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Original Research Report

An Inducible Transgene Expression System for Regulated Phenotypic Modification of Human Embryonic Stem Cells

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ABSTRACT

Self-renewing pluripotent human embryonic stem (hES) cells are capable of regenerating such nondividing cells as neurons and cardiomyocytes for therapies and can serve as an excellent experimental model for studying early human development. Both the spatial and temporal relationships of gene expression play a crucial role in determining differentiation; to obtain a better understanding of hES cell differentiation, it will be necessary to establish an inducible system in hES cells that enables specific transgene(s) to reversibly and conditionally express (1) at specific levels and (2) at particular time points during development. Using lentivirus (LV)-mediated gene transfer and a tetracycline-controlled trans-repressor (TR), we first established in hES cells a doxycycline (DOX)-inducible expression system of green fluorescent protein (GFP) to probe its reversibility and kinetics. Upon the addition of DOX, the percentage of GFP^+ hES cells increased time dependently: The time at which 50% of all green cells appeared (T_{50}^{on}) was 119.5 ± 3.2 h; upon DOX removal, GFP expression declined with a half-time (T_{50}^{off}) of 127.7 ± 3.9 h and became completely silenced at day 8. Both the proportion and total mean fluorescence intensity (MFI) were dose-dependent (EC_{50} = 24.5 ± 2.2 ng/ml). The same system when incorporated into murine (m) ES cells similarly exhibited reversible dose-dependent responses with a similar sensitivity (EC_{50} = 49.5 ± 8.5 ng/ml), but the much faster kinetics (T_{50}^{on} = 35.5 ± 5.5 h, T_{50}^{off} = 71.5 ± 2.4 hours). DOX-induced expression of the Kir2.1 channels in mES and hES cells led to robust expression of the inwardly rectifying potassium (K^+) current and thereby hyperpolarized the resting membrane potential (RMP). We conclude that the LV-inducible system established presents a unique tool for probing differentiation.

INTRODUCTION

HUMAN EMBRYONIC STEM (hES) cells, isolated from the inner cell mass (ICM) of the human blastocyst, are capable of self-renewing while maintaining the pluripotency to differentiate into the three germ layers (i.e., ectoderm, mesoderm, and endoderm) and their corresponding derivatives [1]. In addition to their potential of regenerating such nondividing, highly specialized cells as neurons and cardiomyocytes for cell-based therapies [2,3], hES cells can also serve as an excellent experimental model for studying the very poorly defined human organogenesis. Among many factors, both the spatial and temporal relationships of gene expression are known to play a crucial role in determining differentiation during development. Indeed, differentiation is a dynamic process that involves the precise regulation of numerous genes, proteins, transcription factors, etc; often, the same genes are repeatedly turned on and off with distinct roles during different developmental stages [4–6]. To obtain a better understanding of hES cell differentiation, it will be necessary to establish an inducible system

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in hES cells that enables specific transgene(s) to reversibly and conditionally express (1) at specific levels and (2) at particular time points during development so as to dissect the underlying molecular mechanisms. Recently, we discovered the presence of several specialized ion channels in pluripotent murine (m) and hES cells whose functional activities influence proliferation [7]. Here we conjecture that a dose-dependent, ligand-inducible system [8,9] can provide a flexible approach for controlling ectopic, for example, a reporter protein such as green fluorescent protein (GFP), or endogenous transgene expression in hES cells.

Using lentivirus (LV)-mediated gene transfer [10,11] and a tetracycline (tet)-controlled trans-repressor (TR) from *Escherichia coli* [12], we initially established an inducible expression system of GFP in mES and hES cells to probe in detail its reversibility and kinetics and to reveal any species similarities and differences. Subsequently, we studied the functional consequences of conditionally expressing Kir2.1 channels, also called the inwardly rectifying potassium (K^+) current or I_{K1}, a key player that governs cellular excitability [13] in pluripotent hES cells. During the preparation of this work, a tet-inducible expression system was similarly and independently established in hES cells [14]. Our present study differs in several major ways: (1) The detailed dose-dependence and kinetics of transgene expression in both mES and hES cells were reported here but not in the previous study. This information is crucial for designing future experiments. (2) We provide the first demonstration that an endogenous gene can be inducibly expressed in hES cells for altering their functional phenotypes. (3) We emphasize electrophysiology of mES and hES cells, built on our own previous report.

METHODS

Lentiviral vector plasmid construction and production

The plasmids pLV-THM-GFP and pLV-TR-KRAB-IRES-dsRed (Fig. 1A) were kind gifts of Dr. Didier Trono (University of Geneva, Geneva, Switzerland). The drug-inducible system was a two-vector one that has been previously described [8]. Briefly, TR-KRAB is a tetracycline-controlled fusion protein that contains the TR fused to the Krüppel-associated box (KRAB) domain of human Kox1 [12]. KRAB, a 75-amino-acid transcriptional repression module in many zinc finger-containing proteins, suppresses transcription within 3 kb from its binding site in an orientation-independent manner [12,15–17]. When fused to the DNA-binding domain of TR, KRAB can modulate transcription from an integrated promoter juxtaposed with the *tet* operator (*tetO*) sequence [15–17]. In the absence of doxycycline (DOX), TR-KRAB binds specifically to *tetO* and thereby suppresses any nearby promoter(s). By contrast, the presence of DOX will sequester TR-KRAB away from *tetO* to enable transgene expression (Fig. 1B) [8].

