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Inducible Transgene Expression in Mouse Stem Cells

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Summary

Embryonic stem (ES) cells serve as a potentially unlimited source of cells and tissues to treat a number of genetic and malignant diseases. The differentiation of these cells into specific cell types is an area of very active investigation. One method of manipulating ES cell differentiation is through the alteration of gene expression. There are a multitude of different methods for expressing a target gene in ES cells, but most are limited in their ability to provide spatial, temporal, and quantitative control of gene expression. These properties are important because many developmentally interesting genes are regulated in at least one of these ways. This chapter will address these limitations through the use of an ES cell line with a doxycycline-inducible transgene system. A characterization of this inducible transgene system will be discussed, as well as the use of this system to develop ES-derived long-term engrafting hematopoietic stem cells. This demonstration is one of many possible uses for this powerful and versatile system.

Key Words: Embryonic stem (ES) cell; genetic modification; inducible transgene; tet operon; rtTA; hematopoiesis.

1. Introduction

1.1. ES Cells as Research Tool and Potential Therapy

Since their identification and isolation in 1981 by Evans and Kaufman ***(1)*** and Martin ***(2)***, there have been major advances in genetically modifying ES cells, allowing for the ability to manipulate cellular processes involved in embryonic development. These innovations not only provide for a very powerful research tool to study development but also the capability to develop cellular therapies for a myriad of diseases. The key to unlocking the potential of ES cells is the ability to control the development of these cells into the desired cell or tissue type. The most popular strategies to control ES cell fate are to manipulate culture conditions or to alter gene expression. The former is accomplished with variations in exogenous factors, including cytokines,

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media, serum, or growth in different spatial organizations, such as through embryoid body (EB) formation. The latter can be done through a combination of different gene transfer modalities, including viral vectors, homologous recombination, and/or recombinase-based approaches. This chapter will discuss the development of a doxycycline-dependent inducible gene expression system using a combination of targeted homologous recombination and Cre-lox recombinase techniques. We will also discuss the use of this inducible expression system in the context of generating and propagating long-term engrafting hematopoietic stem cells (HSCs) from ES cells.

1.2. Comparison of Different Gene Expression Systems

There have been a number of different approaches developed for expressing a desired gene of interest in ES cells. One of the most common and easiest approaches for transferring a gene is through viral vector systems. The first genetic modifications of ES cells were done with retroviruses ***(3,4)***. Recombinant retroviruses integrate into the ES cell genome, making a provirus that is able to express the gene of interest in cells derived from these cells; however, upon further development and differentiation, proviruses are often found to be silenced through two major mechanisms: 1) methylation of the provirus genome, and 2) transacting repressive factors that bind to the long terminal repeats of the viral promoter ***(5)***. There are a number of groups who have attempted, with varying degrees of success, to develop modified retroviruses ***(6–8)*** and lentiviruses ***(9–13)*** to circumvent the issue of gene silencing.

Another limitation of using retroviruses is the inability to effectively control the quantitative, spatial, and/or temporal expression of the gene of interest. There have been several viral systems that have been developed to control gene expression with the addition of a chemical inducer. One inducible system developed by Jin and Blau uses the F36V mutant of FK506-binding protein's (FKBP's) dimerization domain fused to the cytoplasmic domain of c-Mpl, which in the presence of AP20187 causes dimerization of the fusion protein and activation of c-Mpl ***(14,15)***. Other groups have incorporated the more frequently used tetracycline inducible system in their retroviral constructs, as will be discussed in more detail ***(10,13,16)***.

