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Generation of an Inducible, Cardiomyocyte-Specific Transgenic Mouse Model with PPAR β/δ Overexpression

Teayoun Kim, Olga Zhelyabovska, Jian Liu, and Qinglin Yang

Abstract

Peroxisome proliferator-activated receptors (PPARs) consist of three subtypes, each displaying distinctive tissue distribution. In general, the three PPAR subtypes exert overlapping function in transcriptional regulation of lipid metabolism. However, each PPAR subtype possesses distinctive functions in different tissues dependent on their expression abundance, endogenous ligands, and the PPAR coregulators in a specific tissue. Transgenesis is an invaluable technique in defining the in vivo function of a particular gene and its protein. Cre/LoxP-mediated gene targeting has been extensively used to explore the tissue-specific function of PPARs. While this tissue-specific loss-of-function approach is extremely useful in determining the essential role of a PPAR, the tissue-specific gain-of-function approach is another important technique used to understand the effects of PPAR activation in a particular tissue. Transgenic overexpression of PPAR in a specific tissue has been used. However, this conventional technique requires generating the transgenic models individually for each target tissue. In this chapter, we describe the methodology for a more efficient generation of transgenic mouse models with a constitutively active form of PPAR β/δ in different tissues.

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) consist of three subtypes, each displaying distinctive tissue distribution. While it is well established that the three PPAR subtypes exert overlapping function in transcriptional regulation of lipid metabolism, it is recognized that each PPAR subtype possesses distinctive functions in different tissues, dependent on their expression abundance, endogenous ligands, the PPAR coregulators, and/or other unknown factors in a specific tissue. Therapies targeting PPARs are clinically used and PPARs remain the main drug targets for a growing number of diseases and conditions. However, our understanding of the tissue-specific actions of each PPAR subtype remains poor, hindering a broader clinical use of PPAR agonists in specific groups of patients. For example, Avandia has been extensively used for the treatment of type II diabetes. However, recent reports of the potential risk of increasing heart attack incidences in patients using Avandia dramatically raise the concerns of the potentially fatal side effects of this PPAR γ activator [1, 2]. Therefore, preclinical studies on animal models to identify tissue-specific actions of a PPAR agonist are pivotal in designing novel PPAR agonists with minimal side effects and in the treatment of specific patients.

It is challenging to define the tissue-specific effects of PPAR activation in the intact animal. Cre/LoxP-mediated gene targeting has been extensively used to explore the tissue-specific function of PPARs. While this tissue-specific loss-of-function approach is extremely useful in determining the essential role of a PPAR subtype, tissue-specific gain-of-function approach is also important to fully understand the effects of PPAR activation in a specific tissue. Transgenic overexpression of PPAR in a specific tissue can be achieved and has been

extensively used. However, this technique requires generating a transgenic model individually for each tissue to be assessed. In this chapter, we describe the methodology for a more efficient generation of transgenic mouse models with a constitutively active form of PPAR β/δ in the heart. The constitutively active PPAR β/δ was achieved by fusing the VP16 transactivation domain with the wild type PPAR β/δ . There are many reports using the same strategy on studying LXR α and PPARs with VP16 [3–5]. We first generated a ubiquitous and inducible transgenic model driven by the CAG promoter (human cytomegalovirus immediate early enhancer/chicken β -actin promoter), in which the expression of the fusion protein VP16-PPAR β/δ was silenced with a stop sequence (a bacteria chloramphenicol acetyltransferase gene, CAT) before the initial codon of the VP16-PPAR β/δ . The stop sequence can then be removed by excising one of the LoxP sequences flanking it by the Cre recombinase specifically expressed in the particular tissue being assessed. This method has been successfully used [6, 7]. By crossing the above transgenic line with another transgenic mouse line carrying Cre, the overexpression of VP16-PPAR β/δ can then be achieved as desired. In this chapter, we will demonstrate the successful establishment of transgenic line with cardiomyocyte-specific overexpression of the VP-16-PPAR β/δ fusion protein in cardiomyocytes.