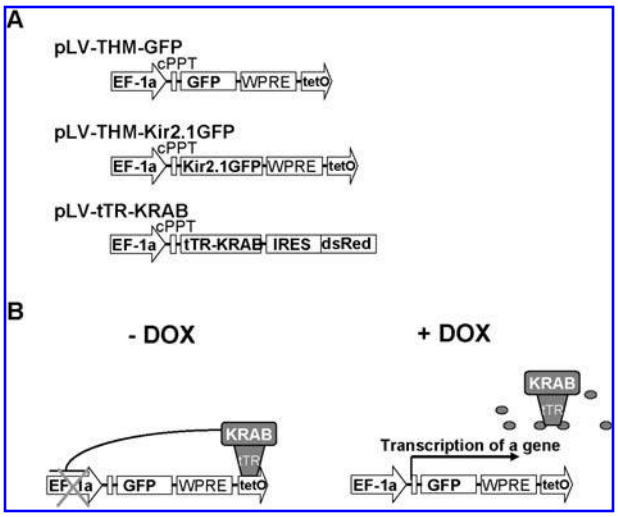

FIG. 1. (**A**) Schematic representation of the LV-based DOX-inducible transgene expression system. (**B**) In the absence of DOX (i.e., −DOX), TR-KRAB binds to *tetO* and thereby suppresses EF-1 α-mediated transcription. In the presence of DOX (+DOX), TR-KRAB cannot bind to *tetO* and hence allows gene expression.

In all cases, the ubiquitously active promoter EF-1α was chosen to drive the transgene to avoid silencing in undifferentiated hES cells. To generate pLV-THM-Kir2.1-GFP (Fig. 1A), the GFP of pLV-THM-GFP was replaced with the fusion protein Kir2.1-GFP. The recombinant lentiviruses were produced by transient transfection of HEK293T cells, as previously described [18]. Briefly, the lentiviral plasmids pΔ8.91, pMD.G, and pLV-THM-GFP or pLV-THM-Kir2.1GFP or pLV-TR-KRAB-dsRed (2:1:3 mass ratio) were co-transfected into HEK293T cells seeded at a density of 6×10^6 cells per 10-cm dish 24 h prior to transfection. The supernatant containing lentiviral particles were harvested at 24 and 48 h post-transfection and stored at −80°C before use.

Maintenance and transduction of mouse and human ES cells

The H1 (WiCell, Madison, WI) hES cell line was maintained on irradiated mouse embryonic fibroblasts (MEFs) and propagated as previously described [1,10]. Briefly, the culture medium consisted of Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen Corp., Carlsbad, CA) supplemented with 15% knockout (KO) serum replacer, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acids, and 4 ng/ml fibroblast growth factor-β (FGF-β). Cells were grown in six-well plates and passaged by collagenase digestion and mechanical dissociation. The resultant suspension was plated on fresh MEFs at a ratio of 1:5. The culture medium was changed daily. The D3 mES cell line was cultivated on irradiated MEFs in the presence of leukemia inhibitory factor (LIF), as previously described [7,19]. The culture medium consisted of DMEM supplemented with 15% fetal bovine serum (FBS) serum (Hyclone, Logan, UT), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acids, 20

U/ml penicillin, and 20 μg/ml streptomycin (Invitrogen, Carlsbad, CA).

For stable lentiviral transduction, a single-cell suspension of mES cells and a small-cluster (~1,000 cells per cluster) culture of H1 hES cells were generated by trypsin and collagenase treatment, respectively. Viral supernantants were added at a final concentration of 100,000 TU/ml with 8 mg/ml Polybrene; mES and hES cells were incubated in suspension for 2–4 h, followed by plating on MEFs. LV-TR-KRAB-IRES-dsRed and LV-THM-GFP or LV-THM-Kir2.1GFP were co-introduced into mES and hES cells successively in the same order. dsRed$^+$ and/or GFP$^+$ cells were identified by their epifluorescence and sorted by MoFlo (Dako, Ft. Collius, CO). Co-transduced mES and hES cells were cultured in the presence or absence of DOX (Sigma) at the concentrations specified.

FACS analysis

The expression of GFP was assessed by flow cytometry using FACScan ($CyAN_{ADP}$, Dako-Cytomation). The percentage of GFP$^+$ cells and mean fluorescence intensity (MFI) of GFP were measured. For analyzing stage-specific embryonic antigen-1 (SSEA-1) expression, the single-cell suspension was stained by mouse anti-SSEA-1 monoclonal antibody (Chemicon), followed by staining with allophycocyanin- (APC) conjugated rat anti-mouse immunoglobulin M (IgM) monoclonal antibody (BD Pharmingen). Purified mouse IgM, κ monoclonal immunoglogulin isotype was used as negative control.

Electrophysiological characterization

Electrophysiological experiments were performed using the whole-cell patch-clamp technique with an Axopatch 200B amplifier and the pClamp9.2 software (Axon Instruments Inc., Foster City, CA). A xenon arc lamp was used to view GFP fluorescence at 488/530 nm (excitation/emission) [7,20]. Patch pipettes were prepared from 1.5-mm thin-walled borosilicate glass tubes using a Sutter micropipette puller P-97 and had typical resistances of 4–6 MΩ when filled with an internal solution containing 110 mM K^+ aspartate, 20 mM KCl, 1 mM $MgCl_2$, 0.1 mM Na-GTP, 5 mM Mg-ATP, 5 mM Na_2-phosphocreatine, 1 mM EGTA, and 10 mM HEPES, pH adjusted to 7.3 with KOH. The external Tyrode's bath solution consisted of 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH adjusted to 7.4 with NaOH. Voltage- and current-clamp recordings were performed at 37°C. To record the resting membrane potential (RMP), cells were held at 0 pA. For eliciting ionic currents, cells were held at a −30-mV potential and pulsed from 0 mV to −140 mV with 10-mV increments for 2 sec, followed by a 1-sec −100-mV pulse. I_{K1} was defined as 1 mM Ba^{2+}-sensitive currents.

