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*Communication*

# Construction of a Regulatable Cancer-Specific Adenoviral Expression System Using Human Telomerase Reverse Transcriptase Gene Promoter

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**We constructed a novel cancer-specific regulatable adenoviral expression system comprising two vectors: one expressing rtTA, a reverse tetracycline transactivator regulated by the human telomerase reverse transcriptase (*hTERT*) gene promoter, the other expressing the target gene regulated by the tetracycline response element (TRE). rtTA transactivates target gene expression in the presence of doxycycline. Using these vectors, we constructed an adenoviral expression system, the Tel-On system, which enables highly efficient target gene transduction. This system enabled efficient and regulatable cancer-specific gene expression, and can be used in targeted cancer gene therapy.**

**Key words:** *hTERT*; Tet-On system; adenovirus; cancer

Control of gene expression using tissue- and cell-specific promoters has been tested extensively as a means of targeting transgene expression. Here we propose a regulatable cancer-specific adenoviral expression system that utilizes a cancer-specific promoter, a tetracycline-regulation system, and an adenoviral gene-expression system. For cancer-specific gene expression, we used the promoter region of the human telomerase transcriptase (*hTERT*) gene.[1)] The *hTERT* gene is known to be expressed in most malignant tumors but not in normal human tissues.[2–4)] We hypothesize that the *hTERT* promoter can be used in cancer-specific expression of transgenes.

Next we used the Tet-On system to enable the regulation of the target gene following the addition of doxycycline (Dox), a tetracycline derivative.[5)] The Tet-On system comprises two vectors: one expressing a reverse tetracycline transactivator (rtTA) and the other expressing the target gene under the control of the tetracycline response element (TRE). rtTA binds to TRE in the presence of Dox and transactivates the expression of the target gene. We then combined these elements and constructed adenoviral expression vectors.

The human diploid fibroblast cell line (TIG-1; Institute of Development, Aging, and Cancer, Tohoku University, Miyagi) was cultured in MEM medium (Nissui, Tokyo, Japan) supplemented with 10% Tet-system approved fetal bovine serum (FBS; Takara, Shiga, Japan) and HeLa in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS at 37 °C in 5% $CO_2$.

The 5′-flanking region of the *hTERT* gene (−289 to −25) was amplified by polymerase chain reaction (PCR), as described previously.[1)] The promoter activity of this region is known to be activated in cancer cells but repressed in normal cells.[1)] The cytomegalovirus (CMV) immediately early promoter (CMVp) of the pShuttle vector (Takara) was replaced with the PCR-amplified *hTERT* promoter (hTERTp) flanked by the *Mfe*I and *Nhe*I sites (phTERTp). After digestion with *Not*I and *Kpn*I, PCR-amplified rtTA, for which pTet-On (Takara) had been used as the template, was inserted into the sites, and we designated this vector phTERTp_rtTA (Fig. 1). pShuttle was digested with *Not*I and *Kpn*I, and rtTA was inserted as described above. We designated this vector pCMVp_rtTA (Fig. 1). The CMVp of the pShuttle vector was replaced with CMV*-1p, which was PCR-amplified using pTRE as the template (pCMV*-1p). CMV*-1p contains a Tet-responsive element just upstream of the minimal CMV promoter. We prepared PCR-amplified enhanced green fluorescent protein (EGFP) flanked by the *Xba*I and *Not*I sites and inserted it into pShuttle, phTERTp, and pCMV*-1p. The resulting vectors were designated pCMVp_EGFP, phTERTp_EGFP, and pCMV*-1p_EGFP respectively.

We produced recombinant adenovirus using the Adeno-X Expression System (Takara) according to the manufacturer's protocol. Briefly, after digestion of the pShuttle vectors (Fig. 1) with PI-*Sce*I and I-*Ceu*I, the coding regions were cloned into pAdeno-X viral DNA.

