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An efficient system to establish multiple embryonic stem cell lines carrying an inducible expression unit

Shinji Masui*, Daisuke Shimosato, Yayoi Toyooka, Rika Yagi, Kazue Takahashi and Hitoshi Niwa

Laboratory for Pluripotent Cell Studies, RIKEN Center for Developmental Biology, Minatojima-minamimachi 2-2-3, Kobe, Japan

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ABSTRACT

The growing use of mouse embryonic stem (ES) cells in research emphasizes their importance in studies of molecular mechanisms that maintain pluripotency and direct cellular differentiation. Although systems for regulatable transgene expression are essential for fine analysis of cellular processes at the molecular level, a strategy for the establishment of multiple ES cell lines carrying any of these systems has not yet been described. Here, we report our development of the ROSA-TET system, an effective system for the establishment of multiple ES cell lines carrying a tetracycline (Tc)-regulatable transgene at the *Gt*(*ROSA*)*26asSor* (*ROSA26*) locus. This system contains a knock-in step of a construct carrying both *lox*P and its mutant sequences into the *ROSA26* locus, followed by a subsequent exchange step that introduces a cDNA to be Tc-regulated to the locus using the recombinase-mediated cassette exchange reaction. Both steps are demonstrated to give desired clones with high efficiency, suggesting that this system can be introduced readily into any ES cell lines, leading to the simultaneous establishment of multiple cell lines carrying different Tc-regulated cDNAs. We believe that use of this system will strongly accelerate molecular biological research using ES cells.

INTRODUCTION

The pluripotency and immortality of mouse embryonic stem (ES) cells have made them attractive for basic studies of regenerative medicine, as well as for gaining molecular insight into cellular differentiation at early developmental stages, which are much more difficult to assess by *in vivo* approaches. Artificially regulated transgene expression systems are indispensable tools for studying the molecular biology of the differentiation process. Tc-regulated transgene expression systems, known as Tet-off and Tet-on systems, have been widely applied to a variety of biological materials, including mammalian cells (1,2). The Tet-off system is based on a Tc-regulatable transactivator (tTA), which induces transcription in the absence of Tc or its analog doxycycline (Dox) through binding to the hCMV*-1 promoter. This promoter is composed of a Tc-responsive element (TRE) followed by a minimal promoter of the human cytomegalovirus (hCMV) immediate early gene. The tTA protein is a fusion protein composed of the TRE-binding domain of Tc repressor protein and the herpes simplex virus VP16 activation domain (3). Alternatively, the Tet-on system uses a reverse Tc-regulated transactivator (rtTA) which binds TRE and induces transcription of the transgene in the presence of Dox (4). The Tet-on system seems to be superior to the Tet-off system, in that the former only requires administration of Dox for the induction of expression, but the Tet-off system has other advantages, including the lower level of leaky expression as background and the lower concentration of Tc or Dox required for regulation of expression (5,6). Although studies based on these systems have been performed in ES cells, it is difficult to establish independent ES cell lines harboring Tc-regulatable systems. One problem is that ES cells are very sensitive to the toxicity of proteins carrying strong transactivation domains, such as VP16 (S. Masui and H. Niwa, unpublished data). Although this toxicity can be reduced to some extent by using transactivators with iterated minimum transactivation domains (7), the success rate for generating cell lines stably expressing tTA or rtTA is very low (8). In addition, randomly integrated transgenes tend to be unstable in the durability and uniformity of their expression due to the positional effect, which depends on the local chromatin structure, especially in ES cells.

*To whom correspondence should be addressed. Tel: +81 78 306 1930; Fax: +81 78 306 1929; Email: masui@cdb.riken.jp

Moreover, it is laborious and time-consuming to perform repeated electroporations, which use large numbers of cells and large quantities of plasmid DNA, and subsequently evaluate the large number of clones generated for the magnitude and uniformity of induced expression. These findings suggest that a knock-in strategy may be better for ensuring a stable level of transgene expression.

The *ROSA26* locus was first described as a gene-trapped locus on chromosome 6, from which β-geo was shown to be expressed ubiquitously in the whole body of a mouse (9). This locus is now regarded as one from which proteins can be expressed ubiquitously at a moderate level. A knock-in strategy into the *ROSA26* locus, however, still requires homologous recombination by electroporation and genomic Southern analysis of large number of clones to confirm the identity and location of the inserted gene(s).