Measurement of membrane potential by using FLIPR

The FLIPR membrane potential assay kit (Molecular Devices, Sunnyvale, CA) was employed to assess the RMPs of mES and hES cells. Cells were loaded for 15 min with FLIPR Membrane Potential (FMP) dye, which is superior to the conventional bisoxonol fluorescent dye DiBAC(4)(3) [21], according to the manufacturer's protocol, and the fluorescence was measured by the microplate reader (TECAN, SAFIRE). The fluorescence intensity of FMP reflects the membrane potential of FMP-loaded cells [21]. $\Delta_{\text{fluorescence}}$ is the difference in fluorescence between treated and untreated cells and was used as an index for changes in the RMP.

Cell proliferation assay

A total of 1×10^5 mES cells were cultivated on irradiated MEFs in 12-well plates in the presence of LIF. After 24 h of incubation, cells were digested into single cells, and the cell counts were assessed by the cytometer chamber. Cell viability was determined in 96-well plates using a colorimetric 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) kit (Chemicon). Briefly, 50,000 cells were plated in 96-well plates at 37°C for 24 h. Then 10 μl of MTT labeling reagent (5 mg/ml in PBS) was added to each well, followed by incubation at 37°C for 4 h. Solubilization solution (100 μl) was added to dissolve the formazan crystals that formed. Absorbances at 570 nm were read by a spectrophotometer.

Statistics

All data are presented as mean ± SEM. Statistical significance was defined as $p < 0.05$ by unpaired Student's *t*-test.

RESULTS

Reversibility and kinetics of doxycycline-induced GFP expression in mES cells

As a first step, we stably co-transduced mES cells with LV-TR-KRAB-IRES-dsRed and LV-THM-GFP (cf. Fig.1). Figure 2A shows that untransduced, control wild-type (WT) mES cells did not fluoresce. By contrast, co-transduced mES cells were always dsRED$^+$, as anticipated from the constitutive promoter activity of EF-1α. In the absence of DOX, co-transduced mES cells did not display any GFP expression. When the same cells were treated with DOX (1 μg/ml), GFP$^+$ mES cells could be observed as soon as 24 h later; in the presence of DOX, the percentage of GFP$^+$ mES cells continued to increase in a time-dependent manner before a plateau of ~80% was reached after 4–5 days (Fig. 2B; from three independent different reactions). The time at which 50% of all green cells appeared (T_{50}^{on}) was 35.5 ± 5.5 h (n = 3). Upon DOX removal, GFP expression started to decline after ~36 h with a half-time (T_{50}^{off}) of 71.5 ± 2.4 h and became completely silenced 4 days later (n = 3, Fig. 2A,C).

Dose-dependent DOX-induced GFP expression in mES cells

Figure 3, A and B, shows the effect of a range of DOX concentrations on the GFP expression of LV-TR-KRAB-

