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In Vitro- and In Vivo-Induced Transgene Expression in Human Embryonic Stem Cells and Derivatives

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Abstract

The use of human embryonic stem cells (hESCs) as a research and therapeutic tool will be facilitated by conditional gene expression. Here, we report drug-induced transgene expression, both in vitro and in vivo, from a tet-on hESC line with >95% purity. Using green fluorescent protein as an indicator, we demonstrated that the tet-on system allowed a tight control of the gene expression in both undifferentiated hESCs and differentiated cells of the three germ layers. More importantly, after the cells were transplanted into animals, the gene expression remained to be regulated by an orally administered drug. These results provide a technical basis for regulation of gene expression in hESCs and derivatives in vitro and in vivo.

Keywords

Human embryonic stem cell; Gene modification; Gene delivery systems in vivo or in vitro; Inducible gene expression

INTRODUCTION

A major contribution of mouse embryonic stem cells (mESCs) to biomedical science is the construction of transgenic mouse lines. Similarly, application of human embryonic stem cells (hESCs) [1,2] in human biology and regenerative medicine will depend largely on our ability to genetically modify them. However, many classic molecular biology technologies that have been successful in mESC studies, such as homologous recombination [3,4], cre-loxp recombination [5], and tetracycline-inducible gene expression [6–8], have not been widely used in hESCs chiefly because of low transfection efficiencies [3,9].

Traditional constitutive overexpression is often limited by its inability to precisely control the timing and level of gene expression, which are critical for the function of many genes. It is not uncommon for fate-determining genes to be toxic at inappropriate stages or even have different or complete opposite functions at different expression levels [10]. Thus, efficient conditional

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

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manipulation of transgene expression is necessary for dissecting basic molecular mechanisms underlying hESC biology.

The most common strategy to achieve conditional transgene expression is use of the tetracycline-inducible gene expression systems (tet-on or tet-off) [11,12]. This technique has recently been used in hESCs, first reported by Zhou et al., using a tet-on lentiviral vector system expressing the transcriptional suppression domain [8]. An independent study also indicates that the tet-on transactivator does not impair the self-renewal or pluripotency of hESCs and thus can safely be applied in these cells [7]. Another conditional gene expression system, Cre-ERT2, has also been demonstrated to efficiently control transgene expression in hESCs [6]. However, in these studies, the best-established cell line exhibited only 75% purity in terms of inducible gene expression. Although inducible gene expression was observed after preliminary differentiation of hESCs to embryoid bodies (EBs) [6], it has not been demonstrated after in vitro differentiation of hESCs to functional cells.

Mouse ESCs with an inducible gene expression system have been used to generate transgenic animals, and many important gene functions have been elucidated through in vivo gene regulation via orally administrated drugs [13,14]. For ethical concerns, the only way to extend hESC study in vivo is to transplant the cells into animals. However, evidence is lacking for regulatable gene expression from transplanted hESCs and their derivatives. Apparently, exploration of conditional gene expression from transplanted hESCs is also of value for basic research and for potential future clinical application of hESCs in both gene therapies and cellular therapies [15].

In this study, we report the establishment of a highly homogeneous tet-on hESC line. Using green fluorescent protein (GFP) as an indicator, we showed a tight regulation of gene expression in response to doxycycline treatment for both the hESCs and their differentiated derivatives in vitro. The gene expression remained regulatable by an orally administrated drug after the hESCs were differentiated to multiple cell lineages in the teratoma in vivo and after the hESC-derived neuroepithelial cells were transplanted into the mouse brain.

MATERIALS AND METHODS

DNA Constructs

The cytomegalovirus promoter was removed from the pTet-On vector (Clontech, Palo Alto, CA, <http://www.clontech.com>) by SpeI/EcoRI digestion and replaced with a CAG promoter to generate the pCAG-reverse tetracycline transactivator (rtTA) vector. The neomycin-resistant gene in the pTet-on vector was retained and used for drug selection. The pTRE-enhanced green fluorescent protein (EGFP) vector was generated by inserting an EGFP gene (Clontech) into the multiple cloning site of the pTRE-Tight vector (Clontech).

