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Original Article

Tet-On advanced inducible gene expression system analyzes the biological functions of Trop-2 *in vitro*

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ABSTRACT

The Tet-On advanced inducible gene expression system *in vitro* is known for genarating robust expression of the desired gene in target cells. The system offers many advantages over other inducible mammalian gene expression systems, such as high specificity, high inducibility, and high absolute expression levels. In this study, the Tet-On advanced inducible gene expression system was applied to induce the expression of the trophoblast cell-surface antigen 2 (*Trop-2*) gene *in vitro* and explore the biological functions of Trop-2. 293/pTet-On-Advanced cell lines were generated, and a recombinant vector containing the *Trop-2* gene was constructed and transfected into stable cell lines to improve Trop-2 protein expression. In the presence of doxycycline (DOX), the proliferation assay, transwell assay, and wound healing assay were performed to analyze the efficacy of Trop-2. The results showed that the Tet-On advanced inducible gene expression system was established successfully in the cell line 293, Trop-2 protein level in cells was significantly increased, and Trop-2 could enhance growth, migration, and aggression in the cell line 293. This study suggests that the Tet-On advanced inducible gene expression system can induce the expression of interest genes specifically and artificially *in vitro* and provides a viable and convenient platform for the study of gene function.

Keywords: Tet-On advanced inducible gene expression system, trophoblast cell-surface antigen 2, gene expression, doxycycline

INTRODUCTION

The Tet-On advanced inducible gene expression system is a severely regulated and highly responsive system that is able to achieve robust expression of the desired gene in target cells as required[1−3]. The Tet-On advanced inducible gene expression system includes the Tet-On-Advanced transactivator and the pTight inducible promoter. In the presence of doxycycline (DOX), the transactivator binds to the tetracycline response element (TRE) in pTight, promoting improved transcription of the desired gene. Here, three kinds of plasmids were applied, including pTet-On-Advanced, pTRE-Tight, and pTRE-Tight-

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luciferase (***Fig. 1A–1C***). The Tet-On advanced inducible gene expression system can achieve maximal expression coupled with extremely low basal promoter activity, with a highly sensitive and concentration-dependent induction level[4]. The system is established in target cells by continuously transfecting the required vectors and selecting stable cell lines. The target cells that express the Tet-On Advanced transactivator and contain recombinant vectors, which integrate the target gene, can express high levels of the target gene when cultured in the presence of the system's inducer, DOX[5–7]. In the presence of DOX, pTet-On-Advanced binds to the TRE in pTRE-Tight and produces high-level transcription of the downstream gene of interest.

The trophoblast cell-surface antigen 2 (*Trop-2*) gene is located in the 1p32 area without the intron gene, encodes Trop-2 protein containing 323 amino acids, and has a molecular weight of 35.7 kDa[8–10]. Trop-2 protein is found at low amounts in normal tissues, but at elevated levels in a variety of epithelial carcinomas, including ovarian cancer, breast cancer, and pancreatic cancer. It is a calcium signal transducer that leads to poor prognosis in many kinds of human carcinomas[11–14]. In triple-negative breast cancer, 90% of patients have high expression of Trop-2 protein. In recent years, Trop-2 has become a popular target for antibody conjugating drugs. Trop-2 protein binds with