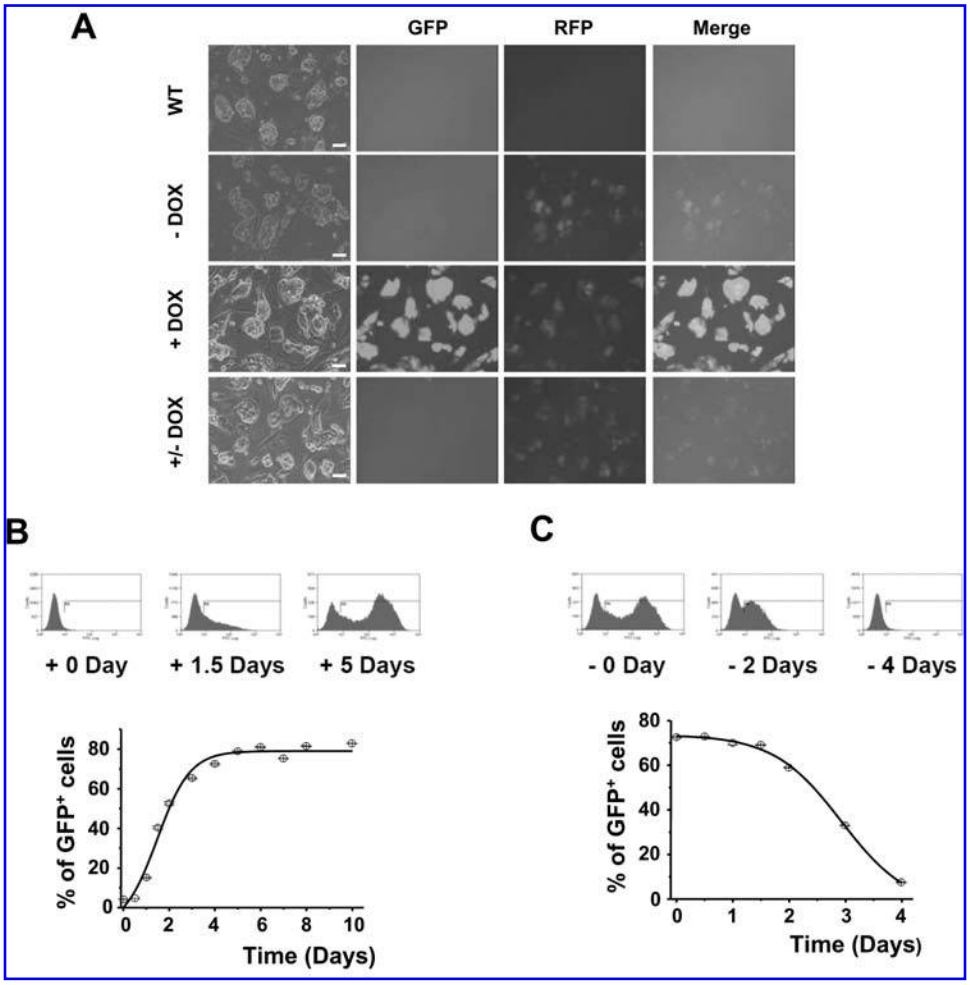

FIG. 2. The LV-based DOX-inducible system in mouse ES cells. (**A**) Representative images of WT and LV-THM-GFP/LV-TR-KRAB-dsRed co-transduced mouse ES cells in presence (+) or absence (−) of DOX (1 μg/ml) as indicated. GFP expression was completely reversible with virtually no expression leakage upon withdrawal of DOX (+/−DOX). Bar, 50 μm. (**B**) Representative FACS analyses (*upper*) and the kinetics (*lower*) of inducible GFP expression of co-transduced mouse ES cells at different time points after DOX treatment (1 μg/ml). (**C**) Representative FACS analyses (*upper*) and the corresponding decay kinetics (*lower*) of GFP disappearance after DOX withdrawal.

IRES-dsRed/LV-THM-GFP co-transduced mES cells when assessed at days 2, 4, and 8. At all of these time points, no GFP expression could be detected for 0, 3, and 10 ng/ml; GFP^+ mES cells were observed with 20 ng/ml DOX, and the percentage increased dose-dependently before leveling at 100 ng/ml. Higher concentrations (300 ng/ml, 1 μg/ml, and 3 μg/ml) did not further increase the percentage of GFP^+ mES cells. Unlike the proportion, the total mean fluorescence intensity (MFI) elevated from 10 ng/ml to 300 ng/ml at all of days 2 (solid squares; Fig. 3C), 4 (open triangles; Fig. 3C), and 8 (solid circles; Fig. 3C). Figure 3D shows the normalized dose-dependent relationships for MFI. The effective concentrations at which 50% of the maximum EFI (EC_{50}) was attained were statistically identical ($p > 0.05$): 47.5 ± 2.3, 46.5 ± 1.0, and 50.4 ± 1.0 ng/ml for days 2, 4, and 8, respectively.

Similarities and differences between murine and human ES cells

Although mES and hES cells share a number of similarities, significant differences are known to exist. To employ the DOX-inducible system described above for hES cell studies, the detailed dose-dependence and kinetics of these cells need to be characterized. Thus, we stably co-transduced hES cells with the same lentiviruses, LV-TR-KRAB-IRES-dsRed and LV-THM-GFP. As anticipated, co-transduced but not control hES cells were always $dsRED^+$ (Fig. 4A). Upon the addition of 1 μg/ml DOX, co-transduced hES cells became GFP^+ time dependently after an initial 3-day lag (vs. 24 h for mES cells). The maximum percentage of ~90% GFP^+ hES cells, comparable to that of co-transduced mES cells, was reached at around day 10 with a T_{50}^{on} of 119.5 ± 3.2 h

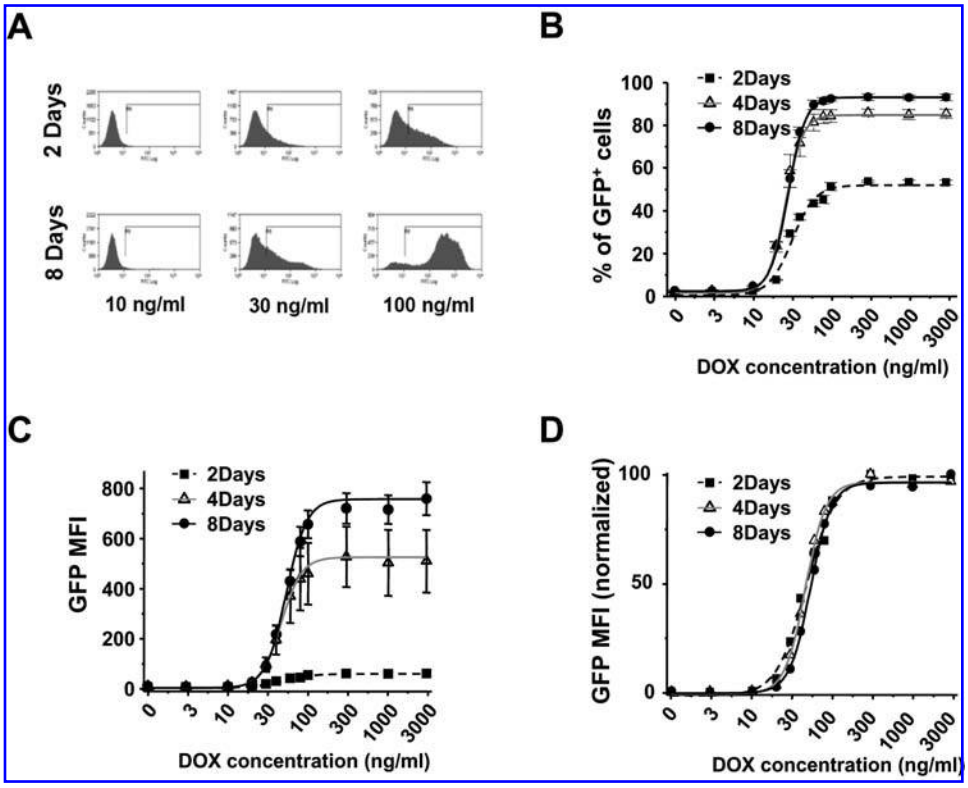

FIG. 3. (**A**) Representative FACS analyses of GFP expression of co-transduced mouse ES cells at days 2 and 8 after treatment with different concentrations of DOX. (**B**) The dose–response relationships for the % of GFP^+ mES cells at days 2, 4, and 8. (**C**) The dose–response relationships for the MFI at days 2, 4, and 8. (**D**) The normalized dose–response relationships for MFI.