The recombinase-mediated cassette exchange (RMCE) system, which uses Cre recombinase and mutant *lox* sequences, has become an alternative to homologous recombination as a method for efficiently introducing a transgene into a cassette-acceptor (CA) locus, previously established to carry *lox*P and mutant *lox* sequences (10,11). Among the *lox*P mutants, *lox*PV (initially designated as *lox*2272) has been shown to be suitable for the RMCE reaction, since its recombination efficiency with wild-type *lox*P was negligible, but its self-recombination efficiency was sufficiently high (12–15). Upon co-transfection with the Cre-expression vector, the *lox*P and *lox*PV sequences on a transfected vector each recombines specifically with the same *lox* sequence on the CA locus, eventually resulting in the replacement of the latter by that derived from the vector in virtually 100% of the cells after both positive and negative selections (14).

Here, we describe the development of the ROSA-TET system, which integrates the inducible expression of the Tet-off system, the ubiquitous expression from the *ROSA26* locus, and the convenience of the transgene introduction by the RMCE system. The ROSA-TET system is thus an easy-to-use method that enables the establishment of multiple ES cell lines carrying inducible transgenes.

MATERIALS AND METHODS

Plasmid construction

Full details of plasmid construction and plasmid maps are available on request. To create the targeting vector pMWROSATcH and the exchange vector pPthC-Oct3/4, we used the homology arms for the *ROSA26* locus and PGK-DTApA from pRosa26-1 (16); the En-2 splice acceptor from pGT1.8 IRESβgeo (17); tTA from pUHD15-1 (3); hCMV*-1 and the beta-globin intron from pUHD10-3 (3) and pLG-1, respectively; the hygromycin-resistant gene from pPGKhygropA (H. Niwa, unpublished data); IRES from pCITE-1 (EMD Biosciences; San Diego, CA; http://www.emdbiosciences.com); Venus from pCS2-Venus (18); the PGK promoter from pPGKhygropA; and PuroTK from pBl-pacΔTK (19). The polyadenylation signal sequences used in these vectors were PGKpA, beta-globin pA for pMWROSATcH; PGKpA, bovine growth hormone pA for pPthC-Oct3/4 (from 5′ most). XhoI-NotI fragments of cDNAs were inserted into the exchange vector pPthC-Oct-3/4, which had been cleaved by XhoI-NotI. pCAGGS-Cre has been described elsewhere (20).

ES cell culture and knock-in experiments

All ES cells were cultured in the absence of feeder cells in Glasgow minimal essential medium (GMEM) (Sigma-Aldrich; St Louis, MO; http://www.sigmaaldrich.com) supplemented with 10% fetal calf serum, 1 mM sodium pyruvate (Invitrogen; Carlsbad, CA; http://www.invitrogen.com), 10^{-4} M 2-mercaptoethanol (Nacalai Tesque; Kyoto, Japan; http://www.nacalai.co.jp), 1× nonessential amino acids (Invitrogen) and 1000 U per ml of leukemia inhibitory factor on gelatin-coated dishes. In targeting experiments, 2×10^7 ES cells were electroporated with 75 μg of linearized pMWROSATcH DNA at 800 V and 3 μF in a 0.4 cm cuvette using a Gene Pulser II (Bio-Rad Laboratories; Hercules, CA; http://www.bio-rad.com) and cultured in the presence of 100 μg/ml of hygromycin B (Invitrogen) without Tc (Tc−) for 7–10 days. Genomic Southern hybridization was performed using Gene Images Random-Prime Labelling and Detection System (Amersham Biosciences; Piscataway, NJ; http://www.amershambiosciences.com). For the 5′ probe, a 0.4 kb fragment was PCR-amplified using the primer pair, 5′-GCAAGGATACTGGGGCATACGCCAC and 5′-TGCCCAGAGAGAAAGGCTCTCCTTCATC, and cloned into pBluescript KS−. For the internal probe, NcoI-BsrGI fragment of pCS2-Venus was used. For the Puro probe, we used the HindIII-BamHI fragment of pBlKS-BspHIpac.

Exchange reaction of the Tc-regulatable unit

ES cells carrying the *ROSA-TET* locus were seeded onto gelatin-coated 24- or 6-well plates at a density of 5 or 10×10^4 cells/well, respectively, in medium containing 1 μg/ml Tc (Tc+; Sigma-Aldrich). When the cells were seeded onto 6-well plates, 5 μg of each circular plasmid DNA of the exchange vector and pCAGGS-Cre and 10 μl of Lipofectoamin 2000 (Invitrogen) were separately mixed with 125 μl of GMEM without serum and combined to make the transfection mixture. After incubation at room temperature for 15 min, 1250 μl of GMEM with serum was added to the transfection mixture, and the resultant transfection medium was added to each well containing cells. The cells were incubated for 3–5 h and re-plated in 10 cm dishes containing Tc+ medium. After 2 days, the medium was changed to Tc+ medium with 1.5 μg/ml puromycin (Sigma-Aldrich). The recombinant cells were maintained in 1.5 μg/ml puromycin, and induced for the transgene expression in 7.5 μg/ml puromycin unless otherwise stated.