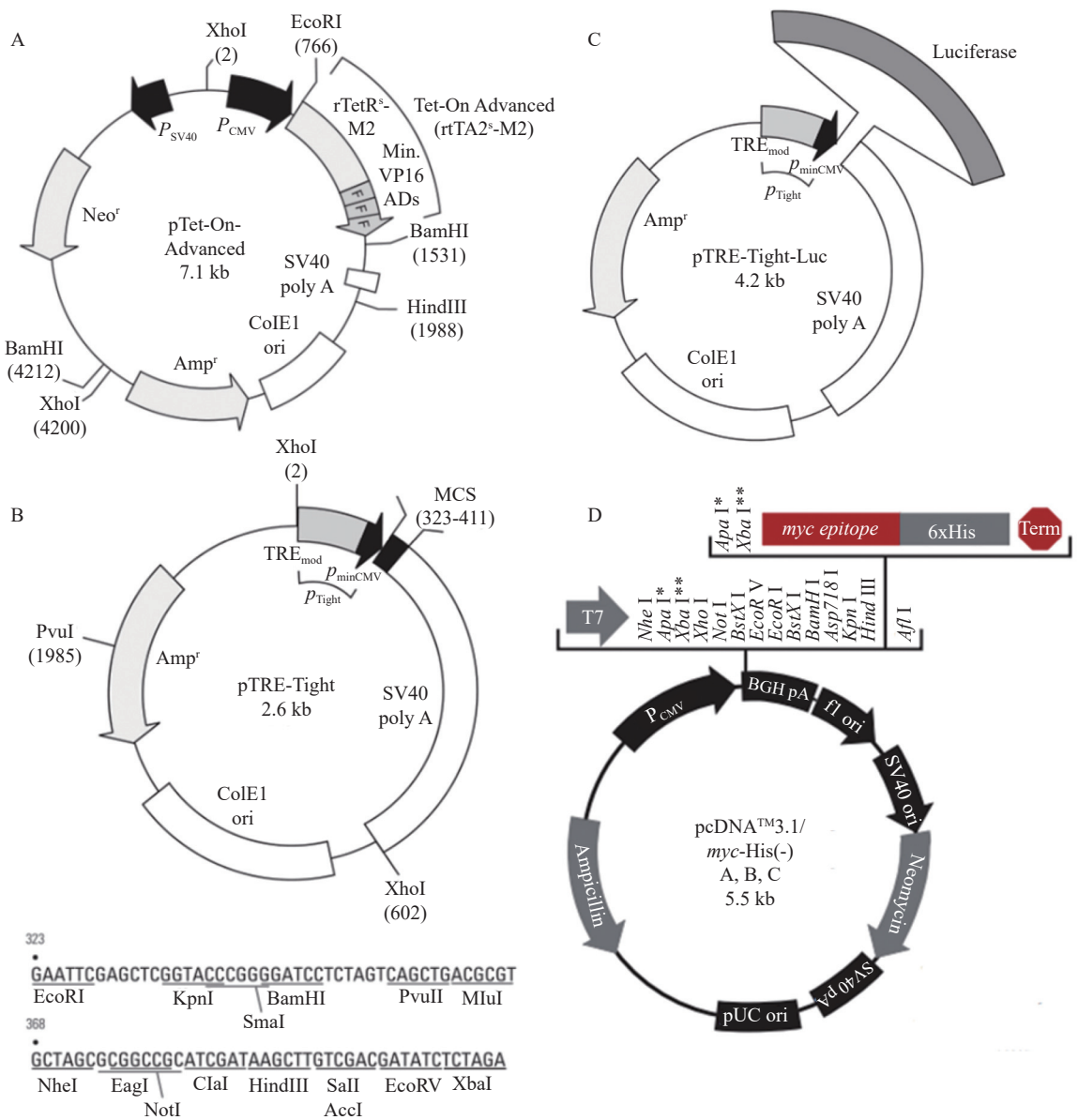

Fig. 1 **The plasmid structure maps.** A: Map of pTet-On-Advanced. B: Map and multiple cloning site (MCS) of pTRE-Tight. C: Map of pTRE-Tight-luciferase. D: Map and MCS of pcDNA3.1/myc-His A.

drugs and tumor cells to induce apoptosis in tumor cells.

Previous analysis of Trop-2 protein depended on systems of *Trop-2* gene expression in cells that were difficult to manipulate. In this study, to explore the effects of the *Trop-2* gene *in vitro* conveniently, the Tet-On advanced inducible gene expression system was established in the human embryonic kidney 293 cell line to increase the Trop-2 protein level by the addition of DOX.

MATERIALS AND METHODS

Cell lines and reagents

Cells from the human embryonic kidney 293 cell line, plasmids including pcDNA3.1/myc-His A, pEGFP-C3, pTet-On-Advanced, pTRE-Tight, and pTRE-Tight-luciferase, and *Escherichia coli* (*E. coli*) DH5α were maintained in the Key Laboratory of Antibody Techniques of National Health Commission, Nanjing Medical University. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were supplied by Lianmai Biological Engineering (Shanghai, China). G418, DOX, and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA, USA). The Firefly Luciferase Assay Kit was obtained from Promega Biotechnology (Beijing, China). DNA Marker, T4 DNA ligase, the restriction enzymes *EcoR*I and *Xba*I, and PrimeScript RT Master Mix were purchased from TaKaRa (Otsu, Japan). Primers were synthesized by GenScript Biotechnology (Nanjing, China).

Cell culture

The 293 cells were previously cultured in our laboratory and maintained as monolayer cultures in DMEM supplemented with 10% FBS in a humidified chamber with 5% CO_2 at 37 ℃.

Determination of the optimal screening concentration for G418

The 293 cells were plated at equimolar amounts of 8×10^3 cells/well in 96-well plates (triplicate wells per data point). G418 was added after 24 hours, and the concentration range for selecting positive 293/pTet-On-Advanced cell lines was 50–800 μg/mL. The G418 growth curve was drawn using an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. The lowest concentration of G418, in which 293 cells could not survive on the sixth day after the addition of G418, was selected as the screening concentration, and the maintenance concentration was half of the screening concentration.

Reverse transcription PCR (RT-PCR)

The cells (3×10^5 cells/well) were seeded in 6-well plates and incubated in media for 24 hours. They were then lysed, and the total RNA was isolated using TRIZOL reagent (Invitrogen) in accordance with the manufacturer's instructions. RNA was reverse-transcribed with Prime Script RT Master Mix (TaKaRa). The resulting cDNA was then taken as the template for PCR amplification using specific primers designed and synthesized by GenScript. Results were visualized on an agarose gel.