(Fig. 4B); GFP silencing did not occur until 3 days after DOX was withdrawn, and was completed at day 8 with a T_{50}^{off} of 127.7 ± 3.9 h (Fig. 4C). Collectively, these results indicate that the responses of the hES cells to the addition and removal of DOX were significantly slower than their mouse counterpart.

We also investigated the effects of different DOX concentrations on LV-TR-KRAB-IRES-dsRed/LV-THM-GFP co-transduced hES cells (Fig. 5). No GFP^+ hES cells were observed for 0, 3, 6, and 8 ng/ml but 10 ng/ml or higher at days 7 and 14; the percentage of GFP^+ hES cells increased dose-dependently and leveled at 30 ng/ml (Fig. 5A,B). Similar to mES cells, higher DOX concentrations (i.e., 60 ng/ml, 100 ng/ml, 300 ng/ml, and 1 μg/ml) did not further increase the total percentage of GFP^+ hES cells (Fig. 5A–C). However, the total MFI increased from 10 to 100 ng/ml at both days 7 and 14 (Fig. 5C). The EC_{50} values were 26.0 ± 2.2 and 23.7 ± 1.7 ng/ml, respectively ($p > 0.05$; Fig. 5D). Taken collectively, the inducible system mediated by LV-TR-KRAB-IRES-dsRed and LV-THM-GFP when incorporated into hES cells exhibited reversible dose-dependent responses with a sensitivity similar to mES cells, albeit the kinetics were much slower. All the parameters assessed for mES and hES cells are summarized in Table 1.

Altering the electrophysiological properties of ES cells via conditional expression of Kir2.1 channels

Recently, we reported the presence of functional ion channels in mES and hES cells [7]. To employ the inducible system described above for conditionally altering electrophysiological phenotypes, we co-transduced mES cells with LV-TR-KRAB-dsRed2 and LV-THM-Kir2.1-GFP (see Materials and Methods). The endogenous gene Kir2.1, which encodes for the inwardly rectifying K^+ current (I_{K1}), was chosen because I_{K1} is known to play a pivotal role in development (e.g., synaptogenesis) [22] as well as the properties of various terminally differentiated cell types, from neurons [23] to cardiomyocytes [24,25]. Functionally, I_{K1} exerts its effects by stabilizing the RMP, which in turn governs cell cycle [26], excitability [27], and other related processes [13]. Figure 6A shows that GFP signals were observed only in LV-THM-Kir2.1-GFP/LV-TR-KRAB-dsRed2-co-transduced mES incubated with DOX (1 μg/ml). Ba^{2+}-sensitive I_{K1} could be recorded only in DOX-treated co-transduced mES cells but not WT or co-transduced mES without DOX (Fig. 6B). With DOX treatment, the RMP became significantly hyperpolarized ($p < 0.001$) from 0.9 ± 1.6 mV ($n = 16$) of control mES cells (i.e., both WT and untreated co-transduced, whose RMPs were sta-