Estimation of copy number by real-time RT–PCR

Total RNA samples purified by TRIZOL reagent (Invitrogen) were reverse-transcribed by ReverTra-Ace alpha (Toyobo; Osaka, Japan; http://www.toyobo.co.jp). A 451 bp fragment of GAPDH was PCR-amplified using the primers, 5′-ACCACAGTCCATGCCATCAC and 5′-TCCACCACCCTGTTGCTGTA, and a 328 bp fragment of Venus was PCR-amplified using the primers, 5′-AGCTGACCCTGAAGCTGATCT and 5′-ACGTTGTGGCTGTTGTAGTTGT. The PCR products were gel purified and their DNA concentrations were titered, and a dilution series of each DNA fragment was

mixed with the cDNA samples, following which real-time RT–PCR was performed using iTaq SYBR Green Supermix for DNA amplification and iCycler iQ (Bio-Rad) for signal detection. Basically, the results of this reaction showed a linear relationship between the logarithm of the copy number of the input DNA fragment and the cycle number at which log-phase of PCR amplification started. At the dilution at which the copy number of each DNA fragment was nearly equal to the cDNA sample, the cycle number was dissociated from the anticipated one, yielding an estimated copy number for each cDNA sample.

Western blotting

Whole-cell lysates were extracted by lysis buffer [10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% NP-40] containing 1% (v/v) of proteinase inhibitor cocktail (Sigma-Aldrich). Twenty micrograms of total protein from each sample were fractionated on 10% SDS–PAGE gels and electroblotted onto PVDF membranes. After treatment in blocking buffer {1 × TTBS [10 mM Tris–HCl (pH 7.4), 137 mM NaCl, 2.7 mM KCl and 0.1% Tween-20]} plus 3% skimmed milk], the membranes were incubated with anti-Gata6 (SC-7244; Santa Cruz Biotechnology; Santa Cruz, CA; http://www.scbt.com) or anti-Oct4 (SC-5279) and then with horseradish peroxidase-coupled anti-goat IgG (SC-2020) or anti-mouse IgG (61-6520; Zymed Laboratories; South San Francisco, CA; http://www.zymed.com), respectively, and developed using ECL reagents (Amersham Biosciences).

FACS analysis

Cells were trypsinized and resuspended in medium with 5 μg/ml propidium iodide, and analyzed for green fluorescent protein (GFP) fluorescence by FACSCalibur (Becton, Dickinson and Company; Franklin Lakes, NJ; http://www.bd.com).

Neural induction by SDIA method

The PA6 stromal cell line was a gift of Dr Sasai (RIKEN CDB, Japan). ES cells carrying the ROSA-TET system were induced to differentiate into cells of the neuronal lineage according to the essentially SDIA method (21). Briefly, ES cells were seeded onto a PA6 layer at a density of 1×10^3 cells per cm^2, and cultured in GMEM supplemented with 10% KSR (Invitrogen), 2 mM glutamine, 1 mM pyruvate, 0.1 mM nonessential amino acids and 10^{-4} M 2-mercaptoethanol.

Immunostaining

Cells that differentiated into neurons were fixed with 4% paraformaldehyde for 2 h at 4°C, washed with PBS containing 0.3% Triton X-100 for 45 min at room temperature, and incubated with anti-TuJ (PRB-435P; CRP; Denver, PA; http://www.crpinc.com) and anti-GFP (GF-090R; Nacalai Tesque) antibodies.

Fluorescent microscopy

The fluorescent images of Venus and secondary antibodies were captured using an IX51 microscope (Olympus; Tokyo, Japan; http://www.olympus.co.jp) and a DP70 digital camera (Olympus).

Induction of transgene expression

The incomplete removal of Tc has been reported to result in insufficient induction of the Tet-off system (22). Therefore, in this study, the cells to be induced were washed twice with PBS, cultured for more than 3 h in GMEM without Tc, trypsinized and re-plated onto new dishes. Cells differentiated by SDIA were washed twice with PBS and incubated for 3 h in the absence of Tc.