Establishment and selection of stable 293/pTet-On-Advanced cell lines

The pTet-On-Advanced plasmid was transfected into 293 cells in equimolar amounts in 6-well plates using Lipofectamine 2000 reagent (Invitrogen). G418 was added after 24 hours for selection. Surviving cells after G418 treatment were plated in 96-well plates for selecting monoclonal cell strains. The positive cell lines were harvested through RT-PCR assay by detecting tetracycline response transcription activating factor (rtTA) from optioned monoclines, and *β*-actin gene was used as the reference gene (primers of *rtTA* gene: forward 5'-ACAAGGAAACTCGCTCAAA-3', reverse 5'-GGCATAGAATCGGTGGTAG-3'; primers of *β*-actin gene: forward 5'-CTGGGACG-ACATGGAGAAAA-3', reverse 5'-AAGGAAGG-CTGGAAGAGTGC-3'). Lastly, the plasmid pTRE-Tight-luciferase was transfected into those positive cell lines, and DOX was used to induce the expression of luciferase. Stable 293/pTet-On-Advanced cell lines were obtained by measuring luciferase values with a Firefly Luciferase Assay Kit (Promega) in accordance with the manufacturer's instructions.

Luciferase induction assay

Stable 293/pTet-On-Advanced cell lines (5×10^4 cells/well) were plated in a volume of 2–3 mL of complete culture medium into 24-well plates (triplicate wells per data point), and the pTRE-Tight-luciferase plasmid was transfected into those stable cell lines. DOX was added at final concentrations of 0, 0.1, 1.0, 10, 100, or 1000 ng/mL. The cells were maintained in 24-well plates for 48 hours (37 ℃, 5% CO_2). Each sample was assayed for luciferase activity using a standard luciferase assay, and a curve was drawn.

Establishment of the recombinant vector

The complete sequence of Trop-2 mRNA was obtained from GenBank (gene serial number: NM_002353). Then, Trop-2 mRNA was used as a

template to synthesize cDNA by reverse transcriptase catalysis, and the cDNA was cloned into the pcDNA3.1/myc-His A plasmid (***Fig. 1D***) to construct the recombinant vector pcDNA3.1/myc-His A-Trop-2 through introducing the restriction enzymes *EcoR*I and *Xba*I, and the recombinant vector was stored in the laboratory. The *Trop-2* gene segment was amplified from the pcDNA3.1/myc-His A-Trop-2 plasmid (primers of *Trop-2* gene: forward 5'-CGGAATTCCCACCATGGCTCGGGGCCCCGGC-C-3', reverse 5'-GCTCTAGACTACAAGCTCGGT-TCCTTTCTCA-3'). Then, the *Trop-2* gene segment was cloned into the pTRE-Tight vector by the restriction enzymes *EcoR*I and *Xba*I, and the recombinant vector pTRE-Tight- Trop-2 was constructed.

The pTRE-Tight-Trop-2 plasmid was added to the competent cells of *E. coli* DH5α, and the mixture was put on ice for 30 minutes. The mixture was then transferred into 700 μL of Luria-Bertani (LB) broth. The culture was incubated at 37 ℃ for 1 hour and centrifuged for 10 minutes at 3000 rpm. Transformed *E. coli* DH5α was plated on LB agar plates containing 100 μg/mL ampicillin. The plates were incubated at 37 ℃ until colonies became visible. Monoclonal colonies were selected to undergo PCR for the *Trop-2* gene.

Analysis of the stable cell lines transfected with recombinant vector pTRE-Tight-Trop-2 *via* flow cytometry

The recombinant vector pTRE-Tight-Trop-2 was transfected into stable 293/pTet-On-Advanced cell lines, and Trop-2 expression was induced by continuously adding 1000 ng/mL DOX to the culture medium for 2 weeks. Cells were then harvested and sorted by flow cytometry to determine the different levels of Trop-2 expression.

Cell proliferation assay

The cells transfected with the recombinant vector were seeded at 1×10^3 cells/well in 96-well plates (three replicated wells per data point). Cell proliferation was measured every 48 hours using MTT colorimetric assay, and cell growth curves were calculated.

Cell monolayer wound healing assay

Wounds were generated in confluent cell monolayers grown at 1×10^5 cells/well in 24-well plates with media containing 10% FBS using a sterile pipette tip. Healing was observed at 0, 24, and 48 hours along the scrape line and a representative field for each cell line was photographed.

Transwell migration and invasion assay

Cells were cultured in 6-well plates and transfected with the recombinant vector for 48 hours. In the transwell migration assay, cells were resuspended in the serum-free medium (200 μL) and added to the upper chamber of the transwell insert (2×10^5 cells/well). Cell culture supernatants, gathered separately and stored in a chamber at −20 ℃, were added to the lower chamber. Cells were incubated at 37 ℃ for 48 hours. The cells in the upper chamber were removed by cotton swabs, while the cells on the lower surface of the polycarbonate membrane were fixed with 4% formaldehyde in phosphate buffered saline (PBS) and stained with 2% crystal violet in 2% ethanol. The MTT assay was performed to measure the cells in the lower chamber.

