

A DNA vector system for MMTV-promoter-based, doxycycline-inducible transgene expression and verified by expressing Mst4 gene ⁽¹⁾

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Abstract

A simplified tetracycline-on (Tet-on) system including all the required regulating elements was constructed in a single vector (poMRT_{neo}/GFP plasmid). The single vector contains a Tet-controlled transactivator gene, rtTA2s-M2, a glucocorticoid-responsive mouse mammary tumor virus (MMTV) promoter, a green fluorescent protein (GFP) reporter gene under the control of Tet operator sequences (tetO) flanked with a minimal CMV promoter (TetCMVmin) and a mammalian selection marker neomycin resistant gene. When tested in NMuMG, a mouse mammary epithelial cell line, the expression of GFP was low without treatment and could be efficiently elevated with treatment of doxycycline (Dox, a Tet analogue). Importantly, MMTV-promoter-based Tet-on inducible vectors enabled the transgene not only to express in a mouse mammary cell line, but also be tightly regulated under the simultaneous treatment with Dox and dexamethasone (Dex, a glucocorticoid analogue), when culture media without steroid. Next, in order to verify the construction strategy is correct, we replaced GFP with Mst4 gene, a sterile 20-like kinase, including a full length wild-type cDNA form and two c-terminus deletion mutants in this inducible system (plasmids poMRT_{neo}/HA-Mst4, poMRT_{neo}/HA-Mst4 Δ 272, poMRT_{neo}/HA-Mst4 Δ 297, poMRT_{neo}/GFP-Mst4, poMRT_{neo}/GFP-Mst4 Δ 272 and poMRT_{neo}/GFP-Mst4 Δ 297). The resulting vector could display the Mst4 expression with lower basal level, higher inducibility and its nucleocytoplasmic dissection. These data demonstrated that we have created a MMTV-promoter-based, doxycycline-inducible transgene system and the simple system is suitable for expressing foreign gene in mammary cells.

Key words: Gene regulation, MMTV promoter, Tetracycline inducible system.

Introduction

Uncontrolled over-expression of transgene products within cells may also have undesired side effect or toxic effects. This temporal and spatial specific expression of transgene is essential for studying transgene effect to the desired tissue. There are various techniques for generating animals with inducible expression of genes (Mills, 2001). At present, the Tet inducible system has proven to be successful for inducible expression of luciferase in HeLa cells (Gossen and Bujard, 1992). The system has been studied extensively for gene expression in a variety of organisms (Ewald *et al.*, 1996; Gallagher *et al.*, 2003; Gunther *et al.*, 2002; Howe *et al.*, 1995; Thomas *et al.*, 2000). Some reports using tissue- or cell-specific promoters to achieve spatial control such as neuron-specific or mammary epithelial-specific promoter to drive the production of tetracycline transactivator. They really set up the inducible and cell-specific gene expression system (Michalon *et al.*, 2005; Gallagher *et al.*, 2003; Gunther *et al.*, 2002).

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However, a stable clone contains both pTetracycline-on (Tet-on) regulator plasmid and an conditional expression plasmid for desired gene under the control of Tet operator sequence (tetO) is needed. Reverse tetracycline-controlled transcriptional activator (rtTA) encoded by regulator plasmid binds to tetO in response to Dox (Urlinger *et al.*, 2000). The expression of desired gene can be optimally controlled by two components (rtTA and tetO) of Tet-inducible system incorporated into separate vectors. With two plasmids in one system can control the ratio of rtTA to tetO that is important for the efficient expression of desired gene (Gossen and Bujard, 1992; Gossen *et al.*, 1995). The establishment of Tet-regulated transgenic animal by using Tet-on system require to generate two transgenic strains, one carrying the transgene of interest under the control of tetO, while the other strain carries the transactivator transgene (Ryding *et al.*, 2001). A progeny with both transgenes is generated by crossing the both lines. However, the selection process that finds stable clones displaying Tet-dependent regulation or analyzing transgenic animals of specific line is costly and time consuming. Therefore, the generation of a single Tet-regulated expression cassette containing both regulator and cis-response element exhibit advantage for the generation of inducible transgenic cell lines or animals.