RESULTS AND DISCUSSION

The ROSA-TET system consists of a knock-in step and an exchange step. The *ROSA26* locus was targeted by the knock-in vector, pMWROSATcH, making the *ROSA-TET* locus a CA locus, and the region encompassing *lox*P to *lox*PV on the *ROSA-TET* locus was exchanged for the corresponding region of the exchange vector, carrying a Tc-responsive promoter followed by the desired cDNA (Figure 1). Schematically, the promoter-trap insertion resulted in transcription of tTA from the endogenous *ROSA26* promoter (Figure 2A). Upon removal of Tc, tTA binds to the hCMV*-1 promoter and induces the expression of the desired cDNA as well as Venus, whose fluorescence is utilized as a reporter.

Generation of the *ROSA-TET* locus

The knock-in step to generate the *ROSA-TET* locus is desired to be highly efficient to realize feasible introduction into any ES cell line. Soriano has shown that *ROSA26* locus has high efficiency for homologous recombination (16). In addition, as a structure of the targeting vector pMWROSATcH, the expression of tTA which binds to the hCMV*-1 promoter to induce the expression of the hygromycin-resistant gene is driven by endogenous *ROSA26* promoter by promoter-trapping manner (Figure 1A). Taken together, quite high targeting efficiency can be expected using this targeting vector. pMWROSATcH was electroporated into two different ES cell lines, MGZ5 and EB3, the derivatives of MG1.19 (23) and E14tg2a (24) cells, respectively. The correct targeting events were verified by genomic Southern analysis using the 5′ probe (Figure 1B) and the internal probe (data not shown). We found that the targeting efficiencies for MGZ5 and EB3 cells were 40.4 and 83.3%, respectively, which were sufficiently high to obtain the desired clones repeatedly (Figure 1C). The correctly targeted clones turned to be hygromycin sensitive in the presence of Tc (data not shown). Since a single targeting step was sufficient to generate a functional *ROSA-TET* locus in host ES cells, which is ready for introducing desired cDNAs, our results suggest that the ROSA-TET system can be feasibly introduced into ES cells that have previous genetic modifications, such as gene targeting.

Exchange of the expression cassette on the *ROSA-TET* locus

To establish ES cell lines that express the desired cDNA under the control of the Tc-regulatable promoter, we constructed exchange vectors carrying various cDNAs, and co-transfected these plasmids, along with the Cre-expression vector pCAGGS-Cre, into MGZRTcH2 and EBRTcH3 cells, which had been derived from MGZ5 and EB3 cells,