The procedure for the transwell invasion assay was similar to the transwell migration assay. In the transwell invasion assay, matrigel (100 μL) and serum-free medium (200 μL) were mixed, and solidified matrigel (100 μL) was added into the chamber before cell inoculation. After 48 hours, cells on the lower surface of the polycarbonate membrane were measured by crystal violet staining and cell counting methods.

Statistical analysis

Quantitative results are shown as mean ± standard deviation (SD). Statistical analysis was done using the Student's *t* test for paired data between the control and the Trop-2 group. $P<0.05$ was considered statistically significant.

RESULTS

Establishment of stable 293/pTet-On-Advanced cell lines

The lowest concentration of G418 (300 μg/mL), in which 293 cells could not survive, was selected as the screening concentration of G418, and the maintenance concentration of G418 (150 μg/mL) was obtained (***Fig. 2A***). 21 monoclonal strains were harvested through selection with G418 (300 μg/mL), and 10 monoclonal cell strains that tested positive for the *rtTA* gene were selected (numbered 1–10, ***Fig. 2B***). Two groups, including group A (DOX 1000 ng/mL added to the culture medium) and group B (without DOX), of the 293/pTet-On-Advanced cell lines containing the pTRE-Tight-luciferase plasmid were generated. The luciferase values of group A (except for No. 4) were much higher than those of group B

(P<0.05), and four monoclonal cell strains (No. 1, 2, 6, and 9) were considered stable 293/pTet-On-Advanced cell lines (luciferase values >3000 relative light units [RLUs]) (***Fig. 2C***). Considering background interference, the ratio of luciferase values was applied to reflect the transfection efficiency in a more objective way (***Table 1***), and cell strain No. 1 was taken to be the 293/pTet-On-Advanced cell line with the highest stability, and then it was utilized in subsequent assays. In the No. 1 cell strain, the

