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Tet-On lentiviral transductants lose inducibility when silenced for extended intervals in mammary epithelial cells

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ABSTRACT

Silencing of virally transduced genes by promoter methylation and histone deacetylation has been a chronic problem both experimentally and therapeutically. We observed frequent silencing of the tetracycline-inducible Tet-On promoter borne by the Tripz lentivirus in mammary epithelial cell lines. We found that silencing could be prevented by continuous induction, but uninduced Tet-On gradually became uninducible, suggesting promoter modification. Accordingly, silencing was reversible by a common inhibitor of histone deacetylases, sodium butyrate. The effect was cell-line dependent, as HEK293 cells exhibited only moderate silencing that could be partly reversed by extended induction. These results indicate the need to test individual cell lines prior to using this system for studies that require induction after long periods of repression such as in animal models or RNA interference screens.

1. Introduction

Downregulation of transgenes following viral transduction continues to slow application of transgenic technologies (Papadakis et al., 2004; Johansen et al., 2002). Strong viral promoters in particular are susceptible to silencing by cellular antiviral responses, leading to methylation, deacetylation, and chromatin condensation (Papadakis et al., 2004). One motivation for the development of tetracycline-regulated promoters was the hope that they might escape this mechanism while repressed, thus allowing robust expression upon induction (Johansen et al., 2002).

Since their invention by Bujard and Gossen over 20 years ago, many variations of tetracycline-regulated gene expression systems have been developed and employed widely in genetic engineering of living systems (Gossen and Bujard, 1992; Bockamp et al., 2002). The classic Tet-Off system relies on the bacterial tet repressor fused to the VP16 transactivator domain, known as tTA. Tet operator segments are embedded in a CMV core promoter, and expression is constitutive in the absence of tetracycline or its analog doxycycline. Tetracycline binding blocks this activity. In the Tet-On version, reverse tTA (rtTA), the tet repressor has been modified so that tetracycline induces rtTA binding to the operator and subsequent transcriptional activation. Additional variants have been developed that greatly enhance induction levels, such as rtTA3 (Zhou et al., 2006). While rtTA was originally encoded on a separate vector from its target, necessitating two rounds of antibiotic selection and screening of clones for well regulated expression, more recently both elements have been combined into a single vector in a lentiviral backbone allowing selection of a population of such cells and obviating cloning (Szulc et al., 2006). Since lentiviruses tend to integrate into active chromatin, this design would theoretically be expected to minimize silencing by adjacent heterochromatin, a problem that limited utility of the original system (Ciuffi, 2008).

The single-vector, doxycycline-inducible shRNAmir lentiviral system Tripz (Fig. 1) (http://dharmacon.gelifesciences.com/uploadedfiles/resources/ptripz-inducible-lentiviral-manual.pdf) developed by OpenBiosystems has many desirable features and is widely used in biomedical research, with 565 citations listed by Google Scholar. The synthetic transcription factor rtTA3 is expressed constitutively from the UBC promoter to drive universal expression. Puromycin-resistance is encoded by the same transcript, ensuring that any resistant cell also encodes rtTA3. The target of rtTA3, the TRE promoter, drives expression of red fluorescent protein (RFP) with a gene-specific small hairpin RNA (shRNA) encoded in its tail. Thus red cells must express the shRNA and downregulate the target gene. Conversely, removal of the

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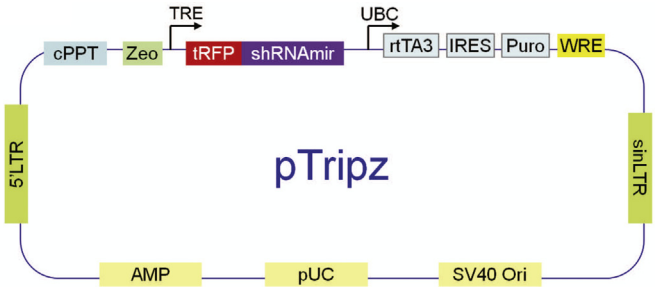

Fig. 1. Structure of pTripz Plasmid. The synthetic transcription factor rtTA3 is activated by tetracycline or doxycycline to bind the tetracycline response element (TRE). TRE drives expression of RFP and shRNA in the same transcript. Puromycin resistance is constitutively driven by the UBC promoter independently of doxycycline.

inducer should result in rapid loss of RFP and reversal of knockdown; and re-addition of the inducer should restore RFP expression and knockdown. Theoretically, this strategy allows the expansion of cells in the absence of toxic effects of knockdown and allows comparison of populations that differ only in attenuation of a single gene. In our experience reported here, the system lost inducibility in cells of mammary epithelial lineage.

2. Methods

MCF7, MDA-MB-231, HEK293, and HEK293T cells were purchased from ATCC. The immortalized human mammary epithelial cell line HMLE was a kind gift from Robert Weinberg. The lentiviral vector Tripz containing shRNA inserts was purchased from OpenBiosystems and packaged by co-transfection into HEK293T with p8.74 and pMD2.G (kind gifts from Didier Trono). Lentiviral supernatants were collected 48 h later, filtered, and applied to cells in the presence of 8 μg/ml polybrene. Target cells were infected at a multiplicity of infection of about 0.5 infectious units per cell. After 48 h, 0.5 μg/ml doxycycline was added to determine the infection efficiency and maintained unless otherwise noted. After 72 h, puromycin was added (1 μg/ml) and maintained throughout the experiments unless otherwise noted. Uninfected cells were killed by 4 days of selection, and all surviving cells were RFP-positive. Puromycin-resistant cells were pooled and analyzed as populations rather than clones. Sodium butyrate (Sigma) was resuspended in water, filtered, and applied at a final concentration of 1 mM. Transcriptional induction was measured by RT-qPCR as described (Walia et al., 2011). *P* values were obtained by Student's two-tailed *t* test.