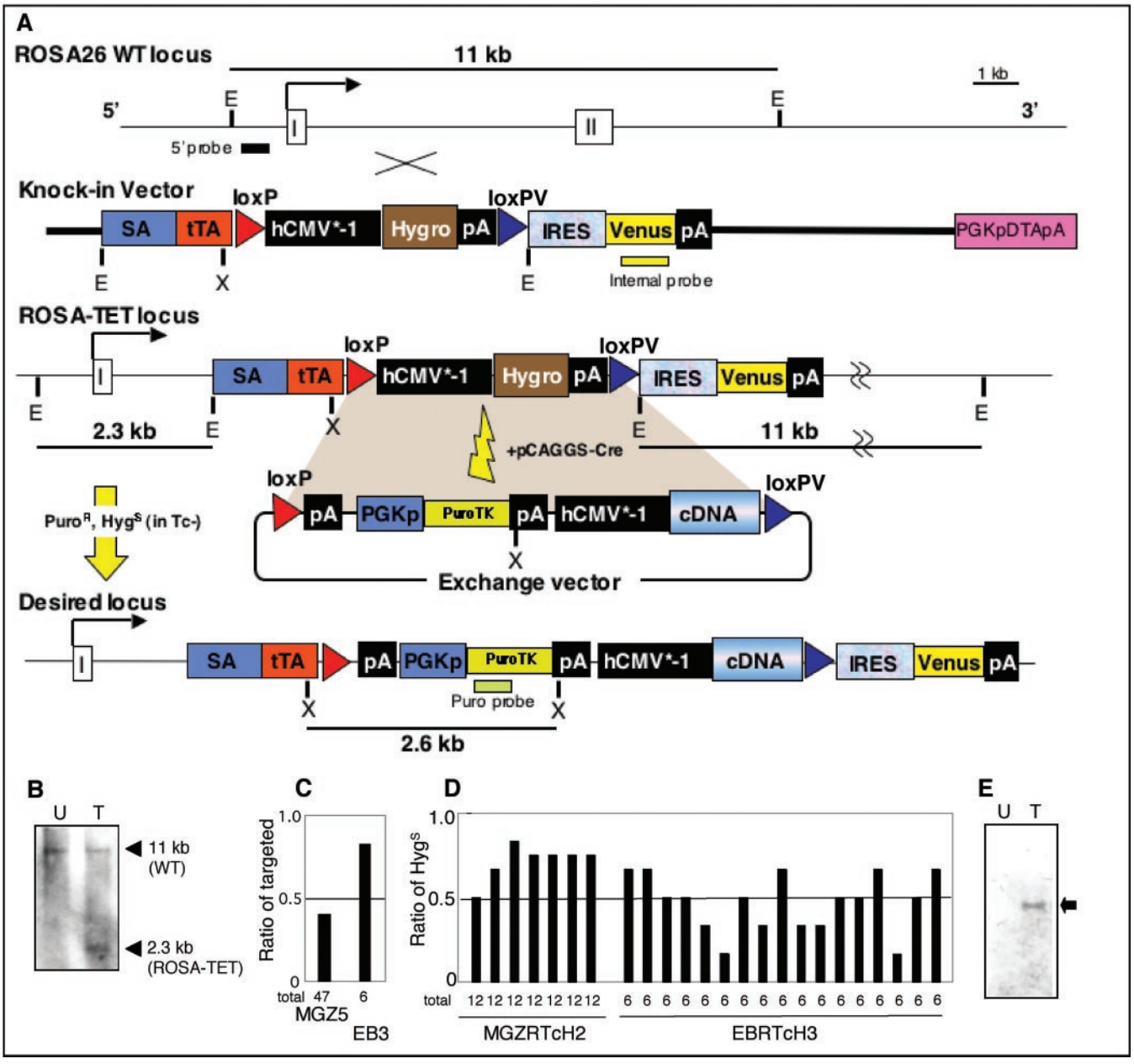

Figure 1. Development of the ROSA-TET system. (**A**) Experimental strategy to generate the *ROSA-TET* locus and the desired locus. The *ROSA26* locus was targeted by the knock-in vector pMWROSATcH, and the region from *lox*P to *lox*PV of the resultant locus was replaced by the exchange vector. Puromycin selection and subsequent assay for hygromycin sensitivity was used to isolate desired clones. E, EcoRV; and X, XbaI. (**B**) Representative results of Southern analysis using a 5′ probe; results using an internal probe are not shown. (**C**) Success ratio of the knock-in step from Southern analysis, measured as the numbers of correctly targeted clones relative to total hygromycin-resistant clones. The total numbers of hygromycin-resistant clones analyzed are shown. (**D**) Efficiency of RMCE reaction on the *ROSA-TET* locus, measured as the ratio of hygromycin-sensitive clones (in Tc− medium) to total puromycin-resistant clones. Each bar represents the results using the exchange vectors carrying different cDNAs. The total numbers of puromycin-resistant clones analyzed are shown. (**E**) An example of Southern analysis of hygromycin-sensitive clones using a Puro probe. The 2.6 kb bands are indicated by an arrow. U, Untargeted clone; and T, Targeted clone.

respectively, by the above knock-in step. We employed the lipofection method to reduce the number of cells and amount of plasmid DNA required per transfection, compared with those required for electroporation. The RMCE reaction replaced the hygromycin resistance gene on the parental *ROSA-TET* locus, making the resultant cells sensitive to hygromycin in Tc− medium. Puromycin-resistant colonies were therefore picked and assayed for hygromycin sensitivity. Subsequent genomic Southern analysis using the Puro probe showed that it hybridized to a 2.6 kb band in 81 of 84 hygromycin-sensitive clones derived from MGZRTcH2 using 10 exchange vectors, and in 42 of 47 hygromycin-sensitive clones derived from EBRTcH3 cells using 7 vectors, indicating that about 90% of hygromycin-sensitive clones had been correctly targeted (e.g. Figure 1E). The RMCE reaction using *lox*P and *lox*PV has been shown to occur at an efficiency of nearly 100%