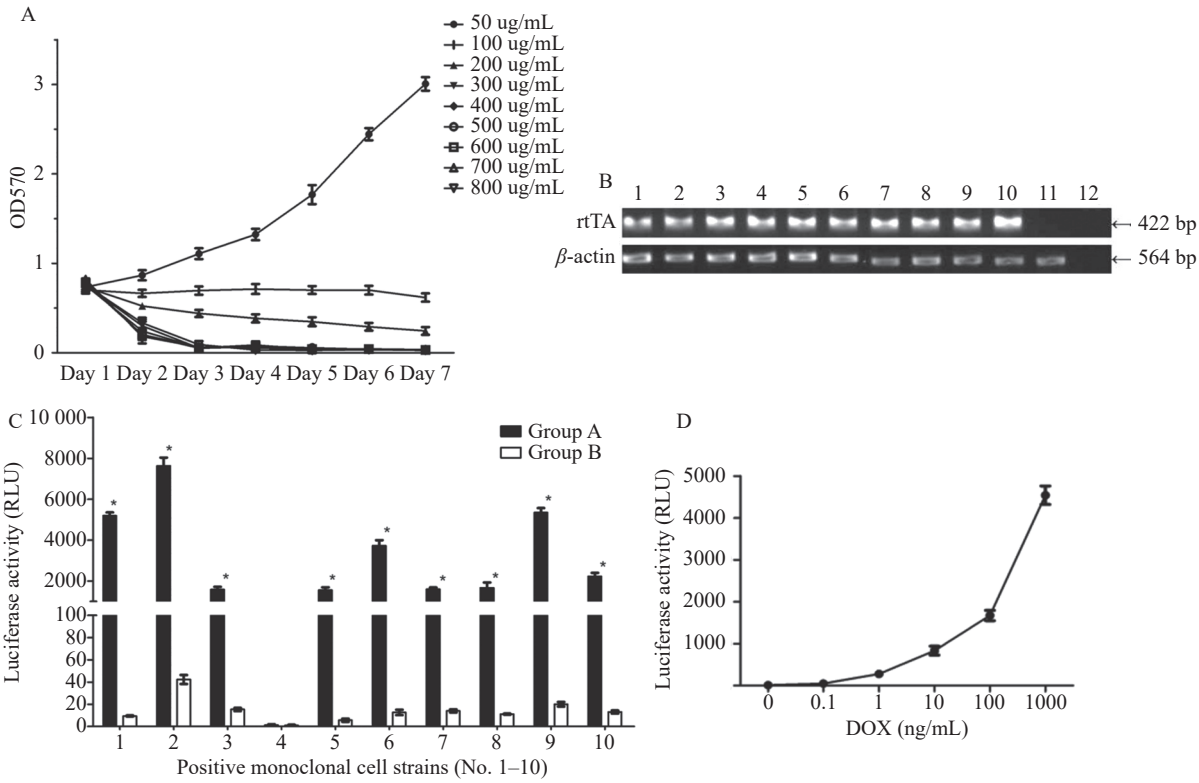

Fig. 2 **Establishment of stable 293/pTet-On-Advanced cell lines.** A: G418 growth curve. Cell counts were measured by MTT assay to obtain the screening concentration of G418 (300 μg/mL) and the maintenance concentration of G418 (150 μg/mL) (n=3). B: Reverse transcription PCR (RT-PCR) amplification of *rtTA* gene segment. Lanes 1–10: positive cell strains (No. 1–10). Lane 11: 293 cell line. Lane 12: negative control. C: Two groups of 293/pTet-On-Advanced cell lines containing the pTRE-Tight-luciferase plasmid were generated. Luciferase values of groups A and B were measured to assess the differences among cells inducing luciferase expression and luciferase activity (n=3). *P<0.05. D: Effect of different concentrations of doxycycline (DOX) on the expression of luciferase. Luciferase activity was assessed to describe the association between the expression of luciferase and concentrations of DOX (n=3).

Table 1 **Comparison of luciferase values between cells of group A and group B ($\bar{x} \pm s$, n=3)**

Cell strain	Group A	Group B	P	Group A/B
1	5193.33±161.67	9.44±0.51	<0.05	549.93
2	7629.67±402.47	42.33±4.01	<0.05	180.23
3	1589±127.43	15.36±1.46	<0.05	103.41
4	1.37±0.64	1.08±0.49	3.77	1.27
5	1555±137.82	5.64±1.37	<0.05	275.71
6	3733±265.25	12.67±2.45	<0.05	294.71
7	1605.67±81.21	13.99±1.43	<0.05	114.77
8	1671.33±256.04	11.16±0.44	<0.05	149.81
9	5344.67±216.15	20±2.05	<0.05	267.23
10	2232±167.65	13.01±1.39	<0.05	171.60

Group A: 293/pTet-On-Advanced cell lines containing the pTRE-Tight-luciferase plasmid with doxycycline (DOX) (1000 ng/mL). Group B: 293/pTet-On-Advanced cell lines containing the pTRE-Tight-luciferase plasmid without DOX. Group A/B: the ratio of luciferase values between group A and group B.

expression of luciferase was correlated with the DOX concentration (***Fig. 2D***).

Establishment of the recombinant vector pTRE-Tight-Trop-2

The *Trop-2* gene segment was cloned into the pTRE-Tight vector to construct the recombinant vector pTRE-Tight-Trop-2. The pTRE-Tight-Trop-2 plasmid was added to the competent cells of *E. coli* DH5α. Two monoclonal positive strains containing gene *Trop-2* were obtained. Gene sequences of the plasmids extracted from the two monoclonal strains were verified to be correct, and the plasmid was named pTRE-Tight-Trop-2.

Adjustment of Trop-2 expression in 293 cells by DOX

Three different cell groups were generated through cell strain No. 1: untreated cells (group Ⅰ); cells transfected with pTRE-Tight-Trop-2 plasmids in the presence of DOX (1000 ng/mL, group Ⅱ); and cells transfected with pTRE-Tight-Trop-2 plasmids without DOX (group Ⅲ). The cells of groups Ⅰ and Ⅲ were grown in DMEM with 10% FBS and 150 μg/mL G418. The cells of group Ⅱ were maintained in the same medium but with the addition of 1000 ng/mL DOX. To detect the expressions of Trop-2, groups Ⅰ and Ⅲ were used as controls in subsequent assays.

RT-PCR and flow cytometry were performed to assess whether there were any differences in the Trop-2 mRNA levels or Trop-2 protein expressions. Trop-2 mRNA was detected in groups Ⅱ and Ⅲ but could not be detected in group Ⅰ (***Fig. 3A***). However, the level of Trop-2 mRNA in group Ⅱ was much higher than that in group Ⅲ. In addition, Trop-2 protein

