TÜBİTAK

Original Article

Turk J Med Sci
2011; 41 (5): 821-825
© TÜBİTAK
E-mail: medsci@tubitak.gov.tr
doi:10.3906/sag-1008-1057

An efficient method for stable transfection of mouse myogenic C2C12 cell line using a nonviral transfection approach

Mehmet Deniz AKYÜZ, Burcu BALCI HAYTA, Pervin R. DİNÇER

Aim: To describe in detail the use of the T-REx™ System (a tetracycline-regulated mammalian expression system; Invitrogen) for rapid, easy, and effective generation of stable C2C12 cell clones. Tetracycline-inducible stable cell lines are useful tools to study the function of various genes in different types of mammalian cells. However, the generation of stable cell lines is a time-consuming, technically difficult, and expensive process.

Materials and methods: Generation of a stable C2C12 cell line was performed by stable transfection of the cells with the pcDNA6/TR plasmid vector, which contains the tetracycline repressor and the blasticidin drug resistance genes. The establishment of the stable cell line and the efficiency of the tetracycline-inducible gene expression system were shown with the transient transfection of reporter plasmid pTO-EGFP and observation of the GFP expression by fluorescence microscopy.

Results: We established a stable T-REx™ C2C12 cell line that can be used for muscle research studies by transfecting the gene of interest with a second plasmid.

Conclusion: The T-REx™ system is useful in the development of stable cell lines for protein production or regulation of gene expression.

Key words: C2C12 cell line, stable transfection, inducible expression, tetracycline resistance gene

C2C12 fare miyoblast hücre hattında viral olmayan transfeksiyon yöntemi ile kalıcı transfeksiyonun gerçekleştirilmesi

Amaç: Tetrasiklinle uyarılabilir kalıcı hücre hatları, farklı memeli hücrelerindeki çeşitli genlerin fonksiyonunun aydınlatılması amacıyla kullanılan yararlı bir araçtır. Ancak, kalıcı hücre hatlarının oluşturulması zaman alıcı, teknik olarak zor ve pahalı bir süreçtir. Çalışmamızda, T-REx™ Systeminin (tetrasiklin ile uyarılabilir memeli gen ifade sistemi, Invitrogen) kullanılması ile hızlı, kolay ve etkin olarak kalıcı transfekte edilmiş C2C12 hücre klonlarının oluşturulması detaylı olarak anlatılmıştır.

Yöntem ve gereç: Kalıcı transfekte C2C12 hücre hattının oluşturulması; tetrasiklin represör ve blastisidin ilaç direnç genlerini içeren pcDNA6/TR plazmid vektörünün hücrelere kalıcı transfeksiyonu ile gerçekleştirilmiştir. Kalıcı transfekte hücre hattının kurulumu ve tetrasiklin ile uyarılabilir gen ifade sisteminin etkinliği; pTO-EGFP plazmid vektörünün C2C12 hücrelerine geçici olarak transfeksiyonu sonrası GFP'nin ifadesinin floresan mikroskop ile gözlenmesi ile gösterilmiştir.

Bulgular: Çalışmamızda, ilgilenilen genin ikinci bir plazmid ile transfekte edilmesi ile kas dokusu ile ilgili araştırmalarda kullanılabilecek, kalıcı T-REx™ C2C12 hücre hattı oluşturulmuştur.

Sonuç: T-Rex™ sistemi ile oluşturulan kalıcı hücre hatları protein üretimi veya gen ifadesinin kontrolü için kullanılabilmektedir.

Received: 23.08.2010 – Accepted: 15.12.2010

Department of Medical Biology, Rectorate Building - 4th Floor, Faculty of Medicine, Hacettepe University, Sıhhiye, Ankara - TURKEY

Correspondence: *Pervin DİNÇER, Department of Medical Biology, Rectorate Building - 4th Floor, Faculty of Medicine, Hacettepe University, Sıhhiye, Ankara - TURKEY*
E-mail: pdincer@hacettepe.edu.tr