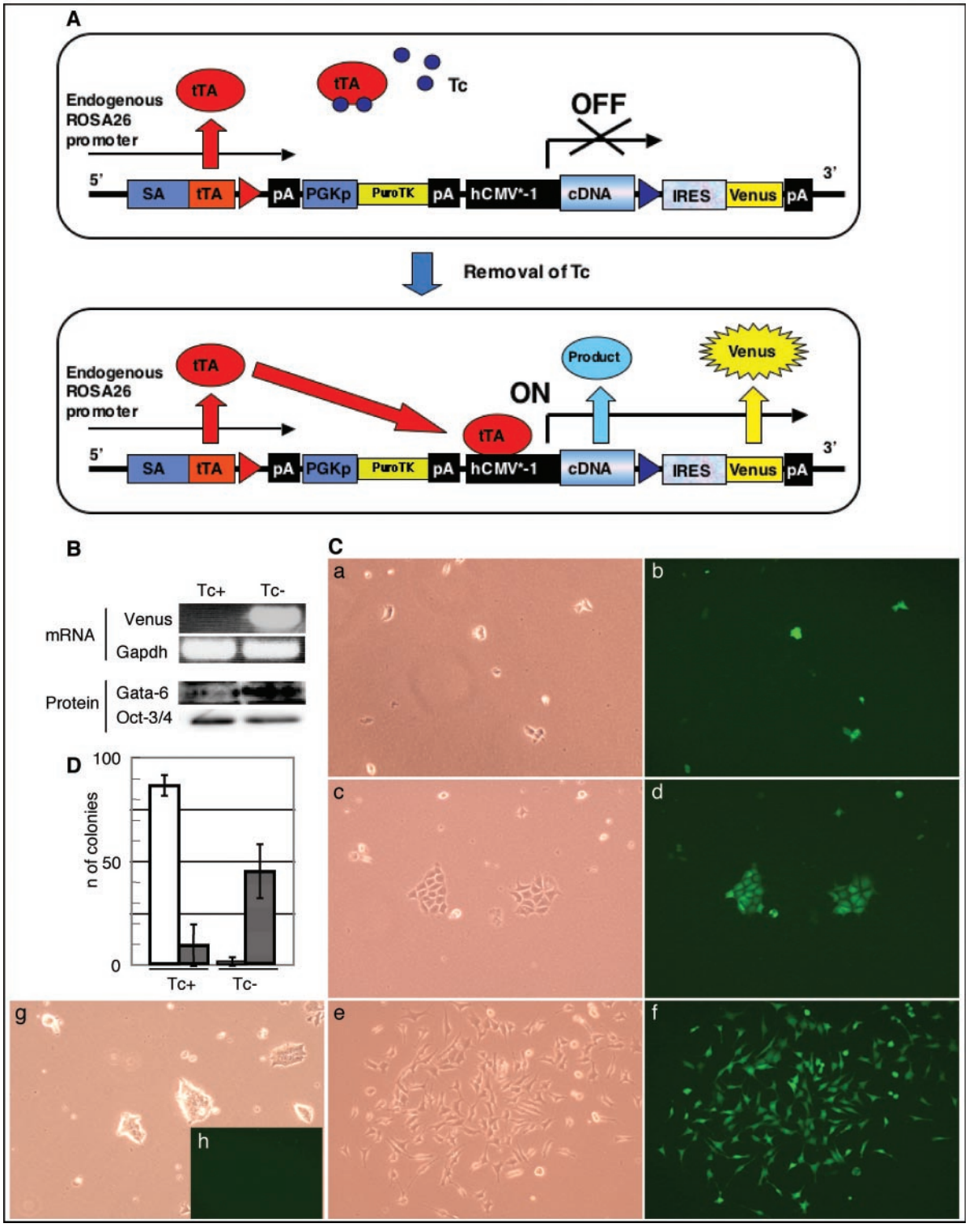

Figure 2. Induction of expression in the ROSA-TET system. (**A**) Schematic representation of the induction of expression. The endogenous *ROSA26* promoter was trapped by SA-tTA. In Tc− medium, tTA binds to the hCMV*-1 promoter, inducing expression of cDNA-IRES-Venus. (**B**) Induction of expression in EBRTcP-GATA6 cells. mRNA and protein samples were prepared at 24 h after the induction. GAPDH and Oct-3/4 are used to normalize expression of Gata-6-specific mRNA and protein, respectively. It is known that endogenous Gata-6 protein is expressed at detectable level even in undifferentiated ES cells, and Oct-3/4 is expressed at the same level within 24 h after the induction of Gata-6 expression (25). (**C**) Morphological changes in EBRTcPGATA6 cells during induction of Gata-6 expression. Cells were observed 24 (panels a and b), 48 (panels c and d), and 72 (panels e and f) h after induction of Gata-6 expression through a bright field (panels a, c, e and g) and through a YFP filter (panels b, d, f and h). In the non-induced cell population, stem cell colonies without fluorescence were observed after 72 h (panels g and h). (**D**) Expression induction in the cellular population. Open and striped boxes indicate undifferentiated and differentiated colonies, respectively. A marked increase in the number of differentiated colonies was observed 6 days after induction, whereas undifferentiated colonies were almost eliminated. Error bars represent mean ± 2 × SEM ($n = 3$).