An alternative to viral gene transfer in ES cells emerged when the constitutively active *hprt* locus was targeted through homologous recombination in 1987 by Doetschman et al. ***(17)*** and Thomas and Capecchi ***(18)***. Further refinements were later made to target nonselectable genes by Mansour et al. ***(19)*** and Schwartzberg et al. ***(20)***. These discoveries revolutionized ES cell genetic manipulation and allowed the generation of numerous transgenic and knockout ES cell lines and mice. Despite its tremendous utility, homologous recombination is a relatively inefficient method and is burdensome when trying to retarget a given locus with different gene insertions. Strategies to improve the latter include the "hit and run" ***(21–23)***, "tag and exchange" ***(24,25)***, and "plug and socket" ***(26)*** protocols, but they are all constrained by the inefficiency of homologous recombination. The discovery of the Cre-lox recombinase system solved this problem of inefficient gene insertion and allowed for effective retargeting of a specified locus ***(27–30)***. The combination of homologous recombination and the Cre-lox system provides a reliable and flexible gene transfer method.

Although this method is more labor intensive and less efficient than viral transduction modalities, there are a couple of key advantages to this system. The first advantage is that homologous recombination can be targeted to a precise locus allowing for control of the gene of interest by an endogenous gene regulatory system. Targeting a tissue specific gene regulatory system allows spatial and temporal control of gene expression. The second advantage is the ability to target the inducible locus to a location that has a lower propensity for silencing.

The latest advance in ES gene expression systems has been the ability to control expression with an exogenously added factor. This has been coupled to the Cre-lox recombination system to generate conditional knockouts and knockins. In the case of the conditional knockout system, a target gene is engineered with flanking loxP sites, and upon Cre-recombinase expression, the gene of interest is excised. A conditional knockin involves the elimination of an artificial stop codon placed in the gene of interest using the same strategy resulting in target gene expression. Both of these systems have been recently modified to allow for control of Cre-mediated recombination by a steroid exogenous factor such as tamoxifen ***(31–42)***. The system works by expressing a fusion protein of Cre and a mutated ligand-binding domain of the estrogen receptor [ER(T)] This fusion protein is retained in the cytoplasm because of the ER(T), but migrates into the nucleus when the exogenous factor tamoxifen is added and binds ER(T). Once in the nucleus, Cre can then mediate excision of the loxP-targeted site. This system allows for both spatial and temporal control of gene expression, but it lacks quantitative control and reversibility. However, this system has less background activity than other inducible systems and can exert a complete knockout of gene expression.

A more common inducible system employed in ES cells uses the *Escherichia coli* tetracycline resistance operon. This system uses the binding affinity of the tetracycline repressor protein (TetR) to its operon (TetO) to deliver a transcription activator to drive the expression of a target gene. The first system developed involved the fusion of the C-terminal portion of the herpes simplex virus transcription activator VP16 to TetR to make the tetracycline transactivator (tTA), which in the presence of doxycycline releases the fusion protein from its target and turns off gene expression ***(43)***. The same group later created a VP16 fusion with a mutated TetR that bound TetO in the presence of doxycycline, which stimulated expression of a target gene ***(44)***. This VP16-mutated TetR fusion was named the reverse tetracycline transactivator (rtTA; *see* **Note 1**). These systems have proven useful in controlling gene expression in eukaryotic cells in vitro ***(43–48)*** as well as in transgenic mice in vivo ***(49–52)***. Although there is some background activity in this system, the level of expression can be accurately titrated in a reversible fashion. These properties of the doxycycline inducible system were most suitable for our applications in hematopoiesis. We will demonstrate how we developed and used the doxycycline inducible gene expression system in the context of generating ES-derived hemotopoietic stem cells (HSCs).

1.3. Implications of Inducible Transgene Expression in the Development of HSCs

There have been various studies on the effect of diminished or eliminated expression of a specific gene on the development of HSCs using gene knockout and/or con-