2. Materials

1. CAG-LoxP-CAT-LoxP-X vector. The CAG-LoxP-CAT-LoxP-X vector is modified based on the CAG-CAT-lacZ vector [7].
2. DH5 α competent cells (Invitrogen).
3. cDNAs for VP16 transactivation domain (pVP16 vector, Clontech).
4. Expand High Fidelity PCR System kit (Roche).
5. Relevant restriction enzymes, ligase, and buffers (New England Biolabs).
6. Antibodies from Santa Cruz Biotechnology: Polyclonal anti-PPAR β/δ antibody (sc-7197); polyclonal anti-GAPDH antibody (sc-25778); HRP-conjugated anti-rabbit IgG (sc-2004).
7. QIAEX II Gel Extraction kit (Qiagen).
8. Titanium Taq DNA polymerase (Clontech).
9. DNA elution buffer: 5 mM Tris-HCl, 0.1 mM EDTA, pH 7.4.
10. Slide-a-lyzer cassettes (Pierce).
11. Dialysis buffer: 10 mM Tris pH 7.2, 0.1 mM EDTA + 100 mM NaCl.
12. DNA extraction buffer: 50 mM Tris-HCl, 1.0 mM EDTA, pH 8.0, 0.5 % Tween-20, and 200 μ g/mL proteinase K.
13. Total RNA extraction: RNeasy[®] Mini kit (QIAGEN).
14. Real-time PCR kit: *Power SYBR[®] Green* PCR master mix. cDNA preparation: Advantage[®] RT-for PCR kit (Clontech).
15. Nuclear protein extraction kit: CellLytic NuCLEAR Extraction Kit (Sigma).
16. Western blot kit: Membranes were visualized by using Kodak image station 4000R (Kodak) with Super Signal West Dura Extended Duration Substrate (Pierce).
17. Nitrocellulose membrane.
18. Real-Time PCR machine.

19. Thermal cycler.
20. The transgenic mouse line of tamoxifen inducible cardiac-specific overexpression of Cre (α -MyHC-Mer-Cre-Mer mice) is a generous gift from Dr. Jeffrey Molkentin's group [8].
21. Tamoxifen (Sigma T-5648).
22. Sunflower seed oil (Sigma).
23. Primers for Real-time PCR: PPAR β/δ : 5'-TCGGGCTTCCA CTACGG3' and 5'-ACTGACACTTGTTCGGTTCT3'; β -actin: 5'-CTGTCCCTGTATGCCTCTG3' and 5'-ATGTCACGCACGA TTTCC3'.

3. Methods

3.1. Generation of a Transgenic Mouse Model of VP-16- PPAR β/δ That is Inducible in Any Tissue by Cre Recombinase

1. Generation of the constitutively active form of PPAR β/δ gene (VP16-PPAR β/δ , or VPD): The constitutively active form of PPAR β/δ is created by fusing the activation domain of the herpes simplex virus Vp16 protein to the N-terminal of mouse PPAR β/δ (Fig. 1). This mutant (VP16-PPAR β/δ) can trans-activate a PPAR β/δ reporter to a magnitude similar to what is observed with the natural receptor in the presence of ligand [3]. More importantly, VP16-PPAR β/δ signals only through PPAR β/δ pathways. Overexpression of this mutant forms of PPAR β/δ should ensure the constitutive transactivation of its target genes in a specific tissue of the intact mice. The cDNA of the VP16 transactivation domain is cloned from pVP16 vector. The cDNA of the mouse PPAR β/δ is cloned from mouse cDNA extracted from the heart of C57BL/6 mice. Overlapping PCR is performed to obtain the VP16- PPAR β/δ using the Expand High Fidelity PCR System kit (Roche).
2. Generation of the transgenic construct of CAG-LoxP-CAT-LoxP-VPD:
The CAG-CAT-LacZ vector [7] is modified by removing the LacZ gene (see Note 1). The human growth hormone poly A (HGH-PA) is inserted at the end of the cloning site for the candidate transgene to stabilize transgenic expression. The vector is engineered to enable a candidate gene be readily sub-cloned into the vector using the Not I site (Fig. 1).
3. To establish transgenic lines of CAG-LoxP-CAT-LoxP-VPD:
After proofreading and orientation confirmation of the DNA sequence, the transgenic construct of CAG-LoxP-CAT-LoxP-VPD is released from the vector using the Sal I restriction enzyme, followed by gel purification (QIAEX II, Qiagen) and dialysis in Slide-a-lyzer cassettes (Pierce). For dialyzing the DNA sample, 4 L of dialysis buffer is used and is kept at 4°C overnight. The purified transgenic DNA construct is microinjected into the pronucleus of fertilized mouse oocytes derived from superovulated C57BL/6 female. Transgenic founder mice are identified using PCR genotyping as described later in Subheading 3.5.