Human ESC Culture and Transfection

Human ESC line H9 [1] (WiCell Research Institute, Madison, WI, <http://www.wicell.org>) was cultured on irradiated mouse embryonic fibroblasts (MEF) in the hESC culture medium consisting of Dulbecco's modified Eagle's medium (DMEM)-Ham's F-12 medium (F12), 20% knockout serum replacer (Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>), 100 μ M minimal essential medium nonessential amino acids, 1 mM L-glutamine, 0.1 mM β -mercaptoethanol, and 4 ng/ml basic fibroblast growth factor (bFGF) (R&D Systems Inc., Minneapolis, <http://www.rndsystems.com>). For transfection, cells were dissociated with dispase II (1 mg/ml) to small clusters of approximately 50 – 100 μ m in diameter. For estimation of cell density, an aliquot of the cell cluster suspension (0.5 ml) was further trypsinized to single cells for counting. For each electroporation, 10×10^6 cells were mixed with 55 μ g of

linearized DNA (5 µg of pCAG-rtTA, 50 µg of pTRE-EGFP) and resuspended in 0.8 ml of hESC culture medium. Cells were then transferred to a 4-mm electroporation cuvette and exposed to a single 320-V, 200-µF pulse using a Bio-Rad Gene Pulser II (Bio-Rad, Hercules, CA, <http://www.bio-rad.com>) [3,16,17]. After electroporation, cells were plated on MEF at a high density in three wells of a six-well plate. G418 selection was applied 48 hours later, starting with 50 µg/ml and increasing to 100 µg/ml 2 days later. Two weeks after selection, G418-resistant colonies were picked and transferred to an MEF-coated 24-well plate for expansion.

Neural Differentiation In Vitro

Neural differentiation of hESCs was performed following a chemically defined protocol [18, 19]. ESCs were first grown in suspension in the ESC culture medium to form aggregates for 4 days. The aggregates were then transferred to a neural medium (DMEM-F12, nonessential amino acids, neural supplement N2, 10 ng/ml bFGF) and grown in suspension for another 3 days before they were attached to a plastic culture surface. After 7 days, the columnar cells in the neural tube-like structures were detached and grown in suspension to form neuroepithelial clusters. For neuronal differentiation, neuroepithelial clusters were expanded for 7 days in suspension and then dissociated with Accutase (Innovative Cell Technologies Inc., San Diego, <http://www.innovativecelltech.com>), plated on polyornithine- and laminin-coated coverslips, and further cultured until they were used for immunocytochemistry.

Cardiomyocyte Differentiation In Vitro

Cardiomyocyte differentiation of hESCs was performed as described previously [20], with slight modification. Briefly, undifferentiated hESCs were dispersed to small clumps of approximately 100 µm in diameter with 1 mg/ml dispase II. Approximately 5×10^6 cells were transferred to a 60-mm Petri dish and grown in suspension in fetal bovine serum (FBS) medium (DMEM with 10% FBS, nonessential amino acids, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol) for 7 days to form EBs. EBs were then plated on a 0.1% gelatin-coated 24-well plate at a density of five EBs per well. Cells were observed microscopically every day for the appearance of spontaneous contractions, which typically start within 3 days.

Insulin-Expressing Endocrine Cell Differentiation In Vitro

Insulin-expressing endocrine cells were differentiated following the protocol described by D'Amour et al. [21,22]. Cells were cultured on MEF feeder layers in a six-well plate during the whole procedure. To start differentiation, the hESC culture medium was changed to RPMI (Invitrogen) with 100 ng/ml activin A and 25 ng/ml Wnt3a (both from R&D Systems). Two days later, Wnt3a was withdrawn, and 0.2% FBS was added to the culture medium for another 2 days. Then, the concentration of FBS was increased to 2%, and activin was replaced with 50 ng/ml fibroblast growth factor 10 (FGF10) (R&D Systems) and 0.25 µM keto-N-aminoethylaminocaproyl dihydrocinamoyl-cyclopamine (Calbiochem, San Diego, <http://www.emdbiosciences.com>). Three days later, the medium was changed to DMEM with 1% B27 (Invitrogen) supplemented with FGF10, KAAD-cyclopamine, and 2 µM all-trans retinoic acid for 6 days. Cells were then grown in CMRL (Invitrogen) with 1% B27 until they were used for immunocytochemistry study.

Immunocytochemistry

Immunocytochemical staining was performed as previously described [18]. Antibodies used included rabbit anti-Oct4 (Abcam, Cambridge, MA, <http://www.abcam.com>), mouse anti-stage specific embryonic antigen (SSEA)-4 (Chemicon, Temecula, CA, <http://www.chemicon.com>), mouse anti-human nuclei (Chemicon), mouse anti-βIII-tubulin (anti-Tuj) (Sigma-Aldrich, St. Louis, <http://www.sigmaaldrich.com>), mouse anti-cardiac

troponin I (antiTnI) (Abcam), rabbit anti-insulin (Chemicon), and mouse anti-glucagon (Sigma-Aldrich).