ditional knockout systems *(39,53–60)*. However, the use of an inducible system to understand the effects of a specified gene's expression on developmental hematopoiesis has not been extensively used. Era and Witte used the tet-off system to study the effect of Bcr-Abl on hematopoietic differentiation of ES cells *(48)*. The tTA- and Tet-responsive Bcr-Abl were not targeted to a particular gene, but stable integrants were obtained using successive rounds of clone selection. In this system, Bcr-Abl drove proliferation of multipotent cells and myeloid progenitors while suppressing erythroid progenitor development. This effect was reversed when Bcr-Abl expression was stopped with the addition of doxycycline. A distinct tet-inducible transgene system in ES cells was designed by Niwa et al. to investigate the POU transcription factor Oct-3/4 *(47)*. In this study, an ES cell line named ZHTc6 was created from CGR8 ES cells using random integration of both the tTA and the inducible transgene constructs. Doxycycline repressed Oct-3/4 expression within 24 h. After 48 h in the absence of doxycycline, Oct-3/4 expression reached approx 50% higher levels than wild-type ES cells. Experiments performed with this ES cell line showed that a defined range of Oct-3/4 expression regulated the pluripotent or trophoblastic fate of ES cells. In another set of experiments, the same ES cell line was modified with a super-targeting vector to replace Oct-3/4 with STAT3F, a dominant interfering mutant of the transcription factor signal transducer and activator of transcription (STAT)3 *(46)*. STAT3 was shown to be necessary for ES cell self-renewal, which was inhibited by STAT3F expression. An inducible system was necessary for this study because constitutive expression of STAT3F precluded the isolation of a viable ES cell.

Our system differs from the previous two examples in three ways. First, we have targeted the transactivator and the inducible tet operator into loci that have previously been shown to be favorable transgene expression sites. This bypasses the problem of silencing and allows us to maintain a homogenous population of cells with consistent properties. Furthermore, because the targeted sites are known, they can be easily retargeted if the system requires modifications. Second, we use the rtTA instead of the tTA, which allows us to drive gene expression by adding doxycycline, (so called "dox-on") The "dox-on" system offers the advantages of controlling the timing and amount of target gene expression and avoids the potential adverse effects of a constitutively active transgene and/or the toxic effect of continuous doxycycline administration to suppress gene expression. Third, we use a lox-in strategy that affords us the ability to easily and efficiently insert any gene of interest into the inducible locus rather than requiring the inefficient retargeting of a site by homologous recombination.

We have found that the inducible system provided some fortuitous advantages in our attempts to develop long-term engrafting HSCs using HoxB4 as our transgene *(61)*. HoxB4 was selected because it had previously been shown that several homeobox (Hox) genes were expressed in definitive HSCs and not in the nonrepopulating hematopoietic progenitors found in the yolk sac of developing embryos *(62,63)*. In our system, the timing of HoxB4 expression during a critical window was important for developing definitive HSCs in vitro. Interestingly, experiments showed that continued expression of HoxB4 was not necessary after bone marrow engraftment of these cells in vivo. This avoids the need for continuous doxycycline administration to animals, which prevents

the possible in vivo adverse effects of doxycycline, constitutive expression of HoxB4, and/or constant high levels of activated transactivator. In addition to enabling us to model blood transplantation from ES cells, manipulation of HoxB4 suggests it can trigger a cell fate switch from primitive to definitive hematopoietic potential.

In addition to HoxB4, we are also investigating whether the regulated expression of other genes, including Stat5 and SCL/tal-1, in this system will enhance the development of definitive HSCs from ES cells. Stat5 is a signal transducer that has been shown to be important in hematopoiesis by various groups ***(64–71)*** and was particularly interesting to us because of its implication as a downstream effector in the transforming ability of Bcr-Abl ***(72–77)***. These properties made it an attractive target for inducible expression to provide nononcogenic proliferation of ES-derived HSCs. SCL/tal-1 expression was chosen because it has been shown to be important in early hematopoiesis by a number of groups in a variety of species ***(39,54,55,78–88)***. Furthermore, using a tamoxifen-inducible Cre-lox knockin system, SCL/tal-1 has been shown to be critical before d 4 of ES differentiation into hematopoietic cells on OP9 ***(39)***. Another group has recently shown that SCL/tal-1 is essential for the generation of HSCs and differentiation of erythroid and megakaryocytic precursors, but dispensable for certain HSC functions including self-renewal and bone marrow engraftment ***(58)***. The demonstrated importance of the temporal aspects of SCL/tal-1 expression made it an appropriate gene for investigation using our inducible system. These examples delineate the versatility and power of this inducible system to provide important insights into the genetic regulation of hematopoietic development and to advance experimental models of cell transplantation therapies.