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*Abbreviations*: Dox, doxycycline; *hTERT*, human telomerase reverse transcriptase; Tel-On system, telomerase-dependent Tet-On system; TRE, tetracycline response element

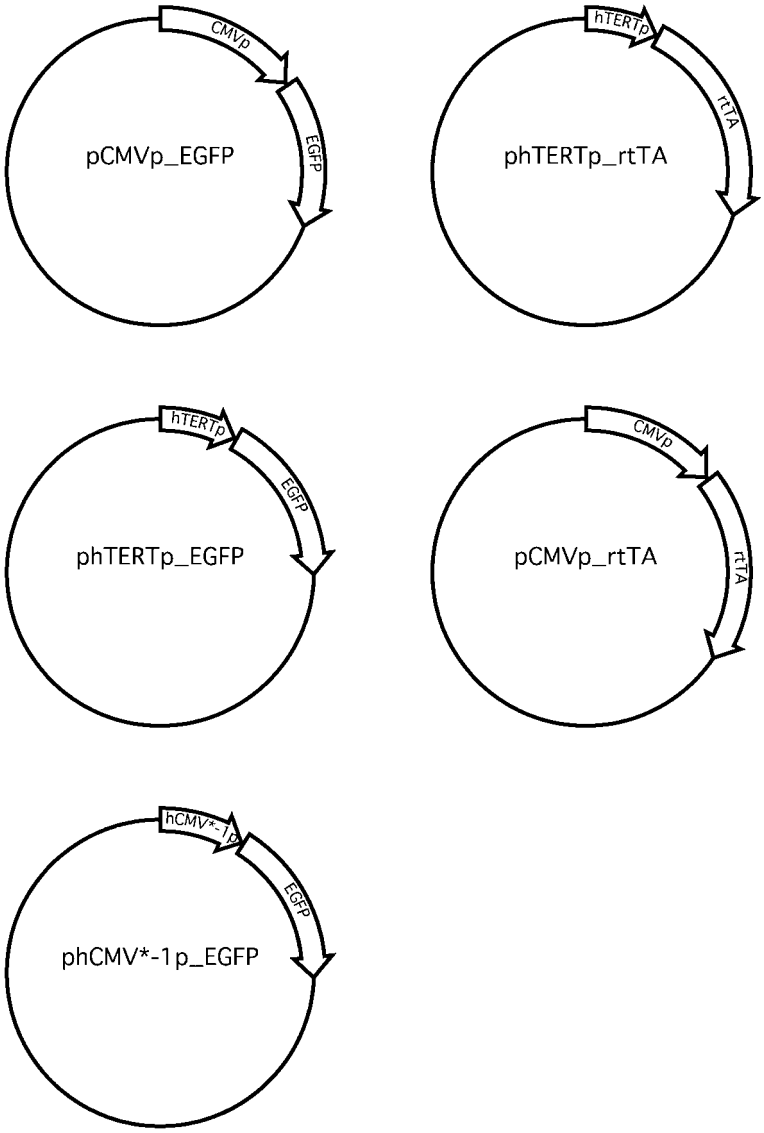


**Fig. 1.** Schematic Diagrams of Vectors Constituting the Regulatable Cancer-Specific Adenoviral Expression System.

Recombinant adenoviruses were prepared by disrupting HEK293 cells transfected with *Pac*I-linearized recombinant adenoviral DNA. The infection and preparation of recombinant adenoviruses was repeated at least 3 times by increasing the number of HEK293 cells in a stepwise manner, thereby amplifying the virus titer to $10^9$–$10^{10}$ pfu/ml. The cells underwent adenoviral transduction for 3 d in the presence and the absence of Dox (1 μg/ml) after infection for 1 h at a multiplicity of infection (moi) of 100.

To assess cancer-specific transgene expression by the *hTERT* promoter in the various human cancer cell lines, we constructed a regulatable adenoviral expression system using Dox. This regulatable system comprises two adenoviruses: Ad_hTERTp_rtTA and Ad_hCMV*-1p_EGFP. First we constructed phTERTp_rtTA, in which rtTA expression is under the control of the *hTERT* core promoter (Fig. 1). rtTA is a fusion of the reverse Tet repressor and the VP16 activation domain. Vector phTERTp_rtTA makes possible the selective expression of rtTA in cells in which *hTERT* expression is up-regulated, such as cancer cells. Vector phCMV*-1p_EGFP expresses EGFP under the control of hCMV*-1p, which comprises TRE and the minimal CMV promoter (Fig. 1). rtTA binds to TRE and activates the transcription of EGFP in the presence of Dox. Thus, EGFP expression is induced in cells that express rtTA and are exposed to Dox. Cells expressing *hTERT*, such as cancer cells, produced rtTA when transfected with phTERTp_rtTA (data not shown). After producing the relevant adenoviruses, we constructed a regulatable adenoviral expression system that enables the regulatable expression of the target gene upon the addition of Dox to cancer cells. We termed it the telomerase-dependent Tet-On system (Tel-On system). Ad_CMVp_EGFP expressing EGFP under the control of the CMV promoter, Ad_hTERTp_EGFP expressing EGFP under the control of the *hTERT* promoter, and