after positive and negative selections (14). In the present case, however, incomplete removal of Tc may result in clones pseudo-sensitive for hygromycin, suggesting that the above reaction efficiencies were too low and were due to the overestimation of the total number of hygromycin-sensitive clones. Further RMCE reactions using a different exchange vector set were performed to determine the ratio of hygromycin-sensitive to puromycin-resistant colonies (Figure 1D). We observed some difference in the efficiency between cell lines and exchange vectors used. Nevertheless, about 50% of the total clones on average were hygromycin sensitive (Figure 1D), indicating that picking 6–12 clones should be sufficient to obtain the desired clones. Since clones that were hygromycin sensitive and become fluorescent in Tc− medium were correctly targeted and were functional clones in all cases, this assay would allow the identification of recombinants without the need for genomic Southern analysis.

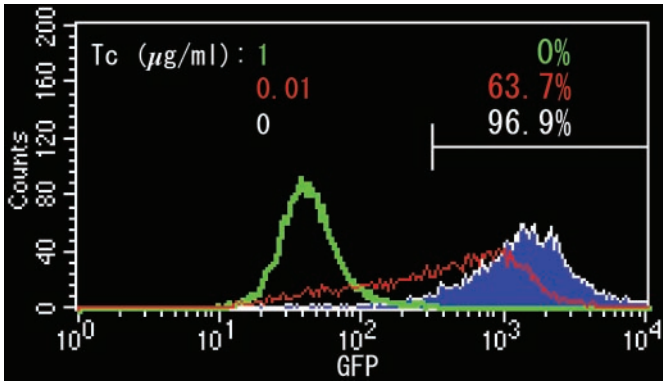

Figure 3. FACS analysis of the induction of the expression. EBRTcPGATA6 cells were cultivated for the indicated concentrations of Tc for 24 h and 1×10^4 cells were analyzed for the intensity of GFP fluorescence. Note that in the absence of Tc 96.9% of cells are GFP positive.

An example using the transcription factor Gata-6

We previously showed that forced expression of the transcription factor Gata-6 was sufficient to induce mouse ES cells to differentiate to parietal endoderm cells (25). To confirm that the ROSA-TET system possesses the property of inducible expression, we introduced Gata-6 into the *ROSA-TET* locus. Using the procedure described above, we established EBRTcPGATA6 cells by co-transfection of the exchange vector pPthC-Gata6 and pCAGGS-Cre. Induction of mRNA from the *ROSA-TET* locus was measured by RT–PCR, using the primers for the Venus sequence. The PCR signal was undetectable in the Tc+ lane, indicating that the background expression level was quite low, whereas a strong signal was obtained in the absence of Tc (Figure 2B). Real-time PCR analysis showed that there were 2.18×10^3 copies of ROSA-TET mRNA per 10^5 copies of GAPDH mRNA. Gata-6 protein induction in Tc− cells was confirmed by western blotting.

Next we addressed whether the induced expression of Gata-6 was sufficient to induce these cells to differentiate. EBRTcPGATA6 cells were seeded at a density of 200 cells per well in 6-well plates and cultured for 6 days in the presence or absence of Tc. Venus fluorescence was observed in virtually all cells cultured in the absence of Tc (Figure 2C, panels b, d and f), whereas no fluorescence was detected in cells cultured in the presence of Tc (Figure 2C, panel h). As described previously (25), morphological differentiation into parietal endoderm cells was evident within 72 h after induction of Gata-6 expression (Figure 2C, panel e), indicating that the level of induced expression from the *ROSA-TET* locus was sufficient to provoke a physiological response in ES cells. Similar levels of induction or repression were observed in other cell lines carrying different cDNAs in their *ROSA-TET* loci (data not shown).

To determine the uniformity of induction in cell populations, we counted the numbers of undifferentiated colonies, which were calculated as an average of 86.0 in the presence of Tc and 1.33 in its absence (Figure 2D), suggesting that Gata-6 expression had been induced in more than 98% of the cells in population Tc− culture. The differentiated colonies observed in Tc+ cultures had originated from spontaneously differentiated, morphologically primitive endoderm-like colonies, whereas those observed in Tc− cultures were parietal-endoderm colonies, suggesting that there was no 'background' differentiation caused by leaky expression of Gata-6. Although the total number of colonies in Tc− dishes was about half that in Tc+ dishes, this was probably due to the toxic effects of overexpressed Gata-6 protein on ES cells (S. Masui and H. Niwa, unpublished data). Finally, the expression induction in the population was analyzed by FACS, which revealed that 96.9% of the cells incubated for 24 h in Tc− medium have significant fluorescence, whereas modest level of the expression can also be achieved by adding 0.01 μg/ml of Tc (Figure 3).