In this study, a simplified single Tet-on vector with MMTV-promoter-based, Tet-mediated inducible system has been generated. This single vector consists of a Tet-controlled transactivator rtTA2s-M2 at the downstream of MMTV promoter and a Tet-responsive minimal promoter. A neomycin cassette (2.3 kb) was flanked the transactivator gene and Tet-responsive minimal promoter to allow for making stable mammalian cell lines. The MMTV promoter is hormonally regulated and is markedly up-regulated in mammary gland during pregnancy and lactation (Hennighausen, 2000; Jager *et al.*, 2003; Munarini *et al.*, 2002; Munoz *et al.*, 1989). The developed single vector system was functional and exhibited low basal expression level for gene (Chao *et al.*, 2012).

For testing versatility of the simplified single Tet-on vector, we replaced GFP with Mst4 gene, a sterile 20-like kinase. Mst4 may be a multiplicate protein to response diverse environment. Lin *et al.* (2001) demonstrated that overexpressing MST4 in Phoenix cells increased growth rate in both serum and serum-free conditions. Sung *et al.* (2003) also demonstrated that overexpression of MST4 potentates in vitro growth in DU 145 cells grown in serum-free conditions. In contrast, Dan *et al.* (2002) demonstrated that overexpressing MST4 in MCF-7 breast tumor cells displayed an apoptotic phenotype. Such discriminate may be because of transfection system; Lin *et al.* (2001) and Sung *et al.* (2003) made stable clones overexpressing the protein, otherwise, Dan *et al.* (2002) used transient transfection system to make cells expressing substantially higher levels of MST4. But recently, Ma *et al.* (2007) also used transient transfection system and determined that MST4 could promote cell growth and transformation by interacts with PDCD10 via modulation of ERK pathway. The mechanism of MST4 regulation and distribution is not yet clear. Tight quantitative and temporal control of MST4 expression is very useful for basic biological research on MST4 function and moreover, may be for its medical research applications.

Mammary gland growth and maturation consist of a series of highly ordered events that are regulated by complex interactions among many steroid hormones and growth factors (Medina *et al.*, 1996; Hennighausen and Robinson, 2001). For developing normal mammary gland, however, there must display a balance between cell proliferation, cell differentiation and cell death throughout the development. These phenomenons display that it is a suitable cell model for dissecting the controversial MST4 function. MST4 may be involved in mammary gland development.

The resulting vector could display the Mst4 expression with lower basal level and higher inducibility. Many reports using the individual components of the Tet-inducible system (Tet-controlled transactivator and tetO) incorporated into separate vectors and MMTV promoter drive Tet-controlled transactivator expression (Gunther *et al.*, 2002; Nguyen and Pollard, 2002; Hruska *et al.*, 2002; Stairs *et al.*, 2005; Vargo-Gogola *et al.*, 2006; Moody *et al.*, 2002; Hsu *et al.*, 2001). To our knowledge, it is the first time to create a single vector for MMTV-based, tetracycline-inducible system that applied in mammary cells and they could display a simplified, highly inducible event that can be easily adapted to study mammary gland biology.

Materials and Methods

I. Construction of Plasmids

A simplified tetracycline-on (Tet-on) system including all the required regulating elements was constructed in a single vector (poMRT_{neo}/GFP plasmids) (Chao *et al.*, 2012). The single vector contains a Tet-controlled transactivator

gene, rtTA2s-M2, a mouse mammary tumor virus (MMTV) promoter, a GFP reporter gene under the control of tetO flanked with a minimal CMV promoter (TetCMVmin) and a mammalian selection marker neomycin resistant gene. For constructing poMRT_{neo}/HA-Mst4, poMRT_{neo}/HA-Mst4 Δ 272, poMRT_{neo}/HA-Mst4 Δ 297, poMRT_{neo}/GFP-Mst4, poMRT_{neo}/GFP-Mst4 Δ 272 and poMRT_{neo}/GFP-Mst4 Δ 297 plasmids, these PCR-amplified of HA- or GFP-Mst4 full length cDNA (416 residues) or its c-terminus deletion mutants (Δ 272, 1-271 residues contain the kinase domain and Δ 297, 1-296 residues) were inserted into poMRT_{neo}/GFP between MluI and SalI to replace GFP.