2. Materials

2.1. Generation of ES Cell Lines With Inducible Transgene Expression From Ainv15 Targeting Cells

1. Standard deoxyribonucleic acid (DNA) restriction enzyme and ligation kits.
2. Targeting plasmid with loxP site in between the Pgk1 promoter-ATG and the gene of interest (*see* **Note 2**).
3. Cre expression plasmid: pSalk-Cre (generously provided by Stephen O'Gorman; *see* **Note 2**).
4. Electroporation apparatus: Bio-Rad gene pulser with capacitance extender.
5. Neo-resistant murine embryonic fibroblasts (store at –80°C).
6. ES cell media: DME, 15% fetal calf serum approved for ES cell maintenance (Stem Cell Technologies, Vancouver, B.C., Canada), LIF 1000 U/mL, 0.1 m*M* nonessential amino acids (Gibco), 0.1 m*M* β-mercaptoethanol, 2 m*M* glutamine, penicillin/streptomycin (Gibco; store at 4°C).
7. Selection agent: G418 (Neo^r).
8. 0.25% Trypsin/ethylenediamine tetraacetic acid (EDTA; store at –20°C).
9. Polymerase chain reaction (PCR) machine and standard PCR kit (Taq based).
10. PCR primers: LoxinF: 5'-ctagatctcgaaggatctggag-3' LoxinR: 5'-atactttctcggcaggagca-3'.

2.2. Characterization of Inducible ES Cell Line

1. Doxycycline powder (Sigma) dissolved in water (store at –20°C).
2. Method for detecting gene expression levels (e.g., fluorescence-activated cell sorting [FACS], immunoblot, or reverse transcription [RT]-PCR).

2.3. Induction of Target Gene HoxB4 and Culture of EB-Derived Cells on OP9 to Develop Definitive Hematopoietic Stem Cells

1. 0.25% Trypsin–EDTA.
2. EB differentiation medium: Iscove's modified Dulbecco's medium, 15% fetal calf serum approved for ES cell differentiation (Stem Cell Technologies, Vancouver, B.C., Canada), 50 μg/mL ascorbic acid (Sigma), 200 μg/mL iron-saturated transferring (Sigma), 4.5 m*M* monothioglycerol (Sigma), 2 m*M* glutamine, penicillin/streptomycin (Gibco; store at 4°C).
3. Eight-well multichannel pipetor.
4. Non-tissue culture-treated dishes, 15 cm.
5. Rotating shaker.
6. Doxycycline powder (Sigma) dissolved in water (store at –20°C).
7. 10X Collagenase/DNAse I mixture: 10 mg/mL collagenase IV (Sigma) + 800 U/mL DNAse I mixture (use as 10X; store at –20°C).
8. Cell dissociation buffer (Gibco 13151-014).
9. OP9 Stroma cell line (store at –80°C; **ref. *89***).
10. OP9 Culture VFTS media: Iscove's modified Dulbecco's medium /10% IFS, 100 ng/mL stem cell factor, 40 ng/mL vascular endothelial growth factor, 40 ng/mL thrombopoietin, and 100 ng/mL Flt-3 ligand (Cytokines from Peprotech; store at 4°C).
11. 0.04% Trypsin–EDTA.

2.4. Therapeutic Transplantation of ES-derived Cells In Vivo

1. MSCV-IRES-GFP retrovirus or other retrovirus expressing a marker that can be followed in vivo.
2. Syngeneic mice 129 Ola/Hsd (Harlan Laboratories).