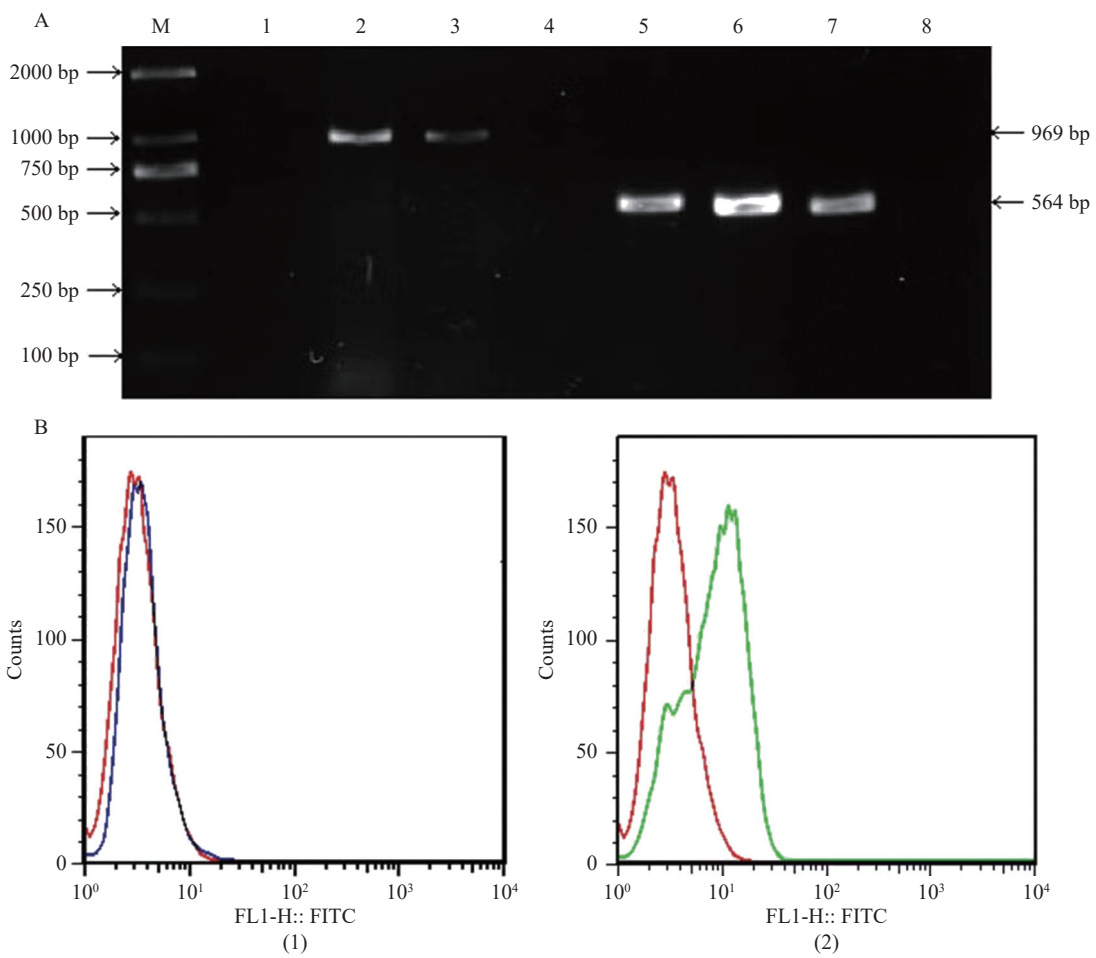

Fig. 3 **Adjustment of Trop-2 expression in 293 cells by DOX.** A: Reverse transcription PCR (RT-PCR) amplification of *Trop-2* and *β*-actin gene segments. Lane M: DL2000 DNA Marker. Lanes 1–4: RT-PCR amplification of *Trop-2* gene segment of groups Ⅰ, Ⅱ, and Ⅲ, and negative control, respectively. Lanes 5–8: RT-PCR amplification of *β*-actin gene segment of groups Ⅰ, Ⅱ, and Ⅲ, and negative control, respectively. B: The Trop-2 protein expression of different cell lines by flow cytometry analysis. The red curve represents Trop-2 protein expression of group Ⅰ; the green curve demonstrates Trop-2 protein expression of group Ⅱ; the blue curve shows group Ⅲ.

expression was present on the cell surface as shown by flow cytometry (***Fig. 3B***). The Trop-2 protein levels of groups Ⅰ and Ⅲ could not be detected, while those of group Ⅱ were remarkably high.

Biological functions of Trop-2 protein

The cells of groups Ⅰ, Ⅱ, and Ⅲ were applied to explore the biological functions of Trop-2 protein. We found that the proliferation of group Ⅱ was significantly greater than those of group Ⅰ or group Ⅲ (***Fig. 4A***). Photomicrographs of the scratch assay showed a narrower zone of wounding in group Ⅱ compared with those in groups Ⅰ and Ⅲ after 24 hours. What's more, the wound was barely visible in group Ⅱ at 48 hours, indicating a completely closed wound, while the wider wound areas of groups Ⅰ and Ⅲ showed the retarded migration of cells (***Fig. 4B***). At the 24th hour and the 48th hour, the migration distances of group Ⅱ were higher than those of groups Ⅰ and Ⅲ (***Fig. 4C***). In the transwell migration assay, through crystal violet staining, cell counts on the lower surface of the polycarbonate membrane of group Ⅱ were higher than those of groups Ⅰ and Ⅲ (***Fig. 4D***), and the MTT assay showed that the OD570 value of group was higher than those of groups Ⅰ and Ⅲ (***Fig. 4E***). In the transwell invasion assay, by crystal violet staining and cell counting methods, cells on the lower surface of the polycarbonate membrane of group Ⅱ were more than those of groups Ⅰ and Ⅲ (***Fig. 4F*** and ***4G***).