II. Cell Culture and Transfections

Mouse mammary epithelial cell line NMuMG were grown in Dulbecco's modified Eagle medium (DMEM, Aldrich, Sigma) contained 10% fetal bovine serum (FBS, Biological Industries), 10 ng/mL insulin, 100 units/mL penicillin and 100 μ g/mL streptomycin in a 37°C humidified incubator containing 95% air and 5% CO₂. Transfection of vector into NMuMG cells was performed by TurboFect transfection kit (Fermentas, PA) as directed by the manufacturer's instructions. Briefly, DNA mixed with 4 μ l TurboFect reagent in 200 μ l Opti-MEM Reduced Serum Media (Invitrogen, CA) and incubated under room temperature for 20 min. After incubation, the DNA/TurboFect mixture (200 μ l) was added into 60 mm cultural dish with cell density of around 60% confluency at 37°C for 3 h. After transfection, cells were washed by PBS buffer (10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, 137 mM NaCl and 2.7 mM KCl, pH 7.4) and replaced with the normal culture medium with mock or Dox for additional 24 h. GFP expression in the transfected cells was monitored by western blot analysis.

Stable clones were selected in media containing 750 μ g/mL G418-sulfate for 2 weeks. Drug-resistant colonies were picked and amplified for further studies. Tet-inducible GFP expression in stable clones was monitored by GFP fluorescence with Leica microscope and Western blot analysis with antibody for actin, HA tag, GFP and MST4 (Santa Cruz Biotechnology, CA) were performed.

III. Fluorescence Imaging

Transiently-transfected poMRT_{neo}/GFP-Mst4, GFP-Mst4 Δ 272 and GFP-Mst4 Δ 297 clones of NMuMG were treated with 1 μ g/mL Dox for 24 h. After treatment, cells were washed twice with 1x PBS (pH 7.4) then subjected to GFP fluorescent image analysis on Leica microscope. To counterstain the nucleus, the paraformaldehyde-fixed cells were treated with 0.2% Triton X-100 at 4°C for 20 min and then stained with 200 ng/ml DAPI (4',6'-diamidino-2-phenylindole) at 4°C for 20 min.

IV. Western Blotting

Cells were harvested and lysed in RIPA buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, 1 μ g/mL leupeptin). Cell lysate (50 μ g) was separated on a 10% SDS-PAGE and transferred to a PVDF membrane by electroblotting. The proteins on membrane were probed by primary antibodies specific against actin, HA tag and GFP and Mst4 (Santa Cruz Biotechnology, CA). The signals were detected by an enhanced chemiluminescence system (Western Lightning PLUS-ECL, PerkinElmer Inc. MA).

Results and Discussion

Because the production of a single tetracycline-regulated expression cassette contains regulatory and responsive unit could provide advantages for the simplified generation of inducible transgenic cell lines or animals. We wanted to generate a single Dox-inducible gene expression vector with both regulator and response operator element to display glucocorticoid-responsive and to eliminate the disadvantage of costly and time consuming from conventional set up. It also has the neomycin resistance (Neo^r) gene, which can be used as a mammalian selection marker. To achieve these goals, we generate a simplified Tet-on vector poMRT_{neo}/GFP (Fig. 1). A neomycin cassette (2.3 kb) was flanked the tetracycline transactivator gene and Tet-responsive minimal promoter with Tet operator to allow for making stable mammalian cell lines.

The single Dox-inducible expression vector was transfected transiently into NMuMG cell to evaluate the efficiency of Dox-inducible transgene expression (Fig. 2). These cells exhibited Dox-inducible and dose-dependent expression of GFP. In contrast, cells without Dox exhibited basal only.