¹The CAG promoter construct has been proven highly effective in the ubiquitous expression of genes. However, expression is not as robust as with some other promoters, such as the cardiac-specific promoter, α -MyHC. However, the transgenic expression driven by CAG is uniform. The relatively modest transgenic expression in mice using this promoter should be enough for most physiologically related studies. However, it may not be strong enough to mimic a situation with a robust overexpression in response to pathophysiological stimulations. Other promoter constructs like CMV may be used to improve the expression level.

3.2. Generation of Transgenic Mice with Inducible, Cardiomyocyte-Restricted VPD Overexpression

To generate transgenic mice with tamoxifen inducible, cardiomyocyte-restricted VPD overexpression, the two founders of CAG-LoxP-CAT-LoxP-VPD mice, designated as VPD mice are crossed with the α MyHC-Mer-Cre-Mer (TMCM) mice [8]. Offsprings of the double transgenic line are confirmed by genotyping as described in Subheading 3.4. Most importantly, we confirmed that the CAG-LoxP-CAT-LoxP-VPD mice are overtly normal with ubiquitous transgene expression without a change in PPAR β/δ transcript expression in any tissue (data not shown). Two VPD lines (line 1 and line 2) with similar transgenic expression levels are established and they show normal life span with no overt phenotype. The cardiomyocyte-restricted expression of VPD is achieved by treating the mice with tamoxifen as described in Subheading 3.5 to remove the CAT and triggered VPD overexpression (Fig. 2).

3.3. Genotyping

Genomic DNA is extracted from tail samples digested with the DNA extraction buffer. Transgenic mice are identified by PCR on the above DNA samples using the following primer pairs.

1. To identify the CAG-LoxP-CAT-LoxP-VPD transgenic mice, the following primer pair was used: 5' TTA CAT GGT GGT AAG CTT3' and 5' CAG TCA GTT GCT CAA TGT ACC3' (94°C 8 min, 50°C 1 min, 72°C 13 min, 35 cycles) (see Note 2).
2. To identify the α -MyHC-Mer-Cre-Mer/CAG-LoxP-CAT-LoxP-VPD double transgenic mice, in addition to primer pair used in Subheading 3.3, **step 1**, a primer pair recognized α -MyHC-Mer-Cre-Mer are used: 5' GTC TGA CTA GGT GTC CTT CT3' and 5' CGT CCT CCT GCT GGT ATA G3' (94°C 5.5 min, 56°C 30 s, 72°C 11 min, 30 cycles).

Figure 4 shows an example of genotyping for the double transgenic line of α -MyHC-Mer-Cre-Mer/CAG-LoxP-CAT-LoxP-VPD (TMVPD) (Fig. 3).

3.4. Induction of Transgenic Expression of VP16-PPAR β/δ Specifically in Cardiomyocytes of Adult Mice

The transgenic mice with both CAG-LoxP-CAT-LoxP-VPD and α -MyHC-Mer-Cre-mer are treated with tamoxifen (Sigma) by intraperitoneal injection once a day for 2 or 5 days at a dosage of 20 mg/kg/day (see Notes 3 and 4).

3.4.1. Preparing the Tamoxifen Stock for IP Injection

1. Dissolve 50 mg tamoxifen in 300 μ L 100 % ethanol. Vortex the vial well, although the solution remains cloudy.

²To increase the accuracy of genotyping, primer pairs that recognize the CAT sequence can be used.