Introduction

The stable transfection method is used to introduce recombinant DNA into eukaryotic cells through integration into the host cells' genomes. It is widely used for versatile reasons such as gene silencing, inducing the expression of a gene of interest and observing the physiological effects of the target protein, target validation, assay development, and compound screening (1-4). There are many different approaches for establishing stable cell lines, depending on the type of expression (inducible or constitutive) and the construct that is being incorporated. Inducible expression systems are widely used for controlling the dosage and temporal expression of a given gene product in a population of cells, either in tissue culture or in an intact organism (5,6). Several inducible systems, based on eukaryotic as well as prokaryotic regulatory mechanisms, are available for research and potential therapeutic applications. One of the most convenient is the tetracycline-controlled transcription activation system (Tet system) (7). The T-REx™ System (Invitrogen) is a tetracycline-regulated mammalian expression system that uses regulatory elements from the *E. coli* Tn*10*-encoded tetracycline resistance operon (8,9). Tetracycline regulation in the T-REx™ System is based on the binding of tetracycline to the Tet repressor and derepression of the promoter controlling the expression of the gene of interest. The components of this system include the regulatory plasmid (pcDNA6/TR) and the inducible plasmid (pcDNA4/TO) (10). The repressor protein (TetR) is expressed from the plasmid pcDNA6/TR and binds to a specific DNA sequence, the Tet operator site (TetO), in the absence of tetracycline, thus preventing transcription of the gene of interest from the second plasmid pcDNA4/TO. Upon addition, tetracycline binds with high affinity to the TetR and causes a conformational change in the repressor that renders it unable to bind to the Tet operator. The Tet repressor-Tet operator complex is unable to form and this allows the transcription of the gene of interest.

Although there are many studies in which the T-REx™ System was used to establish an inducible expression (11,12), a detailed method is hard to find in research papers. In this study we present a detailed and highly efficient method for stable transfection of the mouse myogenic C2C12 cell line, which is a well-established and useful model for the study of myogenic differentiation, using the currently available T-REx™ System. We generated stable C2C12 cell clones with inducible expression of green fluorescent protein (GFP). Using this system, a mouse-derived C2C12 stable cell line was successfully generated. Our method is simple, direct, and efficient.

Materials and methods

Cell culture conditions and transfection of pcDNA6/TR into C2C12 cells

Myoblasts from the mouse skeletal muscle cell line C2C12 (American Type Culture Collection, Manassas, VA, USA) were grown on 35-mm^2 culture dishes (Falcon™). Cells (passage 7) were plated at a density of 1×10^5 cells per well 24 h before transfection, and were expanded in growth medium consisting of DMEM with 10% fetal bovine serum and 2 mM L-glutamine (complete medium) at 37 °C in 95% air and 5% CO_2. C2C12 cells were then transfected with pcDNA6/TR (Invitrogen) for 24 h using FuGENE HD Transfection Reagent (Roche) at a DNA (×1000 ng)-to-FuGENE HD (µL) ratio of 6:14. The pcDNA6/TR plasmid DNA was linearized with SapI restriction enzyme prior to transfection. In order to determine the transfection efficiency, C2C12 cells were also transfected with pEGFP-C1 (Clontech). This was determined by counting GFP-positive cells and total cells from 6 random fields for each condition. The transfection rate was defined as the number of GFP-positive myoblasts divided by the total number of myoblasts within the same field.

Selection of stably transfected cell lines

At 24 h after transfection, cultures were shifted to complete medium containing 2 µg/mL of blasticidin as a selective agent. At 18 days after transfection, cell colonies were picked with cloning cylinders and split into 60-mm petri dishes. Isolated clones were then cultured and expanded. Nontransfected C2C12 cells under the same conditions were used as experimental controls.

Immunofluorescence staining

Immunofluorescence staining was done on the expanded clones and nontransfected control cells. Cells on glass coverslips were fixed in 4% paraformaldehyde for 10 min, washed in 1 × PBS (pH 7.4) and permeabilized with 0.2% Triton X-100 in 1 × PBS for 10 min, and then blocked with 10% goat serum in 1 × PBS for 1 h. To detect the expression of TetR, transfected and nontransfected control cells were immunostained by rabbit anti-TetR antibody (1:500; MoBiTec) for 24 h at +4 °C. Cells were then washed with 1 × PBS and labeled with Alexa Fluor 488 goat antirabbit IgG (Molecular Probes) at 1:1000 for 45 min at room temperature. After washing in 1 × PBS, immunostained cells were mounted and observed under an inverted fluorescent microscopy (Leica DMIL). Appropriate excitation and barrier filters were used to observe fluorescence.