To examine the induction of transcription from the *ROSA-TET* locus in completely differentiated cells, we employed the SDIA method to differentiate ES cells into neuronal cells (21). Derivatives of EBRTcH3 cells were differentiated on PA6 feeder cells, and expression from the *ROSA-TET* locus was induced by removing Tc from the medium. Immunostaining showed that almost all of the colonies were positive for TuJ (Figure 4A and C), the beta III isotype of tubulin and a marker for post-mitotic neurons (26). No fluorescence was observed in colonies of Tc+ cells (Figure 4B), indicating that background expression was negligible in differentiated cells. An anti-GFP antibody signal was clearly detected in virtually all Tc− colonies (Figure 4D), demonstrating that expression from *ROSA-TET* locus can be induced in completely differentiated cells.

To date, there have been several reports about Tet-systems in ES cells. The 'supertargeting' system, which required electroporation and homologous recombination to introduce the desired cDNAs into parental cells constructed in advance by the repeated random integration of the components (27), was successful in undifferentiated cells (25,27,28), but their transgenes were partially silenced in differentiated cells (S. Masui and H. Niwa, unpublished data). Previously described Tet-systems using the *ROSA26* locus (29,30), in which rtTA was introduced into the *ROSA26* locus and the desired cDNA into the *Hprt* locus, involved separate introduction of the required components, thus making it difficult to establish new cell lines carrying these systems. In addition, the desired cDNAs in these systems were integrated using a single *lox*P-based strategy, in which the resultant locus inevitably carried the whole plasmid backbone sequence, which may

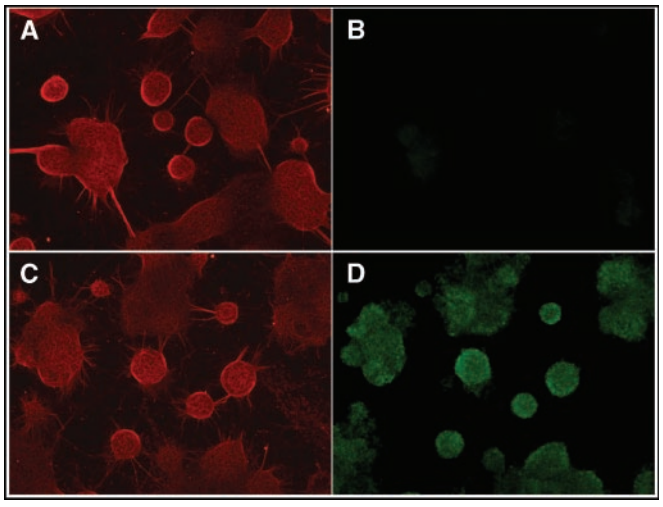

Figure 4. Induction of expression in completely differentiated cells. EBRTcPOct3 cells, constructed by introducing Oct-3/4 cDNA into EBRTcH3 cells, were induced to differentiate into neuronal cells by the SDIA method with Tc for 8 days, and with (A and B) or without Tc (C and D) for an additional 6 days. The cells were fluorescently immunostained with anti-TuJ (**A** and **C**) and anti-GFP (**B** and **D**) antibodies, which showed that almost all the TuJ-positive colonies were GFP positives.

cause transgene silencing (31–34), one of the major obstacles accompanying genetic manipulation in ES cells. Unlike the single *lox*P strategy, the RMCE system eliminates the backbone sequence from the locus, thus minimizing the risk of transgene silencing. Nevertheless, we observed variations in the expression and attenuation of transgene induction level in cells passaged more than five times, as determined by Venus fluorescence. The extent of these silencing effects seemed to depend on the cDNA sequence introduced into the *ROSA-TET* locus, suggesting that epigenetic modification of particular sequence motifs may be involved (data not shown). We observed that elevated concentrations of puromycin, as high as 7.5 μg/ml, which eliminated weakly expressing cells from the PGK-puroTKpA unit, decreased the variation and increased the induction level, which may have been due to the selection of the cells having chromatin of more 'open' states at this locus.

In conclusion, we describe here the establishment of the ROSA-TET system, a Tc-regulatable transgene expression system that can be easily introduced into any ES cell lines, and in which the Tc-responsive expression cassette can be efficiently exchanged. This system will allow the establishment of multiple cell lines harboring Tc-regulatable transgenes, thereby strongly accelerating ES cell research, including the screening and identification of transcription factors involved in cellular differentiation processes into cells of particular lineages, including neurons.

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