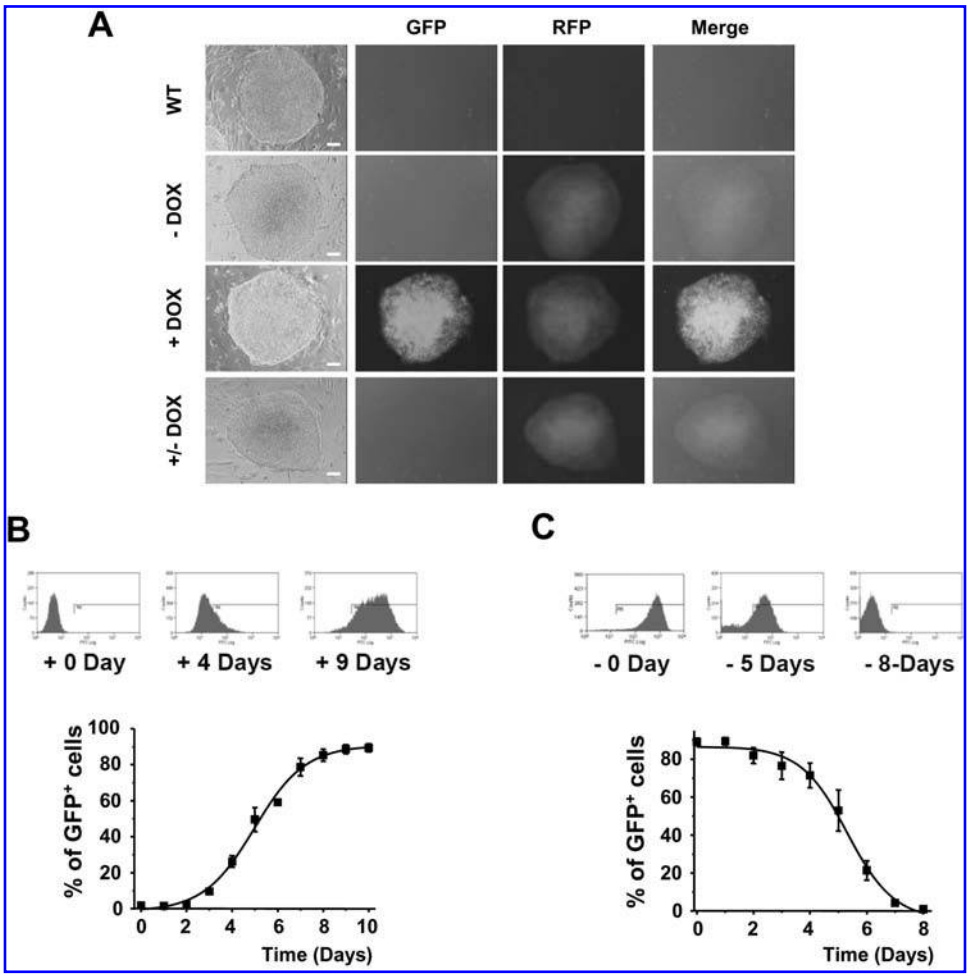

FIG. 4. The LV-based DOX-inducible system in human ES cells. (**A**) Representative images of WT and LV-THM-GFP/LV-TR-KRAB-dsRed co-transduced human ES cells in presence (+) or absence (−) of DOX (1 μg/ml) as indicated. GFP expression was completely reversible with virtually no expression leakage upon withdrawal of DOX (+/− DOX). Bar, 100 μm. (**B**) Representative FACS analyses (*upper*) and the kinetics (*lower*) of inducible GFP expression of co-transduced human ES cells at different time points after DOX treatment (1 μg/ml). (**C**) Representative FACS analyses (*upper*) and the corresponding decay kinetics (*lower*) of GFP disappearance after DOX withdrawal.

tistically identical; $p > 0.05$) to -61.1 ± 1.5 mV ($n = 8$) after Kir2.1 expression was induced by 1 μ/ml DOX (Fig. 6C; squares and triangles, respectively). Similar to the expression of GFP alone (cf. Fig. 2), the functional effect of Kir2.1 expression on RMP was highly DOX-dependent. Figure 6C also shows that the change in FMP fluorescence density increased with elevating DOX concentrations (from 10 to 100 ng/ml). These changes mirrored the changes in electrophysiological properties as assessed by whole-cell current-clamp recordings [21] as well as the level of GFP signal (of the fusion protein Kir2.1-GFP; Fig. 6D). The expression of SSEA-1 was statistically identical before and after DOX treatment (Fig. 6E; $p > 0.05$), indicating that pluripotency was unaffected by Kir2.1-GFP overexpression. Indeed, co-transduced mES cells could be readily differentiated into the germ layer derivatives (including cardiomyocytes; data not shown), indicating that pluripotency was not affected. Additionally, cell proliferation and metabolism were also not different before and after induction by DOX ($p > 0.05$; Fig. 6F,G).

Similar to mES cells, Kir2.1-GFP could be inducibly expressed in hES cells. Electrophysiological recordings of LV-THM-Kir2.1-GFP/LV-TR-KRAB-dsRed2-co-transduced hES cells confirmed that Kir2.1 channels were expressed and functional (Fig. 6H, right). As anticipated, their RMP was also hyperpolarized (-41.4 ± 2.1 mV, $n = 16$ vs. -4.2 ± 1.3 mV, $n = 28$ of control, $p < 0.001$; Fig. 6H).

DISCUSSION

Mammalian ES cells were first derived from murine blastocysts over a quarter century ago [28,29]. This landmark achievement led to the subsequent generation of transgenic