3. Results

We found that when Tripz was transduced into breast cancer cell line MCF7, the system initially produced high fluorescence upon induction with doxycycline (Fig. 2A). As expected, after one week in the absence of inducer, red fluorescence was nearly undetectable. However, upon reintroduction of doxycycline, fluorescence was much fainter than originally observed (Fig. 2A, lower). We found that this silencing effect could be prevented by maintaining the cells in doxycycline inducer continuously (Fig. 2B), suggesting that silencing was due to chromatin modification of the inactive promoter.

To test this, we treated cells with sodium butyrate (Na Butyrate), a well known inhibitor of histone deacetylases (HDAC), that causes histone hyperacetylation, chromatin decondensation, and activation of silenced promoters (Monneret, 2005). We found that

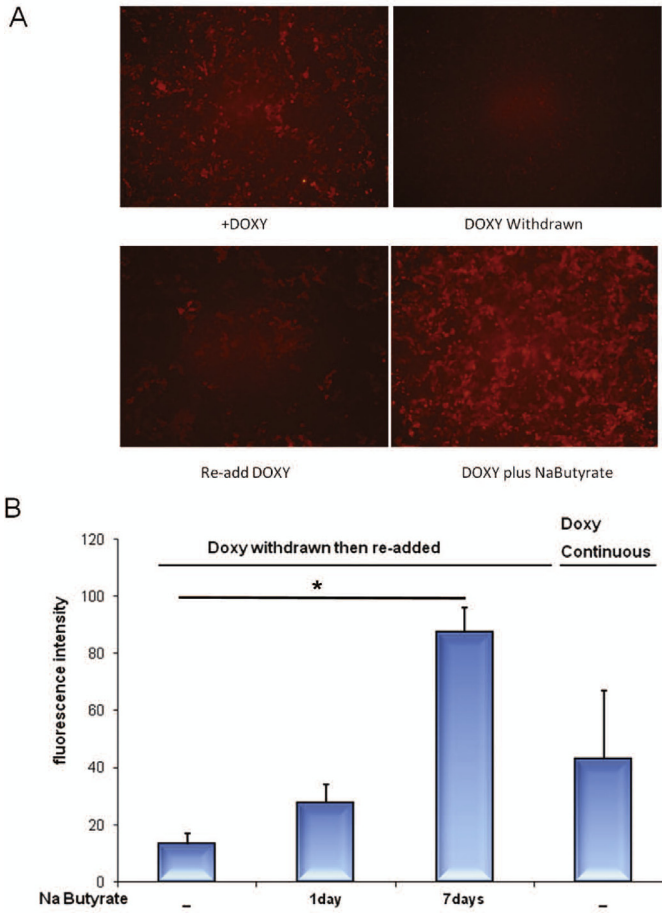

Fig. 2. Rescue of doxycycline-inducibility by sodium butyrate (Na Butyrate). A) Microimages of MCF7 cells transduced with pTripz. RFP expression was induced with 0.5 μg/ml doxycycline (+Doxy), then withdrawn for one week. Cells were then seeded into a 96 well plate, 14,000 per well. Re-addition of doxycycline did not result in full expression unless Na Butyrate was included. All images were collected at the same exposure time and magnification, 200×. B) Fluorimetric quantification of RFP. After doxycycline induction for 24 h, doxycycline was withdrawn for one week then restored in the absence or presence of Na Butyrate for one or seven days. Control had continuous exposure to doxycycline. To measure RFP, 14,000 cells were transferred to a 96 well plate the day before reading it. Puromycin selection (1 μg/ml) was maintained throughout the experiment. *$p=0.04$.

24 h of exposure to Na Butyrate increased RFP expression by twofold, and 7 days exposure increased it by sixfold, completely reversing the silencing effect (Fig. 2B).

A similar loss of inducibility was observed when the Tripz vector was used to express a toxic protein. We replaced the RFP-shRNA cassette with a cDNA encoding hCLCA2 bearing a Flag tag. The resulting lentivirus pTripz-h2-flag was packaged, and HMLE cells were infected and selected with puromycin for approximately one week. Immunoblotting revealed low expression of the transgene after induction (Fig. 3A, lane 3), and less than 1% of the cells were positive by immunofluorescence (Fig. 3B). Addition of 1 mM Na Butyrate for 3 days dramatically increased expression. Similar results were obtained in breast cancer cell line MDA-MB-231 (Fig. 3C). Quantification by RT-qPCR confirmed that induction was at the transcriptional level (Fig. 3D).

These results demonstrate that promoter silencing remains a major problem with transgenes driven by tetracycline-regulated promoters. The cells remained puromycin-resistant so they must still have expressed the rtTA3 transactivator. However, we noticed that if selection was omitted for a few passages then restored, most of the cells were killed, suggesting that the UBC promoter driving puromycin resistance was also subject to silencing. Silencing was