Transfection of pTO-EGFP into C2C12 cells

The responsiveness of a tetracycline-sensitive expression system was evaluated by transient transfection of stably transfected clones with pTO-EGFP at a DNA (×1000 ng)-to-FuGENE HD (μL) ratio of 1:10. TetR promoter activation was achieved 24 h after transfection with the addition of 10 μg/mL of tetracycline. Tetracycline (Sigma) responsiveness was observed and photographed under an inverted fluorescent microscopy (Leica DMIL).

Results and discussion

The transfer of foreign DNA into cultured cells is an essential investigative tool for observing the physiological effects of the change of expression of a target gene (13). Control over the timing and regulation of gene expression levels is essential for both the in vitro and in vivo analysis of gene function and gene therapy with safety and efficiency (14). This control can be obtained with the use of inducible expression systems (15). In this study, we used the T-REx™ inducible expression system to generate a stable C2C12 cell line. We used FuGENE HD, a lipid-based transfection reagent, to transfect the linearized pcDNA6/TR plasmid into C2C12 cells. To determine the transfection efficiency, C2C12 cells were also transfected with pEGFP-C1 plasmid under the same conditions. At 24 h after transfection, the transfection efficiency was easily monitored and detected by counting GFP-positive cells and total cells under a microscope. We achieved a transfection efficiency of approximately 70%.

At 18 days after transfection, 25 individual blasticidin-resistant clones were picked, expanded, and screened for TetR expression by immunofluorescence staining using the anti-TetR polyclonal antibody (16). As shown in Figure 1, the isolated blasticidin-resistant clone TR1 expressed the TetR. In order to understand the tetracycline-inducible gene expression, we transfected the stably transfected clones with the pTO-EGFP plasmid, encoding GFP as a genetic marker. The transfection efficiency was calculated as 40% by counting GFP-positive cells and total cells under a microscope. Out of 25 clones, 4 showed inducibility with tetracycline. As shown in Figure 2, the clone that showed the maximum inducibility and minimum basal gene expression (TR1) was chosen and expanded for further studies.

The amount of the TetR produced from the stable cell line is crucial for minimum background gene expression and inducibility. A major drawback of the T-REx™ system is the difficulty in obtaining clones with high TetR expression. To overcome this problem, different strategies, such as using different reporter genes, replacing the TetR protein with a TetR fusion protein to increase its affinity to the inducible promoter, and the use of the Flp-In method for site-directed transgene integration, have been proposed (17-20). Considering that molecular engineering strategies may take as long as clone screening, and that the stable T-REx™ C2C12 cell line is not commercially available, a stable C2C12 cell line is an essential tool for muscle research.

We generated a stable T-REx™ C2C12 cell line, which can be used for muscle research studies by transfecting the gene of interest with a second plasmid. Therefore, inducible gene expression can be achieved with the inducing agent tetracycline. This is extremely useful in situations where the product of the gene of interest has a toxic or anti-mitotic effect.