3. Methods

3.1. Generation of ES Cell Lines With Inducible Transgene Expression From Ainv15-Targeting Cells

Our system was built by gene targeting through a combination of homologous recombination and the Cre-lox recombinase system. The original ES cell line was created from a male, HPRT-deficient ES cell line E14-Tg5 and was generously provided by Wutz et al. ***(90,91)***. The original system (**Fig. 1**) had rtTA-nls ***(44)*** integrated at the ubiquitously expressed ROSA 26 locus ***(92)*** and used doxycycline-induced gene expression and green fluorescent protein (GFP) co-expression from a bidirectional promoter consisting of seven TetO sites flanked by minimal promoters derived from cyto-megalovirus (CMV) ***(93)***. Although this original system was successful in the study of Xist's role in chromosomal silencing, inducible expression levels of genes that we inserted into the system were significantly reduced in ES-derived differentiated cells. We determined that inverting the orientation of the inducible locus in relation to the HPRT locus and eliminating the co-inducible GFP reporter successfully corrected the problem of transgene silencing with differentiation. The reengineered cell line was named Ainv15 (**Fig. 1**; **ref. *94***) The maintenance of inducible gene expression is evident in the positive expression of HoxB4 in different stages of differentiation as seen by immunoblot analysis (**Fig. 2**).

The targeting vector for Cre-lox recombination (**Fig. 1**) is on a pBS backbone and contains two primary features: 1) A Pgk1 promoter and translation initiation codon (ATG) to restore neo^r function upon recombination into the inducible gene locus,

