Plasmid 69 (2013) 96–103

Contents lists available at SciVerse ScienceDirect

Plasmid

journal homepage: www.elsevier.com/locate/yplas

Construction of a doxycycline inducible adipogenic lentiviral expression system

Q. Liu, P.J. Hill, A. Karamitri [1], K.J.P. Ryan, H.Y. Chen, M.A. Lomax *

School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire LE12 5RD, United Kingdom

ARTICLE INFO

Article history:
Received 2 August 2012
Accepted 15 October 2012
Available online 23 October 2012
Communicated by Prof. Philipp Berger

Keywords:
Tetracycline inducible expression
Lentiviral plasmid
Adipose-specific promoter
Adipocyte

ABSTRACT

To provide a tool for research on regulating adipocyte differentiation, tetracycline inducible (Tet on) lentiviral expression vectors under the control of an adipose-specific promoter were constructed. The lowest basal expression in the absence of doxycycline and most efficient dose-dependent, doxycycline-induced transient overexpression was observed using vectors constructed with a combination of Tetracycline Responsive Element (TRE) and reverse tetracycline-controlled TransActivator advanced (rtTAadv), transfected in white (3T3-L1) and brown (HIB-1B) preadipocytes cell lines. The results demonstrate that doxycycline adipogenic inducible expression can be achieved using a pLenti TRE / rtTA adv under the control of the truncated aP2 promoter in HIB-1B preadipocytes.

© 2012 Elsevier Inc. All rights reserved.

1. Introduction

Adipose tissue is found in two forms, energy-storing white adipose tissue (WAT), and energy expending brown adipose tissue (BAT). It has been proposed that the balance between these two adipose tissue types may contribute to the development of obesity (Klingenberg, 1999; Nedergaard et al., 2001). Adipogenesis is controlled by a complicated regulatory network involving the time-dependent expression of a series of transcription factors, co-regulators and signaling pathways (Morrison and Farmer, 1999; Rosen and Spiegelman, 2000; Yeh et al., 1995). Induction of differentiation in white preadipocytes is orchestrated by the temporal expression of C/EBPβ, C/EBPα and PPARγ while brown adipocytes are thought to emanate from a myogenic progenitor as a result of expression of PRDM16 and C/EBPβ (Kajimura et al., 2009, 2008; Seale et al., 2008). The purpose of the experimental work reported here was to construct an adipose tissue specific promoter (aP2) driving a tetracycline inducible (Tet on) lentiviral expression backbone, so that the vectors can be used in the future to produce lentiviruses for the construction of transgenic cell lines.

Tetracycline has been employed to regulate gene expression by binding to the Tet Operator (tetO) within the tetracycline response element (TRE) to either switch off expression (tet-Off) or switch on expression (tet-On). The Tet On expression system has two essential components, the TRE and the reverse tetracycline-controlled transactivator (rtTA), both of which have evolving design and sequences. The original TRE-based promoter (Gossen and Bujard, 1992) confers inducible expression in the presence of doxycycline but has a relatively high background transcription (“leakiness”). The improved TRE promoter, TRE tight (Clontech) has been suggested to show a greater response to doxycycline coupled with extremely low basal transcriptional activity by redesign of the 7 tetO sequences that make up the TRE and the removal of the potential binding sites of endogenous transcription factors (Clontechniques, 2003). The Tet On transactivator rtTA also has been improved (rtTA advanced also known as $rtTA2^S$-M2) by utilizing human codon preferences, removing cryptic splice sites from the mRNA sequences to increase its sensitivity to doxycycline and significantly diminish residual binding to TREs in the absence of doxycycline (Urlinger et al., 2000). Therefore there are

* Corresponding author. Fax: 44 (0)115 95 16122.
E-mail address: michael.lomax@nottingham.ac.uk (M.A. Lomax).
[1] Inserm, U1016, Institut Cochin, Paris, France

0147-619X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.plasmid.2012.10.001

two versions of TREs and two versions of rtTAs, thus four different combinations were tested in order to determine which backbone gave the best doxycycline inducible expression. Previous studies employing adipose-specific (adipogenic) expression vectors have used the adipose tissue specific promoter aP2 promoter (P_{aP2}). However, the size (5.6 kb) of the aP2 promoter exceeds the maximal insert size (4–5 kb in total) of pLenti6 destination vector (Invitrogen), so a 1.2 kb truncated aP2 promoter (P'_{aP2}) containing only the fat-specific enhancer and the proximal promoter of the original aP2 promoter (Graves et al., 1992), was used to generate the vectors for lentivirus production.

2. Materials and methods

2.1. Cell culture, transfection and luciferase assay

3T3-L1 cells (ATCC) and HIB-1B cells (kindly provided by B. Spiegelman) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (Invitrogen) and 1% (v/v) sodium pyruvate in 5% CO_2. Differentiated 3T3-L1 cells (dif 3T3-L1) were obtained by inducing the cells (2 days post-confluence) by 500 μM 3-isobutyl-1-methylxanthine (IBMX), 250nM dexmethasone (Dex) and 167 nM insulin in growth medium for 48 h and then fed with fresh growth medium supplemented with 167 nM insulin for 8 days (Karamanlidis et al., 2007).