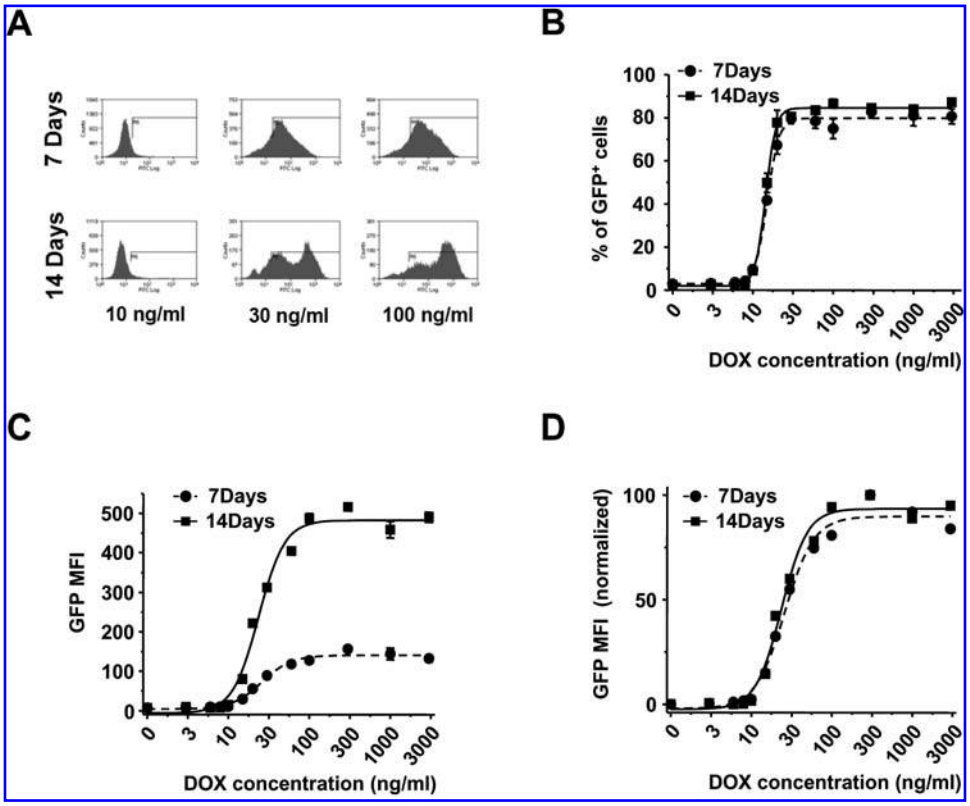

FIG. 5. (**A**) Representative FACS analyses of GFP expression of co-transduced human ES cells at days 7 and 14 after treatment with different concentrations of DOX. (**B**) The dose–response relationships for the % of GFP^+ human ES cells at days 7 and 14. (**C**) The dose–response relationships for the MFI at the same time points. (**D**) The normalized dose–response relationships for MFI.

animals with targeted gene mutations [30], revolutionizing developmental biology, animal disease models, and genetics. In 1998, Thomson et al. [1] reported the first successful isolation of hES cells. Not only do self-renewing pluripotent hES cells offer unprecedented hopes for tissue regeneration, but they can also serve as an invaluable in vitro experimental model for investigating human development and diseases. Clearly, the ability to genetically modify hES cells and/or their tissue-specific derivatives further provides a flexible approach for probing the role(s) of specific gene(s) in human development and may even lead to "custom-tailored" hES cell-derived grafts for transplantation therapies. However, conventional methods for genetic manipulation (e.g., electroporation and plasmid transfection) that are highly effective for mES cells prove to be inefficient or even ineffective for hES cells. By contrast, LV can efficiently transduce a wide range of cell types including post-mitotic and nondividing cells [31]; indeed, long-term LV-mediated transgene expression in hES cells has been demonstrated [10,11,32,33].

The Cre-loxP- or tetracycline (tet)-regulated systems are among the most commonly used inducible gene regulation systems employed for elucidating the (developmental) roles of target genes. Although the Cre-mediated recombination has been successfully established in mES [34] and hES [35] cells and is clearly a robust tool for conditional gene expression, it lacks the reversibility that is needed for temporally switching on and off a given transgene. Using plasmid transfection, the tet-controlled system has been employed to regulate gene expression reversibly in murine

TABLE 1. KINETIC PARAMETERS AND EC_{50} FOR GFP EXPRESSION OF LV-TR-KRAB-IRES-dsRed/LV-THM-GFP CO-TRANSDUCED mES AND hES CELLS

	T_{50}^{on} (hours)	T_{100}^{on} (days)	T_{50}^{off} (hours)	T_{100}^{off} (days)	EC_{50} (ng/ml)	EC_{100} (ng/ml)
mES cells	35.5 ± 5.5	>5	71.5 ± 2.4	>4	49.5 ± 8.5	>300
hES cells	119.5 ± 3.2	>10	127.7 ± 3.9	>8	24.5 ± 2.2	>100

$T_{50}^{on/off}$, the time at which 50% of all green cells appeared/disappeared; $T_{100}^{on/off}$, the time at which 100% of all green cells appeared/disappeared; $EC_{50/100}$, the effective concentrations at which 50%/100% of the maximum EFI was reached.