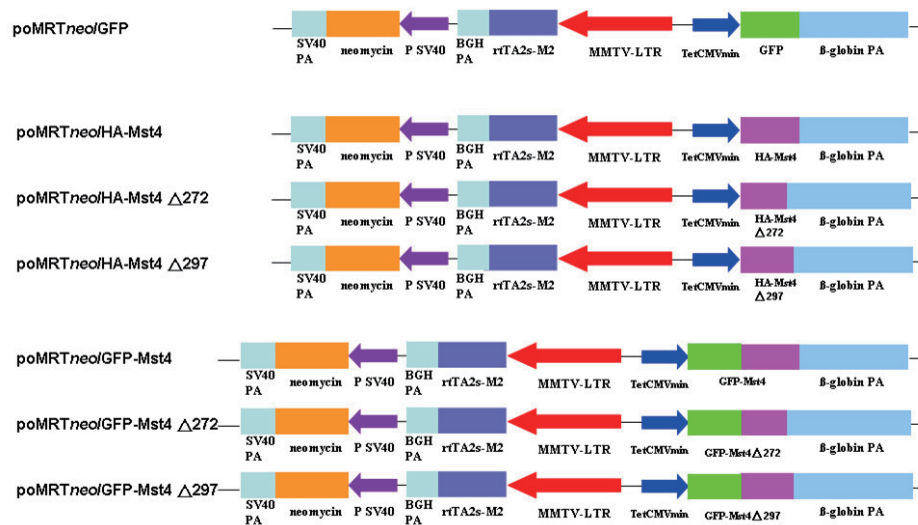


Fig. 1. Simplified map of the MMTV-promoter-based Tet-on inducible systems. The Tet-on single vector inducible systems contain a transactivator rtTA2s-M2 gene, which is a responsive element that comprises a GFP reporter gene under the control of tetO, which is flanked with a TetCMVmin promoter, and a neomycin resistant gene. The regulatory and response elements are arranged in opposite orientation (Chao *et al.*, 2011). MST4 and its c-terminus deletion ($\Delta 272$, 1-271 residues and $\Delta 297$, 1-296 residues) were fused to a HA or GFP-tag.

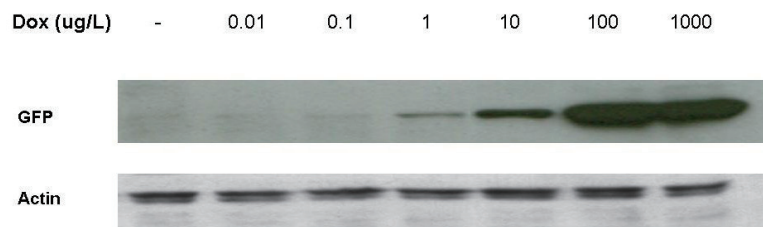


Fig. 2. NMuMG cells were transfected transiently with poMRT_{neo}/GFP vector, and treated with various concentrations or without of Dox for 24 h. GFP expression was determined by Western blot analysis. β -actin was used as an internal control.

MMTV promoter has been demonstrated to respond to steroid hormone glucocorticoid and to induce the tissue- and stage-specific replication of mouse mammary tumor virus *in vivo* (Chandler *et al.*, 1983). Hence, the reactivity of MMTV-based Tet-on inducible systems to the glucocorticoid analogue, Dex, in the Tet-induced GFP expression was studied herein. A charcoal-stripped FBS was used in this experiment to reduce possible interference with the steroids in the normal serum (Cao *et al.*, 2009). As expected, the expression of GFP was extremely low in the poMRT_{neo}/GFP-transfected NMuMG cells that were cultured in the charcoal-stripped FBS without treatment or following treatment with Dex or Dox alone (Fig. 3). However, substantial expression of GFP was induced in cells upon treatment with both Dex and Dox (Fig. 3). This result suggests that the MMTV-based Tet-on inducible single vector can express actually transgene GFP under its glucocorticoid-responsive manner. On the other hand, just added Dox along could get inducible results in other experiments of this article is the cause of the normal FBS containing traces of steroids.

