TAGTCTAGG TATCAGCTCG TCAAGTCAGA
8451 ATCGCAGCTG GCAAGATCCAC TCTGGATTGC CAGGAGCTGG TATCAGCTCG TCAAGTCAGA AATCCAGGGT TAGTCTAGG TATCAGCTCG TCAAGTCAGA
8501 TAGGCGGTCGA TACAGAGGTT GGCCTAACTTC CAAAGGTTCA CCAAGCTTGG CAGGAGCTGG GACGGCGAAT GGCCTATGGA CAGGAGCTGG
8551 AGAAGAGATA TATTTGGTAT CTGCTGTCTT CGGAGGATTG ATCAGGAGTTG TATCAGCTCG TCAAGTCAGA AATCCAGGGT TAGTCTAGG TATCAGCTCG TCAAGTCAGA
8601 AAAAAAACAGG GTAGCTCAGG TATCCGGCAA ACAAACCCAG CACAGAGCTG TATTTGGTAT CTGCTGTCTG CAGGAGGATTG ATCAGGAGTTG TATCAGCTCG TCAAGTCAGA

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8651  GTGGTTTTTT TGTTTGCAGG CACGAGATTA CGGCCAGAAA AAAAGGATCT
       CACCAAAAA ACAAACGTTC GTCGTTCAAT GCGCGTCTTT TTTTCTCTAGA

8701  CAAGAAGATC CTGTTGATCTT TTCTACGGGG TCTGACGCTC AGTGGACGGA
       GTTCTTCTAG GAAACTGAA AAGATGCCCC AGACTGGCAG TCACCTGCT

8751  AAACTCACGT TAAGGGATTT TGTTGCAAGG ATTATCAAAGGAGATCTTCA
       TTTAGCTGCA ATTCCTCATA ACCAGTACTC TAATAGTTTT TCTCTAGAAGT

8801  CCTAGATCCT TTAAATAATTTTAAATGAGTT TTAAATCATTTCAAAATGATATA
       GGATCTAGGA AAATTTAATT TTTACTTCAA AATTTAGTTA GATTTCATAT

8851  TATAGTAAAC GTTGTTCTGA CAGTTACCAAA GTCTTTATCTA GTGAGGACCC
       ATACTCATTT GAAACCAGACT GTCAATGGTTT ACGAAATTAGT CACTCCGTGG

8901  TATTCAGCGG ATCTGTCATAT TTCTGTTCACT CATAGTTGCC TGACTCCCCG
       ATAGAGTCGC TAGACAGATA AAGCAAGTAG GTATCAAGGACTGAGGACGC

8951  TCGTGTTAGAT AACTACGATA CGGGAGGGCT TACCATCTGG CCCCCAGTCT
       AGCAGTACTA TTGAGTGTAT TGGCTCCCGA ATGGTAAGGCGGGGCTCAGA

9001  GCAATGATAC CGCGAGACCC ACGCTCACCAG GCTCCAGAGT TATCAGCAAT
       CTTACTATG GGCGTCTGGG TGGAGTGCGC GGAGGCTAGA ATAGTCGTGCA

9051  AAACCGACCA GCCGGAAGGG CGGAGGCAGG AAGTGGTCTCT GCAACTTTTTAT
       TTTTGTCGTTGGCGCCTCCCTCTGGCCTTCCAGCCA CGTTGAAATA

9101  CGCGCCTCCAT CAAGTCTATTT AATGTGTTGCC GGGAAAGCTAG AGTAAGTATG
       GGCGAGGGTA GTGCAAGTATA TTAACCAACGG CCGACTCGAT CATTCACTTA

9151  TGGTGTCAAGT TATGGTGGCGG CAAGTCTGTTG GCCATTGCTAT CAGGGATAGC
       AGCCAGTCAAT TATCAAAGGCGGTTGCAACCA CAACTGAGAT GCTGTCTCA

9201  GGTTGTCAGCG TCGTCTGTAG GGTCGCTCTC ATTACGCTCCG GTGTTCCCAAC
       CCAGGATGCGGAGCAACCGTCAACCGAAATAGTGAAGTGA GCACCTCCGG

9251  GTGATGACCCG AGTTCAGCTA TCCCCCATGT TGGCAAAAA GCCGTTAGC
       CTAAGTTCCC GTGTAACGCAACGAGCAGATACAGTTTACG GCCAAATCTG

9301  TCCTTCGGCTC TCTGACGAGT TGTGCGAGAT AAGTGCGGCC CAGTGTATC
       AGGAAAGCCAG GAGGCTAGCA ACAGCTTCCA TTCAACCCGGC GTCAAATAGAT

9351  ACTCATAGTTT TACGCGACACG TGCATAATTC TCTTACTGTGCT ATGGCAGTCCG
       TGAGTACCAA TACGCGTCTG TACGTTAGAAG AGATGACACG TACGTTAGGCG
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9401  TAAGATGCTTT GTCTGTGACT GTGAGTACT CAACCAAGTC ATTCTGAGAA ATTCTAGAA AAGACACTGA CCACGATGAA GTTGTTTCAG TAAGACTCTTT