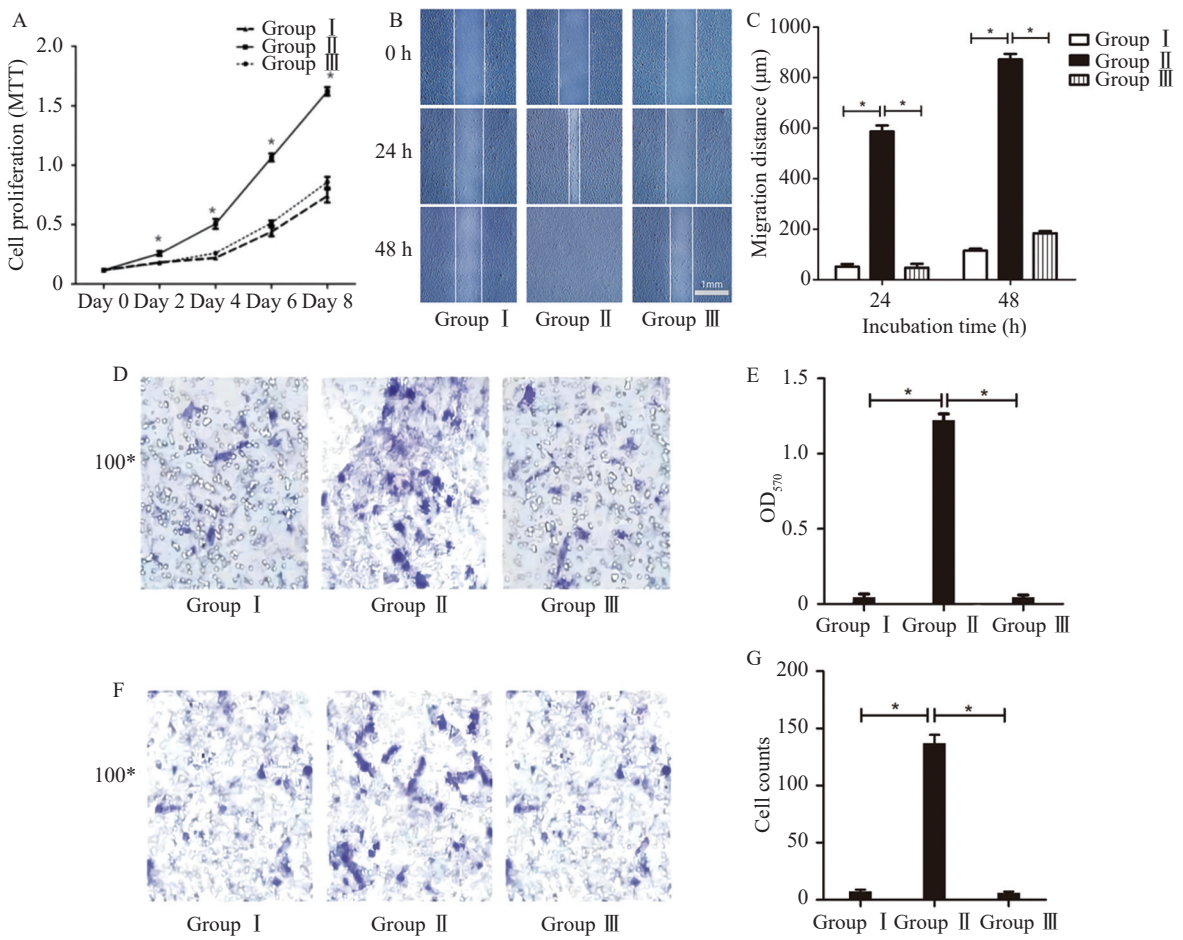

Fig. 4 **Biological functions of Trop-2 protein.** A: Cell proliferation assay by MTT. The data were relative to the number of cells at day 0. MTT assay was performed every 48 hours for the cell growth curve (n=3). *P<0.05. B: The monolayer wound healing assay. Pictures were taken at 0, 24, and 48 hours after the incision (×40). A representative field for each cell line is shown. C: Migration distance. The migration distances of the monolayer wound healing assay were measured to indicate the migration ability (n=3). *P<0.05. D: The transwell migration assay. After 48 hours, cells on the lower surface of the polycarbonate membrane were stained with crystal violet (×100). E: MTT assay. The OD_{570} values of the cells in the lower chamber were measured to indicate the migration ability (n=3). *P<0.05. F: The transwell invasion assay. Cells on the lower surface of the polycarbonate membrane were visualized with crystal violet staining after 48 hours (×100). G: Cell counting. Cells on the lower surface of the polycarbonate membrane were counted to indicate the invasion ability (n=3). *P<0.05.

DISCUSSION

In general, protein concentrations in cells depend on the natural gene expression system of the cell, and it is difficult to manipulate these concentrations by artificial means. This difficulty complicates the study of the biological functions of any particular gene, as it is hard to regulate gene intracellular expression within existing cell lines. The Tetracycline-induced expression system regulates the expression of protein levels *via* artificial controls, making the system useful for the research of gene functions.