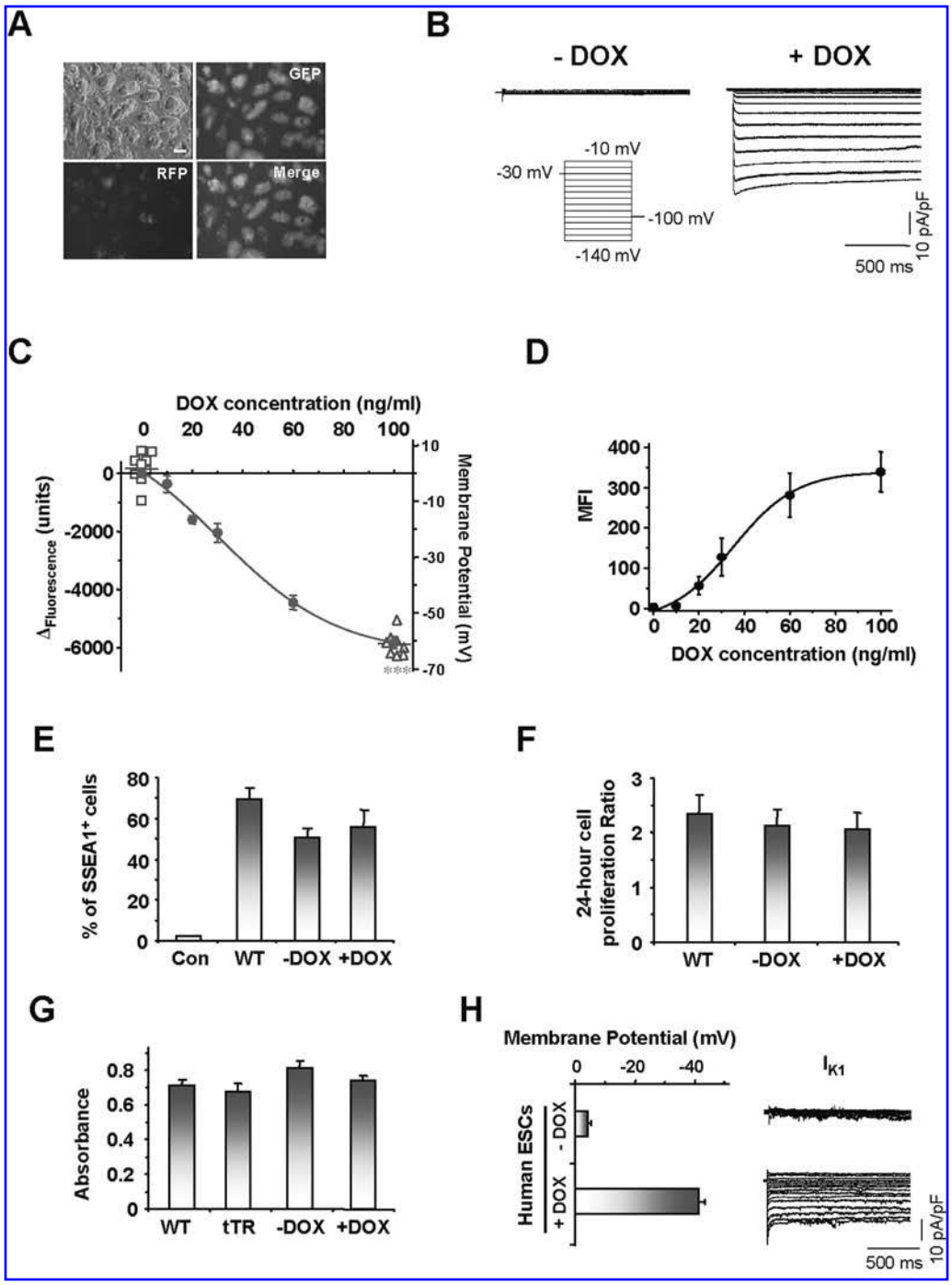

FIG. 6. (**A**) Representative images of LV-THM-Kir2.1-GFP/LV-TR-KRAB-dsRed co-transduced mouse ES cells in presence of DOX. Bar, 50 μm. (**B**) The inwardly rectifying I_{K1} was measured in DOX-treated LV-THM-Kir2.1-GFP/LV-TR-KRAB-dsRed co-transduced but not untreated mES cells. (**C**) The RMP was measured by patch-clamp recordings in the presence (gray open triangles) or absence (gray open squares) DOX. In parallel, the FMP dye was used to measure the changes of membrane potential. $\Delta_{fluorescence}$ (solid circles) between the untreated and DOX-treated mES cells are shown. (**D**) Dose-dependent relationship for MFI of Kir2.1-GFP expression in LV-THM-Kir2.1-GFP/LV-TR-KRAB-dsRed co-transduced cells. SSEA-1 expression (**E**), cell proliferation (**F**), and metabolism (**G**) as assessed by MTT assay of control and LV-THM-Kir2.1-GFP/LV-TR-KRAB-dsRed co-transduced mES cells. (**H**) The RMP (*left*) and I_{K1} (*right*) of LV-THM-Kir2.1-GFP/LV-TR-KRAB-dsRed co-transduced human ES cells in the presence (+) or absence (−) DOX.

[9] and nonhuman primate [36] ES cells. Taking advantage of the demonstrated ability of LV for persistent genetic modification, here we established a reversible LV-based tet-regulated gene expression system in hES cells. During the preparation of this work, such a tet-inducible expression system in hES cells has been independently established and reported [14]. In this recent study, ~57% hES cells could be induced to express GFP upon the addition of DOX. In our experiments, up to 90% of GFP^+ mES and hES cells could be achieved; transgene expression was also tightly controlled by DOX with virtually no leakage.

More importantly, the detailed dose-dependence and kinetics of transgene expression were investigated in our present study. Such information is crucial for designing future experiments in hES cells using this LV-based inducible system. Additionally, we provide the first demonstration that an endogenous gene can be inducibly expressed in hES cells for altering their functional phenotypes. On the basis of our earlier finding that several specialized ion channels are functionally expressed in mES and hES cells [7], we specifically focused on their electrophysiological properties. We have shown that the RMP of undifferentiated mES and hES cells can be hyperpolarized without affecting pluripotency. Indeed, direct injection of pluripotent ES cells after myocardial infarction has been suggested as a means to repair the damaged heart [37]. However, transplantation of cells with undesirable electrical properties (such as a positive RMP) into the heart is known to predispose the recipients to electrical disturbances by generating transmural differences that are substrates of potentially lethal arrhythmias. Developmentally, the ion channel expression profiles of mES and hES cells are known to undergo significant changes during differentiation particularly for excitable cells such as neurons and cardiomyocytes [38]. Whereas the electrical activity per se has been demonstrated to influence the development of neurons heavily [22], similar activity dependence has not been demonstrated for cardiogenesis. In addition, the I_{K1}-stabilized RMP governs cell cycle and excitability, and plays an important role in the development of excitable cells [13,26,27]. The system established here presents a unique tool to investigate the cardiac- and neural differentiation of hES cells and thereby facilitate their future clinical applications.

In summary, we conclude that the LV-inducible system presented here enables investigators to control the level of target gene expression dose dependently at specific time points in hES cells during their differentiation. Although a constitutively active promoter (EF-1α) was employed here for proof-of-concept experiments, promoters that are tissue-restricted (e.g., the myosin light chain 2v,MLC2v, and the Na^+/Ca^{2+} exchanger 1, NCX1, promoters that are specific to the heart) or germ layer-restricted (e.g., the mesoderm-specific brachyury T) can be used for probing lineage-dependent effects.

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