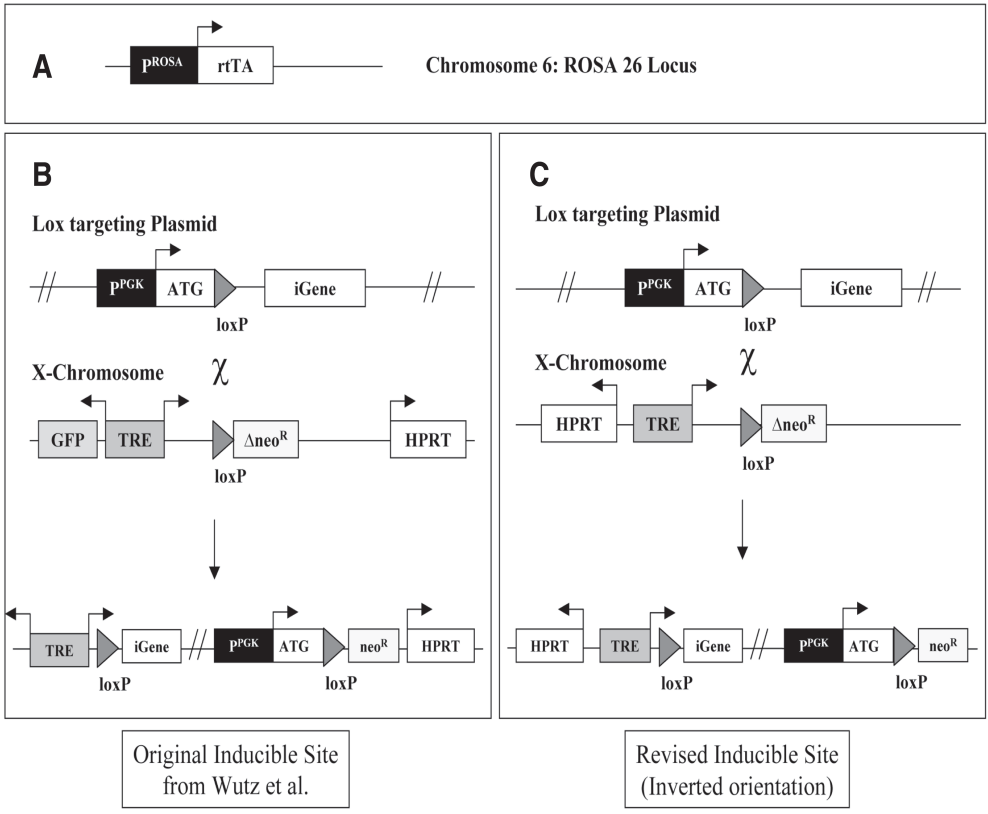

Fig. 1. Comparative schematic of inducible transgene systems. **(A)** Chromosome 6 carries the reverse tetracycline transactivator (rtTA) integrated into the constitutive and ubiquitously expressed ROSA26 locus. **(B,C)** The X chromosome carries the inducible gene locus 5' to the HPRT gene. The general strategy for inserting a gene into the inducible locus involves a lox-in (shown by the symbol chi) of the targeting plasmid. This restores neomycin resistance by providing a start codon (ATG) and the PGK promoter/enhancer to the formerly nonfunctional Δ neoR gene, which provides a method for positive selection. The gene of interest is integrated downstream of the tetracycline responsive element, which provides for doxycycline inducible expression of the gene. **(B)** The original configuration designed by Wutz et al. also has the tetracycline responsive element driving expression of a GFP reporter gene. After integration, the inducible gene locus is separated by several kilobases of plasmid sequence and the neoR gene from the constitutively active HPRT locus. This locus configuration was associated with silencing of gene expression in differentiated products of ES cells. **(C)** To correct this problem, the orientation was inverted relative to HPRT and the GFP reporter was eliminated. This places the inducible gene closer to the HPRT locus and precludes the silencing of the inducible gene. PROSA, ROSA26 enhancer/promoter; PPGK, phospho-glycero-kinase enhancer/promoter; ATG, start codon for the neo gene; loxP, Cre-recombinase recognition sequence; Δneo, deletion mutant of the neomycin (G418) resistance gene; //, plasmid sequence.

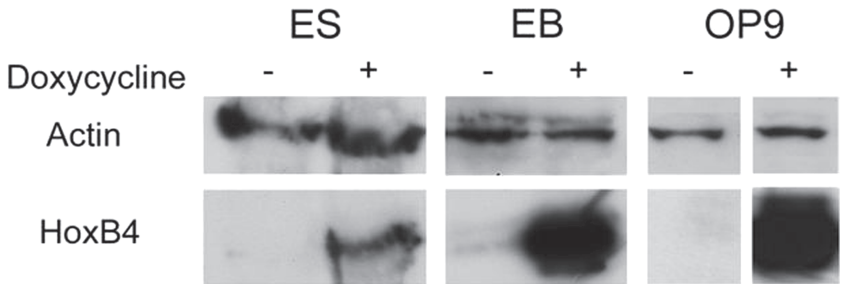

Fig. 2. HoxB4 induction in undifferentiated and differentiated ES cells. Immunoblot of HoxB4 (27 kDa) followed by second probing for actin (42 kDa) from cell lysates of inducible HoxB4 ES cells before and after differentiation in EBs and culture on OP9 stroma cells. HoxB4 expression is insignificant without doxycycline induction in ES cells, EBs, and in OP9 cultures. In the presence of doxycycline at 1 mg/mL, there is marked HoxB4 expression in the ES, EB, and OP9 cultures. This demonstrates that the inducible locus is not silenced upon differentiation of the ES cells.

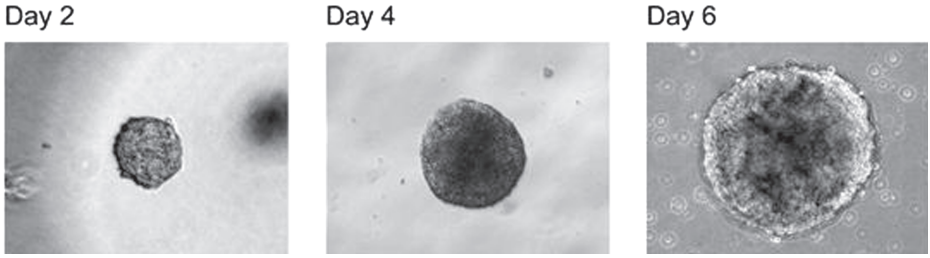

Fig. 3. Growth of EBs at 40X magnification. ES cells are dispersed in hanging drops on d 0 and are allowed to grow until d 2. The EBs are then collected, pooled in a 10-cm dish, and cultured with continuous rotation. At d 4, doxycycline is added to the media to drive expression of HoxB4. At d 6, EBs are harvested and dissociated for plating on OP9. Representative EBs are shown at d 2, 4, and 6 after initial plating.