Fluorescence-Activated Cell Sorting

Cells were trypsinized for 5 minutes, washed once with phosphate-buffered saline (PBS), and resuspended in the BD FACSflow sheath fluid (BD Biosciences, San Diego, <http://www.bdbiosciences.com>). They were then passed through a 70- μ m cell strainer (BD Biosciences) to remove cell clusters before sorting. Fluorescence-activated cell sorting (FACS) analysis was performed on the BD FACSCalibur system (BD Biosciences).

Formation of Teratomas

hESCs were injected subcutaneously into severe combined immunodeficient (SCID) mice (Jackson Laboratory, Bar Harbor, ME, <http://www.jax.org>) on the back. Two months after injection, animals were given 500 μ g/ml doxycycline dissolved in drinking water containing 3% sucrose. Ten days later, mice were sacrificed and teratomas were removed. The teratomas were then postfixed with 4% paraformaldehyde in PBS, followed by cryoprotection in 30% sucrose overnight and cryosectioned to 25 μ m. Histological examination of the sections was performed by hematoxylin and eosin staining. All animal experiments were performed following protocols approved by Institutional Animal Care and Use Committee.

Transplantation

Neuroepithelial clusters were dissociated with trypsin/Accutase (1:1) and resuspended in an artificial cerebral fluid (Harvard Apparatus, Holliston, MA, <http://www.harvardapparatus.com>) at a density of approximately 5×10^4 cells per microliter. For transplantation, neonatal SCID mice were cryoanesthetized with ice. Approximately 2 μ l of cell suspension was slowly injected into each lateral ventricle using a glass micropipette, as previously described [23]. At indicated time points (described in Results), animals were euthanized and perfused transcardially with normal saline followed by freshly made ice-cold 4% para-formaldehyde prepared in PBS. Brains were removed, postfixed in the same fixative for 4 hours at 4°C, and preserved in 20% and then 30% sucrose/PBS solution. Tissues were sliced into 25- μ m-thick coronal sections and stored in a tissue preserving buffer (30% sucrose and 30% ethylene glycol in PBS) at -20°C until immunocytochemical analysis was performed.

Microscopy

Images were collected using a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI, <http://www.diaginc.com>) mounted on a Nikon TE600 fluorescent microscope (Nikon, Tokyo, <http://www.nikon.com>). Cell counting and fluorescence intensity measurement were performed using the Metamorph software (Universal Imaging Corporation). Randomly chosen fields of pictures were taken from at least three independent coverslips for each counting; averages and SDs were calculated using Microsoft Excel (Microsoft, Redmond, WA, <http://www.microsoft.com>). An unpaired *t* test was used to evaluate statistical significance.

RESULTS

Establishment of the inGFP_{hES} Cell Lines

Two vectors were constructed as described in Materials and Methods, the regulator vector pCAG-rtTA and the response vector pTRE-EGFP (Fig. 1A). The CAG promoter was used to drive the rtTA gene for its sustained activity during embryonic stem cell differentiation [24, 25]. After being cotransfected with the two vectors by electroporation, the hESC clones (H9, passage 21) were isolated and subcultured after 14 days of G418 selection. Approximately 200 clones were obtained after transfection with 2×10^7 cells (two electroporations). They were

subsequently characterized for inducible GFP expression, and 10 clones were finally established based on three criteria: no detectable leaky expression in the absence of doxycycline, abundant overexpression upon doxycycline treatment, and homogeneous expression within the clone. The clones were named inGFP_hES and numbered 1–10 individually. Clone 3 (inGFP_hES#3) was used as an example to show inducible GFP expression (Fig. 1B). It had no detectable fluorescence background in the absence of doxycycline. Upon treatment of 1 µg/ml doxycycline, GFP signal started to appear within 8 hours and reached a plateau after approximately 24 hours. After doxycycline withdrawal, GFP signal slowly faded away until it completely disappeared after approximately 96 hours. The GFP signal was quantified using Metamorph software and shown in Figure 1C. Besides temporal control, the GFP expression level can be quantitatively regulated by varying the doxycycline concentration (Fig. 1D). Twenty-four hours after treatment, GFP expression was observed in response to as low as 0.05 µg/ml doxycycline, and increased fluorescence intensity was detected with higher concentrations of doxycycline until it saturated at approximately 0.5 µg/ml doxycycline. The GFP signal responded to doxycycline almost linearly in the range of 0 – 0.2 µg/ml (Fig. 1D).