³The tamoxifen-induced Cre expression has been extensively used for temporal control of transgenic and gene targeting in mice. However, caution should be taken on the dose and duration of tamoxifen treatment. A recent report has demonstrated that tamoxifen-mediated Mer-Cre-Mer nuclear translocation can induce a severe but transient dilated cardiomyopathy, and a dramatic increase of mortality in mice with or without LoxP transgene [10]. However, we have not observed similar phenotypic changes using the protocol presented here. A protocol with 2-day tamoxifen treatment with the same dose has been proposed and suggested minimal effects on cardiac gene expression [11]. Even though a major cytotoxic effect of the 5-day protocol is not observed, the 2-day protocol appears to be feasible with even fewer nonspecific effects.

⁴Considering the potential effects of Cre expression in the heart, it is important to use the littermate mice with Cre expression as a control to exclude any subtle effect of Cre that could alter the phenotypic response of VPD overexpression.

2. Add solution to 4.7 mL sunflower oil (final concentration: 10 mg/mL tamoxifen in 0.06 % ethanol). Sonicate for 15–20 min, vortex briefly for 5–10 min until the solution becomes clear.
3. Aliquot and store the stock solution at -20° . If the stock solution undergoes more than two freeze/thaw cycles it is recommended that it should be resonicated for 5 min prior to use.

3.4.2. Cre Excision

1. Administer 2 mg (200 μ L) tamoxifen once daily per mouse by IP injection.
2. Continue administrations for 5 days. We typically wait at least additional 5 days so that tamoxifen is metabolized and removed from the body.

3.5. Determination of Tissue-Specific Transgenic Expression in Transcript and Protein Levels

3.5.1. Quantitative Real-Time RT-PCR Analyses—Quantitative real-time RT-PCR analysis is carried out using the Roche LightCycler 480 system (Roche).

We mainly use the α -MyHC-Mer-Cre-Mer mice with identical tamoxifen treatment as a control for potential toxic effect of Cre [9]. A stable upregulation of VPD could be detected as late as 5 days after the end of the tamoxifen treatment. The hearts from line 1 displayed a ~twofold increase of the total PPAR β/δ mRNA level (Fig. 4a) with no significant change in PPAR α and PPAR γ mRNA levels compared with the control heart. The expression of total PPAR β/δ in cardiac samples of line 2 was slightly lower (~1.79-fold) than that of line 1 (data not shown).

3.5.2. Western Blot Analysis—To detect the protein expression of the transgenic protein (VPD) and the endogenous PPAR β/δ , Western blots can be conducted using commercially available antibodies (see Note 5). The Immunoblotting images are captured using KODAK image Station 4000R (Carestream Health Inc.) by developing the membranes in Supersignal West substrates (Thermo Scientific), and analyzed with KODAK IM software (Ver 4.5.1).

In isolated cardiomyocytes from line 1, the abundance of VPD protein (see Note 6) is about ~11-fold higher relative to the endogenous PPAR β/δ protein, whereas the endogenous PPAR β/δ level in the TMVPD hearts is similar to that of control hearts (TMCM) (Fig. 4b). Alternatively, VPD protein but not the endogenous PPAR β/δ could be detected by an antibody recognized the trans-activation domain of VP16 (see Note 7).

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References

1. Nissen SE, Wolski K. Effect of rosiglitazone on the risk of myocardial infarction and death from cardiovascular causes. *N Engl J Med.* 2007; 356:2457–2471. [PubMed: 17517853]

⁵Western blot for PPAR β/δ could be challenged. It is easier to perform the Western blot on nuclear protein samples with more enrichment of the protein.

⁶The fusion protein VPD contains the VP16 transactivation domain (80 amino acids). The size for VPD is about 10 KD larger than the endogenous PPAR β/δ . Therefore, Western blot using an anti-PPAR β/δ antibody should reveal two bands (~65 and 55 KD) as shown in Fig. 4.

⁷An antibody recognizing the transactivation domain of VP16 could be used to identify the VPD protein. Its size should be ~65 KD.