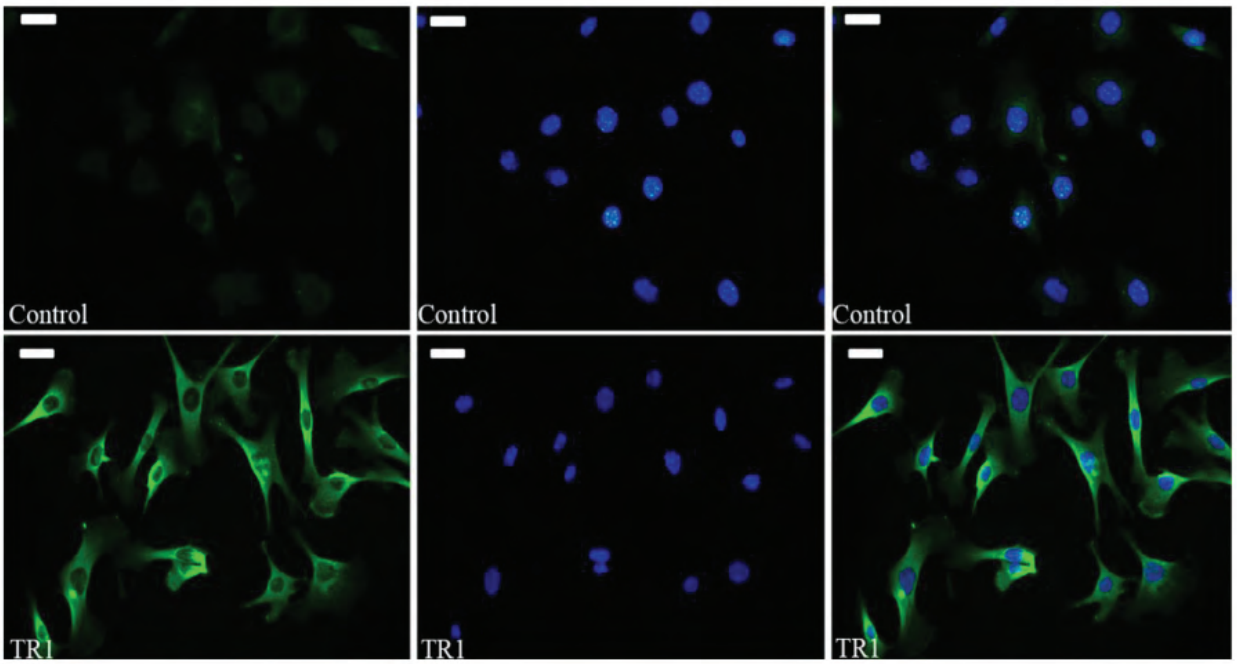

Figure 1. Immunodetection of the TetR in clone TR1. Immunofluorescent staining was performed with Anti-Tet-Repressor polyclonal rabbit antibody (MoBiTec). Untransfected C2C12 cells were used as a negative control. Cells were also incubated with DAPI to show the location of the nucleus. The cells were monitored under an inverted microscope. Bar: 0.1 mm.

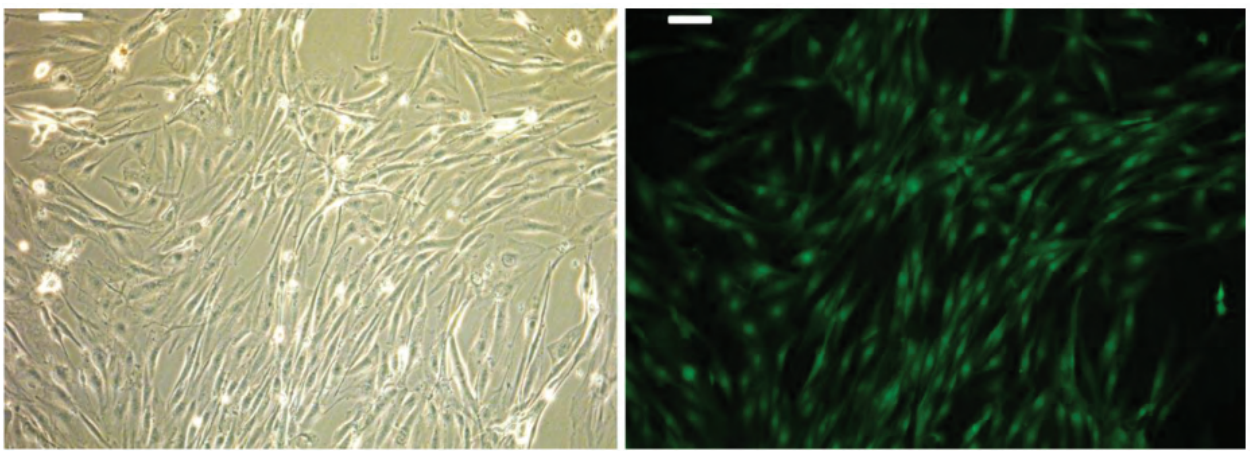

Figure 2. Induction of clone TR1 with 10μg/mL of tetracycline. Left: Bright-field photomicrograph. Right: Fluorescence photomicrograph of the induced TR1 clone expressing GFP reporter protein. Pictures were taken 24 h after induction with tetracycline under an inverted microscope. Bar: 0.1 mm.

Acknowledgements

This study was supported by the Hacettepe University Research Fund (Project No: 03.02.101.009) and the Scientific and Technological Research Council of Turkey (TÜBİTAK; Project No: SBAG-3722, 107S228), Ankara, Turkey. The authors thank Dr. Vasily N. Dobrovolsky for kindly providing the pTO-EGFP plasmid.