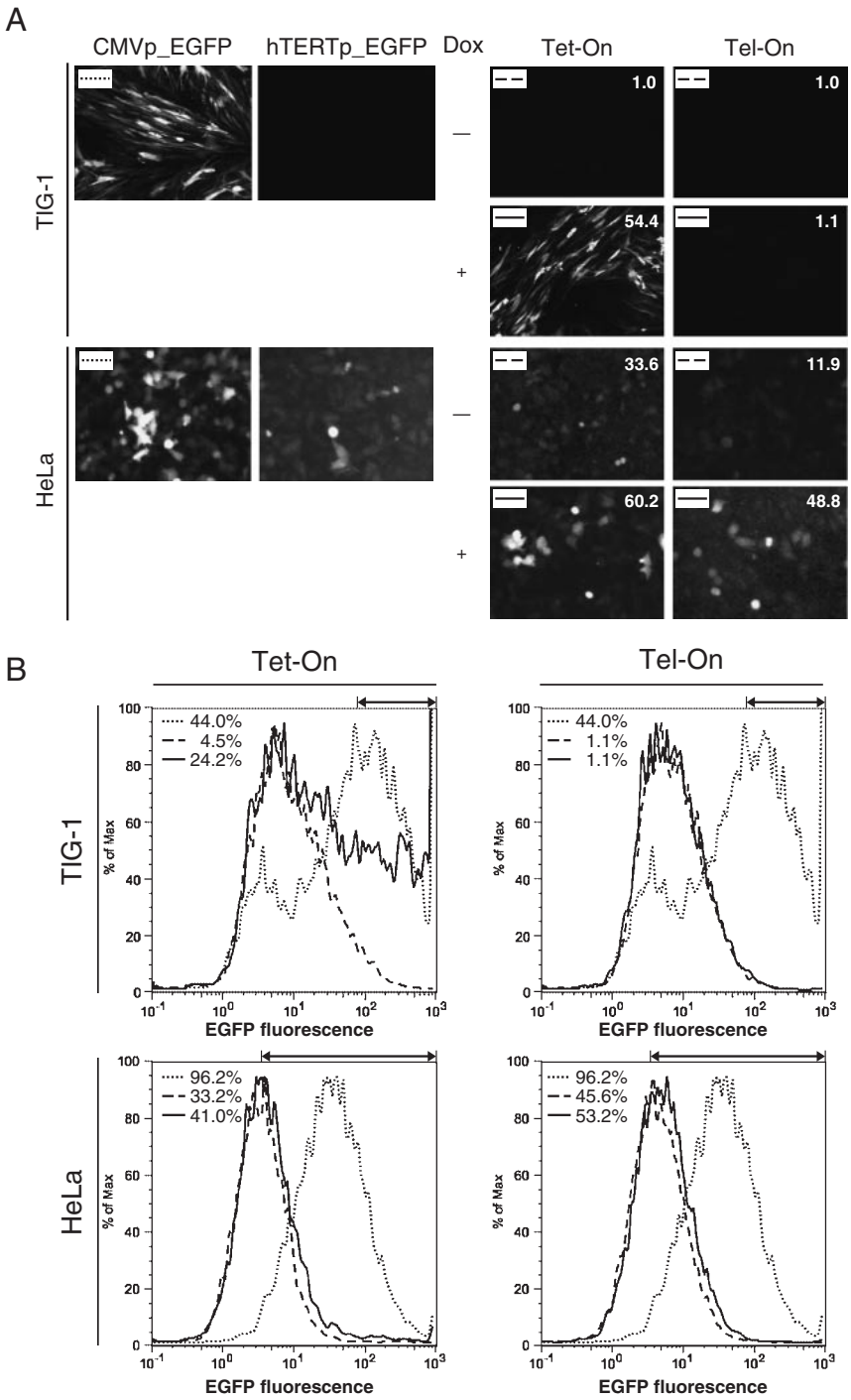


**Fig. 2.** Regulatable Cancer-Specific Adenoviral Expression System.

A, Fluorescence microscopic analysis of the Tet-On and Tel-On systems. Normal cells (TIG-1) and cancer cells (HeLa) were infected with the relevant adenoviruses. Where indicated, Dox (1 μg/ml) was added to the cells. After 48 h, EGFP fluorescence was detected using a fluorescence microscope (IX-50; Olympus, Tokyo). As control experiments, TIG-1 and HeLa were infected with Ad_CMVp_EGFP and with Ad_hTERTp_EGFP (left pannels). In the Tet-On system, cells were infected with Ad_CMVp_rtTA and Ad_hCMV*-1p_EGFP. In the Tel-on system, cells were infected with Ad_hTERTp_rtTA and Ad_hCMV*-1p_EGFP. The lines at the upper left corner of the photographs correspond to those in the histogram of flowcytometer analysis (B). Relative EGFP fluorescence was calculated using ImageJ software, and is shown at the upper right corner of the photographs. B, Flowcytometer analysis of the Tet-On and Tel-On systems. Cells were infected with adenoviruses as described above, and EGFP fluorescence was measured using a flowcytometer (EPCS XL, Beckman Coulter, Miami, FL). Relative numbers of cells in the areas indicated by arrows were measured, and are shown at the upper left corners of the histgrams. Dotted lines indicate histgrams of cells infected with Ad_CMVp_EGFP. Dashed and solid lines showed histgrams of cells infected with the respective adenoviruses and cultured in the absence and the presence of Dox (1 μg/ml) respectively.

Ad_CMVp_rtTA expressing rtTA under the control of the CMV promoter were also prepared.

We tested to determine whether cancer cell-specific expression could be achieved by the Tel-On system. We used telomerase-positive cells of human cancer cell line A549 and HeLa cells and telomerase-negative cells of human diploid fibroblast cell lines, TIG-1, WI-38, and human normal embryonic lung fibroblast MRC-5. The results for TIG-1 and HeLa representative of normal cells and cancer cells are shown in the Fig. 2. Other normal and cancer cell lines demonstrated similar results to TIG-1 and HeLa respectively (data not shown). EGFP fluorescence was detected in all cell lines infected with Ad_CMVp_EGFP, and the infection efficiencies of adenovirus were more than 80% in these cell lines. On the other hand, it was detected only in cancer cells infected with Ad_hTERTp_EGFP. These results indicate that hTERTp can drive the expression of EGFP only in telomerase-positive cancer cells, and further suggests that Ad_hTERTp_EGFP can be used to discriminate between cancer cells and normal cells.