For luciferase assay, HIB-1B and 3T3-L1 cells were passaged and plated in 96- and 24- well plates, respectively, for 24 h before transfection at 70–80% confluence. For selecting the best inducible lentiviral expression backbone, the constitutive or inducible LucGFP lentiviral expression vector (100 ng/well in 96-well plate and 300 ng/well for 24-well plate) was transfected to above cells with FugeneHD® (Roche) according to the manufacturer's instructions. Twenty-four hour after transfection, 1 μg/ml doxycycline was added to the cells to induce LucGFP expression, and treated for 24 h. For selecting the best adipogenic lentiviral expression backbone, the P_{aP2} or P'_{aP2} driving inducible LucGFP lentiviral expression vectors were transfected into the plated cells with or without the co-overexpression of C/EBPβ and PPARγ (50 ng/well for 24-well plate) using FugeneHD®. Twenty-four hour post transfection, 10 μM rosiglitazone and/or 1 μg/ml doxycycline (Dox) was added to the cells and treated for 24 h. If needed, 10 μM forskolin was added to the cells 36 h post-transfection and treated for 12 h. The cells were then assayed by Firefly Luciferase Assay System (Promega). Cotransfection with a Renilla control plasmid was omitted from this series of experiments as each vector was compared with its own no doxycycline control, the plasmid vectors were of similar sizes and no variations in transfection efficiency were predicted, as supported by the relatively small standard error of the mean values.

2.2. Plasmids used in this study

pLenti6/V5 lentiviral expression backbone was from Invitrogen. The templates for cloning TRE and TRE tight, pL3-TRE-LucGFP-2L (Plasmid 11685) and pTRE-Tight miR-1 (Plasmid 14896), respectively, and the template for cloning P_{aP2} and P'_{aP2}, pBS-aP2 (Plasmid 11424) all came from Addgene. The template for cloning rtTA, pTet On was from Clontech and the template for cloning rtTA adv, PB-CA-rtTA advance was from Addgene. The overexpression vectors pcDNA3.1-C/EBPβ (Plasmid 12557) and pcDNA-PPARγ (Plasmid 8895) were also from Addgene.

2.3. Construction of Tet on and fat-specific Tet on Luciferase-GFP (LucGFP) lentiviral expression vectors

The 3-fragment MultiSite Gateway® (Invitrogen) cloning system was used to create Luciferase-GFP (LucGFP) fusion protein lentiviral expression vectors, so the reporter gene LucGFP, transactivator and the promoter driving transactivator could be simultaneously cloned into the modified Tet On lentiviral destination vector. The constructed vectors were identified by restriction digest and vector sequencing. The Tet On vectors worked as illustrated in Fig. 1.

2.3.1. Construction of Tet on lentiviral destination vectors

The tetracycline response element (TRE) and the improved version TRE tight were amplified by PCR from pL3-TRE-LucGFP-2L and pTRE-Tight miR-1 respectively, and *Cla*I and *Spe*I restriction sites were added to the 5′ and 3′ end of the PCR products (primer sequences in Table 1). The digested (*Cla*I + *Spe*I) PCR products and pLenti6/V5 plasmid were then ligated to create new Tet on lentiviral destination vectors, pLenti TRE and pLenti TRE tight.

2.3.2. Construction of short aP2 promoter (P'_{aP2}) template vector

The fat-specific enhancer and the proximal promoter of aP2 were amplified by PCR from pBS-aP2 vector (primer sequences in Table 1), and *Not*I restriction site was added to both 5′ and 3′ ends of the PCR product, which was digested with *Not*I and self-circulated to generate the pBS-aP2′ template vector for amplifying P'_{aP2}.

2.3.3. Entry clones for 3-fragment MultiSite Gateway® recombination

Entry clones of LucGFP, rtTA or rtTA adv and P_{CMV}, P_{aP2} or P'_{aP2} were created by BP reactions according to the protocol from Invitrogen. PCR primers for amplifying each target sequence and adding recombination sites (att sites) were listed in Table 1.

2.3.4. Construction of lentiviral expression vectors

LR reactions were performed according to the protocol from Invitrogen, to construct the lentiviral expression vectors.

Entry clones of LucGFP, P_{CMV} and rtTA and the original pLenti6/V5 destination vector were used to generate constitutive LucGFP lentiviral expression vector, pLenti-LucGFP (Fig. 2A). Entry clones of LucGFP, P_{CMV} and rtTA or rtTA adv and the Tet on lentiviral destination vectors (pLenti TRE or pLenti TRE tight) were used to generate Tet on LucGFP lentiviral expression vectors (Fig. 2B). Entry clones of LucGFP, P_{aP2} or P'_{aP2} and rtTA adv and the destination vector pLenti TRE or pLenti TRE tight were used to

Download English Version:

https://daneshyari.com/en/article/5912934

Download Persian Version:

https://daneshyari.com/article/5912934

Daneshyari.com