References

1. Ramnarain DB, Park S, Lee DY, Hatanpaa KJ, Scoggin SO, Otu H et al. Differential gene expression analysis reveals generation of an autocrine loop by a mutant epidermal growth factor receptor in glioma cells. Cancer Res 2006; 66: 867-74.
2. Tao J, Wendler P, Connelly G, Lim A, Zhang J, King M et al. Drug target validation: lethal infection blocked by inducible peptide. Proc Natl Acad Sci USA 2000; 2: 783-6.
3. Miyagawa Y, Okita H, Nakaijima H, Horiuchi Y, Sato B, Taguchi T et al. Inducible expression of chimeric EWS/ETS proteins confers Ewing's family tumor-like phenotypes to human mesenchymal progenitor cells. Mol Cell Biol 2008; 28: 2125-37.
4. Southan A, Maidment S, Dowler S, Southan AP, Maidment S, Dowle S et al. Using inducible expression vector technology to create stable cell lines expressing KCNQ2/3, KCNQ4, and KCNQ3/5 currents suitable for automated electrophysiology platforms. Biophys J 2009; 96: 177a.
5. Baron U, Bujard H. Tet repressor-based system for regulated gene expression in eukaryotic cells: Principles and advances. Methods Enzymol 2000; 327: 401-21.
6. Lewandoski M. Conditional control of gene expression in the mouse. Nat Rev Genet 2001; 2: 743-55.
7. Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci USA 1992; 89: 5547-51.
8. Hillen W, Berens C. Mechanisms underlying expression of Tn10 encoded tetracycline resistance. Annu Rev Microbiol 1994; 48: 345-69.
9. Hillen W, Gatz C, Altschmied L, Schollmeier K, Meier I. Control of expression of the Tn*10*-encoded tetracycline resistance genes: Equilibrium and kinetic investigations of the regulatory reactions. J Mol Biol 1983; 169: 707-21.
10. Yao F, Svensjö T, Winkler T, Lu M, Eriksson C, Eriksson E. Tetracycline repressor, tetR, rather than the tetR-mammalian cell transcription factor fusion derivatives, regulates inducible gene expression in mammalian cells. Hum Gene Ther 1998; 9: 1939-50.
11. Dobrovolsky VN, McGarrity LJ, Morris SM, Heflich RH. Detection of mutation in transgenic CHO cells using green fluorescent protein as a reporter. Mutat Res 2002; 518: 55-64.
12. Jones J, Nivitchanyong T, Giblin C, Ciccarone V, Judd D, Gorfien S et al. Optimization of tetracycline-responsive recombinant protein production and effect on cell growth and ER stress in mammalian cells. Biotechnol Bioeng 2005; 91: 722-32.
13. Rauth S, Kucherlapati RS. Expression of DNA transferred into mammalian cells. J Biosci 1984; 6: 543-67.
14. Wallenburg JC, Chartrand P. Inducible expression vectors for gene therapy using bone marrow. Transfus Sci 1996; 17: 155-68.
15. Rossi FMV, Blau HM. Recent advances in inducible gene expression systems. Curr Opin Biotechnol 1998; 9: 451-6.
16. Reeves PJ, Callewaert N, Contreras R, Khorana HG. Structure and function in rhodopsin: High-level expression of rhodopsin with restricted and homogeneous N-glycosylation by a tetracycline-inducible N-acetylglucosaminyl transferase I-negative HEK293S stable mammalian cell line. Proc Natl Acad Sci USA 2002; 99: 13419-24.
17. Song P, Wang HCR. Efficient identification of tetR-expressing cell lines for tetracycline-regulated gene expression. Electron J Biotechnol 2004; 7: 210-3.
18. Amar L, Desclaux M, Faucon-Biguet N, Mallet J, Vogel R. Control of small inhibitory RNA levels and RNA interference by doxycycline induced activation of a minimal RNA polymerase III promoter. Nucleic Acids Res 2006; 34: e37.
19. Craig NL. The mechanism of conservative site-specific recombination. Ann Rev Genet 1988; 22: 77-105.
20. Sauer B. Site-specific recombination: developments and applications. Curr Opin Biotechnol 1994; 5: 521-7.