The Tetracycline-induced expression system is a regulatory system based on the essential regulatory components of the *E. coli* tetracycline-resistance operon, and includes Tet-On advanced and Tet-Off advanced inducible gene expression systems. The Tet-Off system was first reported in 1992, and the Tet-On system was first reported in 1995[15–16]. Tet-On advanced and Tet-Off advanced inducible gene expression systems have been optimized for use in mammalian cells. They consist of the regulator vector and the response vector. The regulator vectors, pTet-On-Advanced and pTet-Off-Advanced express one of the tetracycline-controlled transactivators. The response vector, pTRE-Tight, contains an improved TRE within the promoter which induces or inhibits expression of the target gene[17]. The components of the Tet-On advanced induction system are the Tet-On-Advanced transactivator and the pTight inducible promoter. The Tet-On advanced transactivator is a modified protein that is optimized for expression regulation in mammalian cells, with high sensitivity and fidelity[18]. The pTight inducible promoter controls transcription of the target gene[19]. In the presence of DOX, the transactivator binds tightly and specifically to the TRE in pTight, resulting in high-level transcription of the downstream target gene[20]. The Tet-On system generates maximal expression coupled with very low basal promoter activity to yield induction levels that are both highly responsive and concentration-dependent[21]. At present, in addition to the Tetracycline induced expression system, scientists have also developed a variety of gene regulation systems including the Cre-loxp system, Flp-frt system, and Dre-rox system. However, the tetracycline induced expression system has unique advantages, such as extremely tight regulation, high specificity, no pleiotropic effects, high inducibility, fast response time, high absolute expression levels, and well-characterized effector promoter activation. The DOX concentrations required for induction with Tet-On advanced systems are far below cytotoxic levels for either cell culture or transgenic studies[22]. Despite these advantages, the Tet-On advanced induction system also has its limitations, such as high cost, long cycles, complex composition, and high technical requirements.

Trop-2 protein is highly homologous to the cell–cell adhesion molecule Trop-1 (EpCAM, GA733-2)[23–26]. Previous studies showed that Trop-2 exists at elevated expression in various human carcinomas, but is rarely present in normal adult tissues, such as ovarian cancer, breast cancer, and pancreatic cancer, and it is a calcium signal transducer that is associated with poor prognosis in a variety of human carcinomas. Trop-2 is highly expressed in 90% of patients with triple-negative breast cancer. Also, elevated levels of Trop-2 have been shown to strongly induce the mitogen-activated protein kinase activity and promote metastasis in pancreatic cancer. Therefore, Trop-2 has become a popular target for antibody conjugating drugs. For example, Trop-2 is a molecular target for sacituzumab govitecan, an antibody-drug conjugate approved in the United States for the treatment of triple-negative breast cancer and urothelial carcinoma.

In the present study, a stable 293/pTet-On-Advanced cell line was established successfully. Ten positive monoclonal cell strains containing the *rtTA* gene were selected through G418 (300 μg/mL) screening and were successfully transfected with pTRE-Tight-luciferase plasmids. Luciferase values from cell strain No. 4 could not be detected, as the pTRE-Tight-luciferase plasmid in cell strain No. 4 was lost. Therefore, it may have unstable transfection. Four monoclonal cell strains (No. 1, 2, 6, and 9) were considered stable 293/pTet-On-Advanced cell lines. They could markedly increase the levels of luciferase in the presence of DOX. Cell strain No. 1 was chosen as the most stable 293/pTet-On-Advanced cell line from the transfection efficiency in a more objective way, so it was utilized in subsequent assays. Using the Tet-On advanced inducible gene expression system, the level of Trop-2 protein in *vitro* was increased viably *via* transfecting the stable 293/pTet-On-Advanced cells with the recombinant vector pTRE-Tight-Trop-2 and exposing them to DOX. As a consequence, the biological functions of Trop-2 could be investigated easily. Transwell migration assay is usually used to indicate the migration ability of cells able to penetrate the polycarbonate membrane of the transwell insert. However, here the transwell invasion assay was applied to indicate the invasion ability of cells which could degrade matrigel by secreting

matrix metalloproteinases (MMPs), and go through the polycarbonate membrane. From the results of crystal violet staining, cell counts able to go through the polycarbonate membrane in the transwell migration assay were much higher than those in the transwell invasion assay, so MTT assay was used in the previous assay, while cell counting was applied in the latter assay. The results showed that Trop-2 might have an intrinsic ability to foster cell proliferation, migration, and aggression. This study suggests that Tet-On advanced inducible gene expression system is sensitive, controllable, and specific in regulating the expression of target genes in *vitro*, and provides a viable and convenient platform for the exploration of gene functions.

However, this study also had its limitations. Although the Tet-On advanced inducible gene expression system was shown to regulate the expression of *Trop-2* gene *in vitro*, it was not applied *in vivo*. Therefore, it is hoped that an *in vitro* analysis could be combined with an *in vivo* exploration to study the functions of the target genes or proteins comprehensively.

Acknowledgments

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