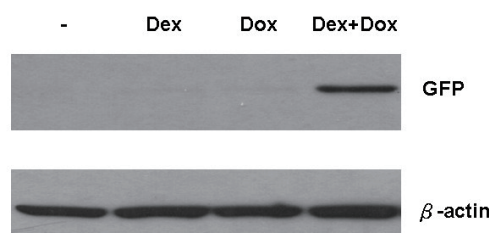


Fig. 3. Glucocorticoid-dependent expression of GFP in NMuMG cells. The poMRTneo/GFP plasmid-stably-transfected NMuMG cells were cultured in the charcoal-stripped FBS and treated with 10 μ g/L Dex, 10 μ g/L Dox or with both 10 μ g/L Dex and 10 μ g/L Dox at 37°C for 24 h. GFP expression was determined by Western blot analysis. β -actin was used as an internal control.

Although, Tet-mediated systems have been reported to display certain limitations, such as basal leakiness and low induction of the genes and have necessitated further development to improve it (Forster *et al.*, 1999; Freundlieb *et al.*, 1999; Markusic *et al.*, 2005; Shaikh *et al.*, 2006). Lee *et al.* (2005) suggested that the individual components of the Tet-inducible system (rtTA and tetO) incorporated into separate vectors could control optimally gene expression. Moreover, the ratio of rtTA to tetO is also very important factor about efficient regulation of gene expression. Our data demonstrated that a simplified Dox-inducible transgene system has been generated and this system can overcome above limitations without complicated modification such combining activation and silencing elements (Forster *et al.*, 1999; Freundlieb *et al.*, 1999). The system displays a negligible basal expression and high inducibility to the reporter gene GFP.

Next, we replaced GFP with Mst4 gene, a sterile 20-like kinase, including a full length wild-type cDNA form and two c-terminus deletion mutants. The resulting vector could display MST4 and its mutants with lower basal activity and higher inducibility (Fig. 4). These result demonstrated this system may also be applied to another gene. Nucleocytoplasmic transport is an important biological process for protein kinases to specifically access to their downstream targets in the nucleus (Faux and Scott, 1996; Hubbard and Cohen, 1993). As shown in the Fig. 5A, removing the c-terminal regulatory domain of MST4 can change the subcellular distribution of MST4. Although the molecular mechanism for the nuclear translocation of MST4 was unclear, the presence of a weaker nuclear localization sequence (NLS) in 272-296 residues and a strong nuclear exporting signal (NES) in 297-416 residues of MST4 was postulated. The nucleocytoplasmic transport results seem to be similar to results of MST1 and MST3, the two members of the sterile 20-like kinase family. MST1 contains a nuclear exporting signal in c-terminal regulatory domain and MST3 contains a NLS in 278-294 residues (Lee *et al.*, 2004; Ura *et al.*, 2001). Upon amino acid sequence alignment, the presence of postulated NLS and NES sequence of MST4 also shown in Fig. 5B. Further investigations by using site-directed mutagenesis may help to determine precisely the presence of NLS and NES in this region.

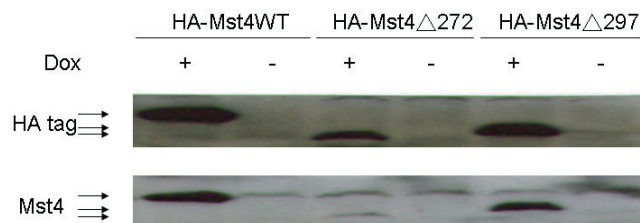


Fig. 4. NMuMG cells were transfected transiently with poMRT_{neo}/GFP-Mst4, poMRT_{neo}/GFP-Mst4 Δ 272 and poMRT_{neo}/GFP-Mst4 Δ 297 vector, and treated with 100 μ g/L Dox for 24 h, then performed Western blotting.