(which provides a selection method for positive loxin of the gene of interest), and 2) The transgene (in our case HoxB4) with polyadenylation sequence so the loxP site is engineered between the Pgk1 promoter-ATG and the gene of interest. Using the lox targeting plasmid, the gene of interest can now be inserted into the inducible locus of Ainv15. This is accomplished through co-electroporation of the targeting plasmid and a suitable Cre-expression plasmid, selection of positive recombinants using **G418,** and confirmation of integrants with PCR (*see* **Note 2**). The procedure is as follows:

1. Add 8×10^6 Ainv15 cells in 800 μL of phosphate-buffered saline (PBS) with 20 μg each of the targeting plasmid carrying the inducible gene of interest and CRE-expression plasmid.

2. Add the mixture to an electroporation cell and electroporate at room temperature on the Bio-Rad gene pulser with capacitance extender using the settings: 200 Ω, 0.25 V.
3. Add the electroporated cells to a 10-cm dish carrying neo-resistant MEFs at 80 to 90% confluence in ES cell media (MEFs are plated the day before).
4. The next day, add **G418** (350 μg/mL) and maintain selection until resistant colonies appear around d 10 to 14. The cells will be very dense the first few days, and medium should be changed twice per day. After most cells have died off, feeding can be done once a day.
5. After 10 to 14 d, flood the dish with 10 to 15 mL of PBS and pick individual colonies using a P20 pipetman. Transfer to 100 μL of 0.25% trypsin–EDTA in an Eppendorf tube and disrupt the colony by gently pipetting up and down. Incubate at 37°C for 2 min and again disrupt any aggregates by pipetting. Add 900 μL of ES cell medium and collect the cells of the colony by centrifugation. Aspirate media and trypsin and resuspend cells in appropriate amount of ES cell medium. Replate the suspension onto fresh MEFs in 12-well dishes.
6. Confirm integration through PCR by using primers that amplify across the loxP site to give a band of approx 420 bp on ethidium bromide agarose gel electrophoresis. The primers are, for LoxinF, 5'-ctagatctcgaaggatctggag-3', and for LoxinR, 5'-atactttctcggcaggagca-3'. The PCR cycle conditions are as follows: 45 s at 95°C, 1 min at 60°C, and 1 min at 72°C; repeat cycle 29X. Promega PCR buffer with 1.25 m*M* $MgCl_2$ should be used with Taq polymerase.
7. Colonies that are positive can be cultured further from the 12-well dishes by using 0.25% trypsin–EDTA and plating on fresh semiconfluent MEFs on an appropriately sized tissue culture dish or flask.

3.2. Characterization of Inducible ES Cell Line

Induction of target gene expression can be started at different times and stages of differentiation; however, the amount and timing of expression can only be controlled if the inducible system has been fully characterized. This characterization was performed using GFP as the inducible gene. One question was whether doxycycline diffusion in different culturing methods was a limiting factor in expression kinetics. EB formation (one method for differentiating ES cells that will be discussed in the next section) creates a multilayer structure that may influence the diffusion kinetics of doxycycline. Images of EBs at different developmental stages are found in **Fig. 3**. Based on confocal images of inducible GFP EBs, doxycycline reaches all cells of the EB by 14 h of incubation in 1 μg/mL of doxycycline (**Fig. 4**). FACS analyses demonstrate faster expression kinetics in monolayer ES cell culture than in EB culture with maximal GFP expression reached in 8 and 14 h, respectively (**Fig. 5A**). This difference in gene expression in monolayer and multilayer culturing conditions should be considered when using this system. Moreover, there may be variations in gene expression in different parts of the embryoid body. The initial lag in GFP positivity is probably the result of three factors: 1) The time required for doxycycline to penetrate the cell membrane and increase intracellular concentration above a critical threshold for rtTA binding; 2) The time required to initiate the transcription and translation of GFP; 3) The time required to synthesize enough GFP to be detectable by FACS. Analysis of GFP expression levels over time shows a sigmoid relationship (**Fig. 5B**), but in this situation the lag in expression is confined to doxycycline membrane diffusion kinetics and the time required to synthesize GFP. The nonlinear increase after the initial lag is either the result of an exponential increase in intracellular doxy-