9451  TAGTGTATGCG CGGAGCGGAG TTTGCTCTTC CCGGCGTCAA TACGGGATAA ATCCACATCG CCGGCTGCTC AACGAGAACG GGCCGCACTC TAGTCAAGC

9501  TACCCGCGCCA CATAGCAGAG AAGCTTAAAT GCTCATCATT GGAAAAAGTT ATGCCGCGGT GATATCGTCTT GAAATTTTCA CAGTAGTAA CTTTTTTGCAA

9551  CTTCGGGGCG AAAACTCTCA AGGATCTTAC CGCTGTTCAG ATCCAGTTCG CAGGGGCGGG TTTTAGAGAT TCCTAGAATG GCGACAACCTC TAGGTCAGGC

9601  ATGTAACCCA CTCGTGCAACT CAACGTATCT TCAGCATCTTT TACTTTCAC TACATTGGGT GAGCACGTGG GTTGACTAGA AGTCGTAGAA AATGAAAGTG

9651  CAGCGTTTCT CGGGAGCGCA AAAACGGAGG GCAAAATGCC GCACAAAAAGG GCGAACTCCT TTTGCTCTTC CTGTATACGG CTTTTTTTCC

9701  GAATAAAGGGC GACAGGAAAA TTGTTGAATAC TCATACATTCT CTTCTTTACA CTTTATCCCT CATCTGTTCCTC TTTAATAGATG CATAACTTATG CTTTTTCC

9751  TATTATGCAA GACATTTATCA GGGTTATTGT CTCATGAGCC GATACATAT TATAACTTCT CTGTATATGT ACCAAATACG GAGTACCGTG CTAGGTCTAA

9801  TGAATGTATT TAGAAATAA ACAAATAGG GTTTCCGCGC ATCTATTTCT TTCTTTCTCT CCGGACGGCG CTGGATGGTT GGAATGAAAAGG

9851  GAAATTGCCC ACCTGAGCCT TAAAGAAACT TTATTATCAT GACATTAAAT CTTTATCCCG TGGACTGCCG ATTTTTAAA GCTGTTATTG CTTTTTATCG

9901  TATAAAAATA GCGGTATCAC GAGGGCTTTCT GCTCTGCGCG GTTTTCTCAG ATATTTTTAT CCGCATAGTG CTGGGAGAAC GCAGAGCGCG CAAAGCCACT

9951  TGAAGGGCTA AACCTCTGAC ACATGCAGCT CCCGGAGCGC GTCAAGCCTT ACTGCCACTT TTGTTAGACTG TGTACGTGGC GGGGCTCTGC CAGTGTCGAA

10001 GTCTGTAAGC GGATGCCGGG AGCAGACAAG CGCGTCAGCG CAGACATTCC CCTACGCGCC TGCTGTTGCTT GGGCAGTCCC GCGCAGTCGC

10051 GGTGTTTGCG GTTGAGCGGCG TTGTGCTGAA TATGGCGGAC TACGTCAGAT CACACGCAGC CACAGGCCTT GACGGAAATTG ATACGCGGAT GTCTGCTGTA

10101 TGCTTACGAGA GTGCGGCACTA TGGGAGGTCG AATACCGCAG AGATGCCGTA AATACGGCAG ATATCCACTC TGTTTTCTGTTT ACGCCACACT TTATGGGCGT GTCACGCCAT
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10151 GGAGAAAAATA CGCCATCAGG CGCCATTCGC CATTCAAGCT GCAGCAACTGT
     CCTCTTTTAT GGCGTAGTCC GCAGTAAGCG GTAAGTCCGA CGCGTTGACA

10201 TGGGAAGGGC GATCGGTGCG GGCCTCTTTG CTATTACGCG AGCTGGCGAAA
     ACCCTTCCCG CTAGCCACGC CCCGAGAAGC GATAATGCAG TCAGCCCGCTT

10251 AGGGGGATGT GCTGCAAGGC GATTAAGTTG GGTAACGGCA GGGTTTTCCC
     TCCCTTACA CGACTTTCCG CTAATTCAAC CCATTGGCGT CCCAAAAGGG

10301 AGTCACGACG TTGTAAAACG ACGGCAGCTG CCAAGCTG
     TCAGTGCTGC AACATTTTG CACCGGTCAC GGTTCGAC
Appendix VI: Standard curves of rtTA and GUSB for statistical analysis.

Figure A.VI: Standard curves of rtTA and GUSB constructed for statistical analysis. Log concentration of 2000, 200 and 20 was used for construction, corresponding to 1:5, 1:50 and 1:500 serial dilutions of each sample. (A), (B) are standard curves for rtTA and GUSB, respectively.
Appendix VII: Amplification curves, standard curve and melting peaks for rtTA.

Figure A.VII: Standard curve optimization for rtTA amplicon using quantitative real-time PCR. (A) Amplification curves of a 10 fold serial dilution were used for construction of a standard curve. (B) Corresponding standard curve with an amplification efficiency of 2.042. (C) Corresponding melting peaks indicate specificity of PCR.
Appendix VIII: Amplification curves, standard curve and melting peaks for GUSB.

Figure A.VIII: Standard curve optimization for GUSB amplicon using quantitative real-time PCR. (A) Amplification curves of a 10 fold serial dilution were used for construction of a standard curve. (B) Corresponding standard curve with an amplification efficiency of 1.97. (C) Corresponding melting peaks indicate specificity of PCR.