Cells were collected 48 hours after treatment with 1 µg/ml doxycycline. FACS analyses indicated that approximately 95% of the cells were able to inducibly express GFP (Fig. 1E). This percentage was likely due to some cells having been buried in the tight colonies and possibly not accessible to doxycycline. The difference in cell accessibility may also explain the left tail of the FACS peak for the cells treated with doxycycline, since inner cells may be shielded from doxycycline molecules by outer cells.

inGFP_hES Cell Line Retains the ESC Property and Has a Normal Karyotype

The pluripotency of the inGFP_hES#3 cell line is maintained, as shown by the expression of two characteristic pluripotent markers Oct4 and SSEA-4 (Fig. 2A). To check whether the cell line is self-renewable and could be expanded for a long term at a normal rate, we passaged it for 20 passages and compared the growth rate with normal H9 cells of equivalent passages. The result showed that the growth rate was not changed by the genetic modification (Fig. 2B). Moreover, we observed only subtle loss of inducible GFP expression during long-term passages, indicating that proliferation-related gene silencing was not significant.

Karyotype abnormalities may develop at high passages [26,27], especially after genetic modifications. We therefore started with a relatively early passage (passage 21) of H9 cells with a normal karyotype. In addition, we passaged the parental cells and the inGFP_hES cells using the mechanical dissecting method [2,26], which has been shown to maintain a stable genetic background for a long time. The inGFP_hES#3 cell line expanded in this way retained the normal karyotype at passage 31 (Fig. 2C).

Differentiated hESC Progenies Retain Inducible GFP Expression In Vitro

Inducible gene expression from fully differentiated hESCs has not been demonstrated, and whether the doxycycline response time changes in differentiated cells remains uninvestigated. In the present study, we examined the inducible GFP expression after the hESCs were differentiated into neurons, cardiomyocytes, and insulin-expressing endocrine cells that developmentally originated from the ectoderm, mesoderm, and endoderm, respectively, following the well-characterized protocols previously published and described in Materials and Methods [19,20,22]. Neurons were characterized by the typical polarized morphology and the expression of Tuj (Fig. 3A), cardiomyocytes were characterized by the expression of cTnI (Fig. 3A) and spontaneous contraction (supplemental online Movie 1), and insulin-expressing endocrine cells were characterized by the expression of insulin and glucagon (Fig. 3A; supplemental online Fig. 1). After the cells were differentiated, doxycycline (1 µg/ml) was applied, and GFP expression was observed in all three different cell types when monitored

every 12 hours (Fig. 3A, middle and right columns show GFP signal before and 48 hours after doxycycline treatment, respectively). However, the doxycycline response time was significantly prolonged as compared with undifferentiated hESCs (Fig. 3B), with a plateau being reached after approximately 48 hours and clearance of GFP at approximately 7 days after doxycycline withdrawal for all three cell types tested.

Although GFP expression was detected in all three differentiated cell types, we noticed that some cells were GFP-negative in the presence of doxycycline, approximately 20%, 35%, and 50% for neurons, cardiomyocytes, and endocrine cells, respectively (Fig. 3C). We also observed that the GFP intensity varied with tissue types (Fig. 3B), with neurons exhibiting the highest GFP level and endocrine cells the lowest. The tissue type-related tendency of gene silencing was the same for all 10 inGFP_{hES} clones we established. Since the clones were established by random DNA integration (using the electroporation method), this result indicates that it is probably a general phenomenon for most of the gene integration loci in the human genome.

GFP Expression Is Inducible in Teratomas by Orally Administered Doxycycline

Teratomas were produced by injection of the synGFP_{hES}#3 cells into SCID mice to further confirm the pluripotency of the cell line as well as test the inducible transgene expression in vivo. Sixty days after injection, teratomas were observed, and the animals were fed with doxycycline in drinking water for another 10 days before teratomas were removed and examined. Neuroepithelium, cartilage, and endodermal epithelium tissues were identified by their typical morphology on the teratoma sections stained with hematoxylin and eosin (Fig. 4A). GFP expression was detected in the tissues representing the three germ layers, although the expression level was not homogenous among all tissues (Fig. 4B, 4C). This result indicates that the orally administered doxycycline was able to get access to the transplanted tissues and turn on the unsilenced inducible promoter.