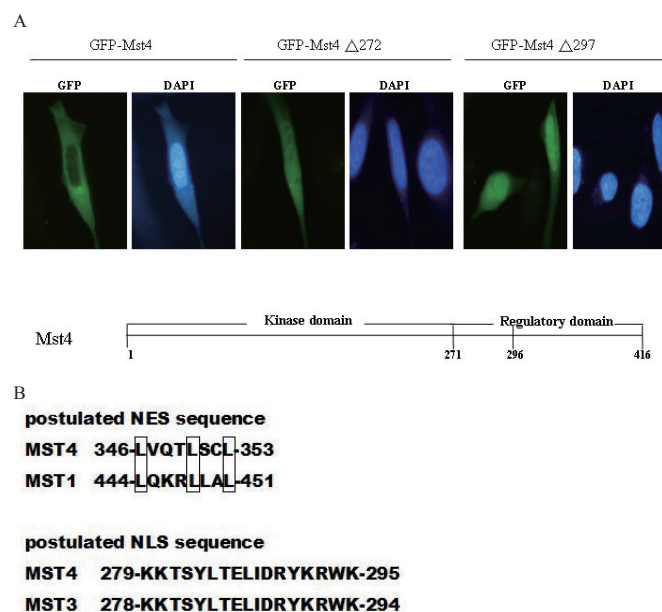


Fig. 5. Mst4 contains NLS and NES signals that promote its nucleocytoplasmic transport. (A) NMuMG cells were transfected transiently with poMRT_{neo}/GFP-Mst4, poMRT_{neo}/GFP-Mst4 Δ 272 and poMRT_{neo}/GFP-Mst4 Δ 297 vector, and treated with 100 μ g/L Dox for 24 h, then subjected to GFP fluorescent image analysis. Nuclei were stained with DAPI. (B) Comparison of postulated NLS and NES sequences with MST1 (Ura *et al.*, 2001) and MST3 (Lee *et al.*, 2004).

These data demonstrated that we have created a MMTV-promoter-based, doxycycline-inducible transgene system and the simply system is highly suitable for mammary cell expression of foreign gene.

Considering the poor transfection efficiency in NMuMG cells, we could not use transient transfection system to determine if MST4 is to promote cell growth or death in NMuMG cells. In the future, we will create these stably-transfected NMuMG cells with MMTV-promoter-based, doxycycline-inducible Mst4 and its c-terminus plasmids to dissect the MST4's real function on Matrigel-wrapped, lumen-forming NMuMG mammary cells resembling the physical mammary gland biology.

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建立以小鼠乳腺腫瘤病毒基因啟動子啟動 可誘導式基因表現 DNA 載體系統並表現 Mst4 基因⁽¹⁾

趙俊炫⁽²⁾⁽³⁾

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摘 要

四環黴素可誘導系統 (tetracycline inducible system) 是目前廣泛使用於調節基因表現的系統之一，當添加四環黴素時即可誘發基因表現；利用四環黴素可調節式基因表現系統需要兩組質體 DNA，分別含有調節性 DNA 片段及反應性 DNA 片段。本研究利用已構築只需一個 DNA 質體就可具有四環黴素可調節式基因表現 (poMRT_{neo}/GFP plasmid)，當轉殖入乳腺上皮細胞株後發現須同時添加四環黴素及醣質皮質素方能誘發表現，證明醣質皮質素確實會作用於 MMTV 啟動子。之後將此質體應用到表現功能性基因上，以 Mst4 蛋白質基酶基因及其 c 端刪除突變序列取代原有 GFP 報導基因，得到 poMRT_{neo}/HA-Mst4，poMRT_{neo}/HA-Mst4 Δ 272，poMRT_{neo}/HA-Mst4 Δ 297，poMRT_{neo}/GFP-Mst4，poMRT_{neo}/GFP-Mst4 Δ 272 and poMRT_{neo}/GFP-Mst4 Δ 297 等質體 DNA。當添加此四環黴素後展現高度誘發 Mst4 表現，並且所表現的 Mst4 蛋白質激酶能正常分佈細胞胞器內。這些結果證明我們已經建構可應用於乳腺上皮細胞之可調節式基因專一表現系統。

關鍵詞：基因調控、小鼠乳腺腫瘤病毒基因啟動子、四環黴素可誘導系統。

(1) 行政院農業委員會畜產試驗所研究報告第 2189 號。

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