Next we tested the suitability of the Tet-On system. The results were that EGFP fluorescence was augmented in both cancer and normal cells infected with Ad_hCMV*-1p_EGFP and Ad_CMVp_rtTA upon the addition of Dox (Fig. 2), indicating that the Tet-On system can be used for both cancer cells and normal human cells. We then tested the suitability of the Tel-On system that we had developed. Photographs of fluorescence microscopy showed that EGFP fluorescence was augmented only in cancer cells, not in normal cells infected with Ad_hCMV*-1_EGFP and Ad_hTERTp_rtTA, upon the addition of Dox. Although the Tet-On system, but not the Tel-On system, functions in TIG-1 cells, systems worked similarly in HeLa cells, as evidenced by fluorescence microscopy. In HeLa cells, expression inducibility upon the addition of Dox was about 2-fold in the Tet-On system, but more than 4-fold in the Tel-On system. To evaluate the results quantitatively, we measured EGFP fluorescence with a flowcytometer. This revealed that induction of EGFP fluorescence upon the addition of Dox was weak but significant in HeLa cells in both systems. Further, we observed intense inducibility of EGFP fluorescence in TIG-1 cells upon the addition of Dox in the Tet-On system, which was also observed by fluorescence microscopy, suggesting that the Tet-On system works more efficiently in normal cells than in cancer cells. All these results indicate that the Tel-On system we developed functions only in telomerase-positive cancer cells. Further, adenoviral gene transduction enables greater, efficient expression of the target gene in various cells. Thus our Tel-On system is thought to increase the regulatable expression of the target gene in a wide variety of cancer cells.

In conclusion, we successfully constructed a regulatable cancer-cell specific adenoviral expression system, the Tel-On system. This system might become a potent therapeutic agent in the treatment of cancer.

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