Transgene Is Regulated in Neural Cells Transplanted into the Mouse Brain

Neuroepithelial cells, differentiated from inGFP_{hES}#3 cells for 3 weeks using our defined culture method [19], were transplanted into the lateral ventricles of both sides of neonatal SCID mice as described [23]. Two months after transplantation, the animals were subjected to a cycle of doxycycline feeding and withdrawing (protocol illustrated in Fig. 5A). Similar to our previous observation [23], most of the grafted cells were localized to the ventricle, with some cells present in the parenchyma tissues surrounding the ventricle (Fig. 5B). GFP expression was observed in grafted cells, labeled by human-specific nuclear protein, 3 days following the oral intake of doxycycline (Fig. 5C). The maximum number of GFP-positive cells was detected 7 days after doxycycline application, which is much slower compared with that in vitro. After withdrawal of doxycycline, the GFP signal gradually decreased until it completely disappeared 2 weeks later (Fig. 5C, 5D). We also observed that the GFP disappeared faster in the neuronal cell body than in neurites, so that 7 days after doxycycline withdrawal, GFP was observed only in the neurites and not in the cell body (Fig. 5C, withdrawal day 7; supplemental online Fig. 2). This is probably caused by the localization of the protein degrading machinery.

DISCUSSION

In this study, we established a highly homogeneous “tet-on” inducible GFP hESC line (inGFP_{hES}). The transgene (GFP) expression is regulatable not only in undifferentiated hESCs but also in differentiated functional cells representative of the three germ layers. Importantly, the transgene is inducible in hESC-differentiated progenies in vivo, in multiple tissue types in the teratoma, as well as in differentiated neuronal cells following engraftment of neuroepithelial cells in the brain. Technically, we have determined the differential kinetics of

the transgene expression in vivo as well as in vitro for undifferentiated hESCs and differentiated functional cell/tissue types. This information will lay down a solid conceptual and technical foundation for future exploration of conditional expression of a functional gene in hESCs.

The inGFPPhES cell lines were established by electroporation [3,17], a random gene integration method, in this study. Transgene expression in stem cells through random integration is almost inevitably downregulated along differentiation, especially in human cells. A previous report showed that the percentage of inducible transgene-expressing cells decreased after 3 days of preliminary differentiation of hESCs to EBs [6]. In our present study, we found that transgene was further silenced after full differentiation, and the extent of gene silencing depended on the cell type. Gene silencing is likely related to local chromatin structure of the integration site. The most common strategy to overcome gene silencing is to knock-in the gene into a specific gene silencing-resistant locus by homologous recombination, as has been proven in mESCs [28,29]. However, gene silencing-resistant loci, such as the ROSA26 locus [30,31] in mESCs, have not been identified in hESCs. Homologous recombination in hESCs remains a technical challenge today, with only a few successful examples reported [3,4]. These technical hurdles need to be overcome before silencing-free transgenic hESC lines can be established.

It is interesting to find different kinetics of transgene induction and clearance between undifferentiated hESCs and differentiated progenies. Although the induction and clearance of the GFP in undifferentiated hESCs are very tight, they are slower to respond to doxycycline in differentiated cells. This is possibly due to a slower metabolic rate in differentiated cells. This finding provides a technical ground for differentially regulating transgene expression in undifferentiated versus differentiated human cells. The clearance of transgene expression following the doxycycline withdrawal should depend on the stability of the protein. Since GFP is stable, with a half-life of approximately 26 hours [32], we expect that the residual protein may be cleared faster for most other expressed proteins. To further accelerate the protein degradation after doxycycline withdrawal, signal peptides may also be used to create fusion proteins with a shorter half-life [32].

Inducible gene transcription in human stem cells in vitro does not necessarily translate to conditional gene expression in vivo. Our in vivo experiment is the first demonstration that gene expression from transplanted hESC derivatives is regulatable through an orally administered drug. This result is inspiring for studying developmental aspects of human cells in vivo and future clinical application of transgenic hESCs. The slower doxycycline response in vivo is expected since orally administered drugs need to be absorbed into the blood, transported to pass the blood-brain barrier, and then released into the cerebral fluid to access to the transplanted cells. Even after the withdrawal of doxycycline from the drinking water, residual doxycycline in the cerebral fluid and blood may sustain the gene expression for approximately 2 weeks, further slowing down the drug responsive rate. Nevertheless, our finding has identified specific parameters for future studies in regulating transgene expression in hESC-derived cells in the brain, which is not currently available.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Establishment of the inGFPPhES cell line