2. Stockl KM, Le L, Zhang S, Harada AS. Risk of acute myocardial infarction in patients treated with thiazolidinediones or other antidiabetic medications. *Pharmacoepidemiol Drug Saf.* 2009; 18:166–174. [PubMed: 19109802]
3. Saez E, Rosenfeld J, Livolsi A, Olson P, Lombardo E, Nelson M, Banayo E, Cardiff RD, Izpisua-Belmonte JC, Evans RM. PPAR gamma signaling exacerbates mammary gland tumor development. *Genes Dev.* 2004; 18:528–540. [PubMed: 15037548]
4. Sugii S, Olson P, Sears DD, Saberi M, Atkins AR, Barish GD, Hong SH, Castro GL, Yin YQ, Nelson MC, et al. PPAR activation in adipocytes is sufficient for systemic insulin sensitization. *Proc Natl Acad Sci.* 2009; 106:22504–22509. [PubMed: 20018750]
5. Lo Sasso G, Murzilli S, Salvatore L, D'Errico I, Petruzzelli M, Conca P, Jiang ZY, Calabresi L, Parini P, Moschetta A. Intestinal specific LXR activation stimulates reverse cholesterol transport and protects from atherosclerosis. *Cell Metab.* 2010; 12:187–193. [PubMed: 20674863]
6. Kisanuki YY, Hammer RE, Miyazaki J, Williams SC, Richardson JA, Yanagisawa M. Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. *Dev Biol.* 2001; 230:230–242. [PubMed: 11161575]
7. Araki K, Araki M, Miyazaki J, Vassalli P. Site-specific recombination of a transgene in fertilized eggs by transient expression of Cre recombinase. *Proc Natl Acad Sci USA.* 1995; 92:160–164. [PubMed: 7816809]
8. Sohal DS, Nghiem M, Crackower MA, Witt SA, Kimball TR, Tymitz KM, Penninger JM, Molkentin JD. Temporally regulated and tissue-specific gene manipulations in the adult and embryonic heart using a tamoxifen-inducible Cre protein. *Circ Res.* 2001; 89:20–25. [PubMed: 11440973]
9. Buerger A, Rozhitskaya O, Sherwood MC, Dorfman AL, Bisping E, Abel ED, Pu WT, Izumo S, Jay PY. Dilated cardiomyopathy resulting from high-level myocardial expression of Cre-recombinase. *J Card Fail.* 2006; 12:392–398. [PubMed: 16762803]
10. Koitabashi N, Bedja D, Zaiman AL, Pinto YM, Zhang M, Gabrielson KL, Takimoto E, Kass DA. Avoidance of transient cardiomyopathy in cardiomyocyte-targeted tamoxifen-induced MerCreMer gene deletion models. *Circ Res.* 2009; 105:12–15. [PubMed: 19520971]
11. Andersson KB, Winer LH, Mork HK, Molkentin JD, Jaisser F. Tamoxifen administration routes and dosage for inducible Cre-mediated gene disruption in mouse hearts. *Transgenic Res.* 2010; 19:715–725. [PubMed: 19894134]

Fig. 1.

Transgenic construct for the generation of the CAG-LoxP-CAT-LoxP-VPD mice. The key cloning site and the specific restricted enzymes are shown. The transgenic construct is released from the Bluescript II vector using *Sal I* restricted enzyme.

Fig. 2.

Schematic illustration for the generation of transgenic mice with tamoxifen-inducible, cardiomyocyte-restricted VP16-PPAR β/δ overexpression. The transgenic mice with tamoxifen-inducible, cardiomyocyte-restricted VP16-PPAR β/δ overexpression are generated by crossing the two parent transgenic lines (CAG-LoxP-CAT-LoxP-VPD and α -MyHC-Mer-Cre-Mer). The tamoxifen-induced translocation of Cre from the cytosol to the nucleus and the Cre-mediated the recombination events are shown.

Fig. 3.

TMVPD genotyping results. PCR products obtained as described in the text were analyzed on 1 % agarose gel electrophoresis.

Fig. 4.

Confirmation of PPAR β/δ overexpression. Hearts from TCM, TMVPD mice, are processed for real-time PCR analysis for transcript and protein expression. **(a)** Transcript expression of PPARs. **(b)** Western blot analysis of protein expression of VPD and endogenous PPAR β/δ (see Note 6). Data are mean $\pm$ SEM. * $P < 0.05$, $n = 4$.