(A): Structures of the pCAG-rtTA-neo and pTRE-EGFP vectors. (B): GFP expression in the inGFPPhES cell after treatment with 1 μ g/ml Doxy. and withdrawal of Doxy. (insets, phase-contrast images). Scale bar = 100 μ m. (C): Quantification of the GFP fluorescent intensity after Doxy. application and withdrawal. (D): Dose-dependent GFP intensity in response to different concentrations of Doxy. Intensities were measured 24 hours after Doxy. treatment. (E): Fluorescence-activated cell sorting analysis of the inGFPPhES clone before (green line) and after (purple filled) 48 hours of Doxy. treatment. Abbreviations: arbitrary unit, a.u.; Doxy., doxycycline; EGFP, enhanced green fluorescent protein; h, hours; rtTA, reverse tetracycline transactivator; t, time.

Figure 2. Pluripotency, expansion, and karyotype of the inGFPhES cell line

(A): Left, BF image of an inGFPhES#3 colony. Right, Oct4 (green) and SSEA-4 (red) staining of the colony. Scale bar = 50 μ m. (B): Comparison of the cell proliferation rates between inGFPhES (red square) and parental H9 (blue triangle) cell lines. Cells were passaged every 6 days for 20 passages. Numbers shown were for one well of a six-well plate. Results are the mean of three independent experiments; bars indicate SD. Statistical analysis showed no difference between the two groups ($p > .5$ by t test). (C): Karyotype of the inGFPhES cell line by standard G banding. Abbreviation: BF, bright-field.

Figure 3. Inducible GFP expression after human ESC differentiation

(A): GFP expression in neurons, cardiomyocytes, and insulin-expressing endocrine cells before and after Doxy. treatment. Left column, characterization of the differentiated cells by lineage-specific markers: Tuj for neurons, cTnI for cardiomyocytes, and insulin for insulin-expressing endocrine cells. Note that although most cells in the region shown are GFP-positive, cells in other regions on the same coverslip may be GFP-negative. The total percentage of GFP-positive cells is given in (C). More characterizations are shown in supplemental online Movie 1 and supplemental online Figure 1. Middle column, GFP expression was not detectable in the absence of Doxy. Insets are bright-field images. Right column, 48-h treatment of Doxy. turned on GFP expression in all three differentiated cells. Insets are bright-field images. Scale bar =

20 μm . **(B)**: Doxy. responsive time for differentiated cells (blue, neuron; red, cardiomyocyte; green, insulin-expressing endocrine cell). Results are the mean of three independent experiments, with indicated SDs. **(C)**: Percentage of GFP-positive cells after Doxy. treatment. Results were averaged from 10 independent coverslips, with indicated SDs. The rest of the cells were GFP-negative due to gene silencing. Abbreviations: arbitrary unit, a.u.; cTnI, cardiac troponin I; Doxy., doxycycline; GFP, green fluorescent protein; h, hours; t, time; Tuj, β III-tubulin.

Figure 4. Inducible green fluorescent protein (GFP) expression in teratoma formed by inGFPPhES cells

(A): Histological examination by hematoxylin and eosin staining showing neuroepithelia, cartilage, and endothelial tissues. Scale bar = 100 μ m. (B): Oral-administered doxycycline induced GFP expression in different tissues. Arrowheads, neuroepithelia; asterisks, cartilage; arrows, endothelia. Animals were fed with doxycycline for 10 days before the tissues were collected. Scale bar = 100 μ m. (C): Magnified view of representative tissues from the three embryonic germ layers. Scale bar = 50 μ m.

Figure 5. Inducible GFP expression in in-GFPhES cell-derived neural cells in the brain
(A): Procedures for transplantation and Doxy. feeding. **(B):** Seven days of Doxy. feeding effectively turned on GFP expression in the inGFPhES-derived cells that were injected into the LV. **(C):** The temporal course of GFP expression during Doxy. feeding and after withdrawal (green, GFP; red, human nuclei). Scale bar = 20 μ m. **(D):** Quantification of the Doxy.-responsive GFP expression in vivo. A total of 31 animals were grafted in both sides of brain in this study. One animal (two LVs) was used for day 0 analysis. Three animals (six lateral ventricles) were used for each of the remaining time points. Abbreviations: Doxy., doxycycline; GFP, green fluorescent protein; hNu, human nuclei; LV, lateral ventricle.