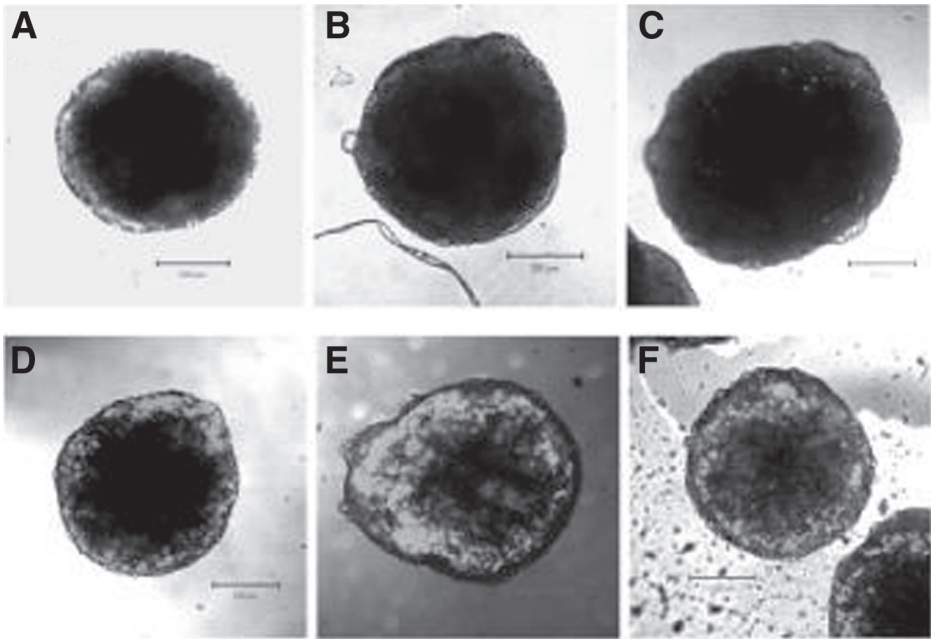

Fig. 4. Midsection confocal image of d 6 EBs from inducible GFP ES cells in the presence of 1 μg/mL of doxycycline for 0 h **(A)**, 4 h **(B)**, 6 h **(C)**, 14 h **(D)**, 24 h **(E)**, and 48 h **(F)**. The above EBs were also dissociated and analyzed by FACs. At 4 h, there was 10% GFP positivity and at 14 h, there was 80% positivity. These data indicate that doxycycline can penetrate into all of the cells of the EB within 14 h. Note that the non-GFP-positive area in the middle of the EB is a developmental cyst. The bar represents 200 μm.

cycline concentration or a cooperative mechanism for rtTA-driven GFP expression. A cooperative mechanism is supported by analysis of the doxycycline concentration dependence of GFP expression (**Fig. 6**). The sigmoidal curve is indicative of a cooperative process and is consistent with the design of the system. There are seven TetO sites located between the minimal promoters that allow for multiple rtTA binding to the bidirectional promoter unit. Because there is only one inducible transgene per cell, the proportional increase of activated rtTA with doxycycline concentration causes a non-linear increase in transgene expression. Lastly, **Fig. 7** shows the decay of GFP expression over time when doxycycline is removed after 24 h in 1 μg/mL. There is an initial rise in GFP expression over 10 h, probably the result of one or more of the following: 1) Transactivator remaining bound to the promoter unit; 2) Residual transcript that continues to be translated; 3) A lag for intracellular concentration of doxycycline to decrease to non-activating levels; and 4) The relatively long half-life of GFP. The induction kinetics between different transgenes is expected to be comparable; however, there will be more variation in the off kinetics between different transgenes because of inherent differences in protein half-lives. This characterization

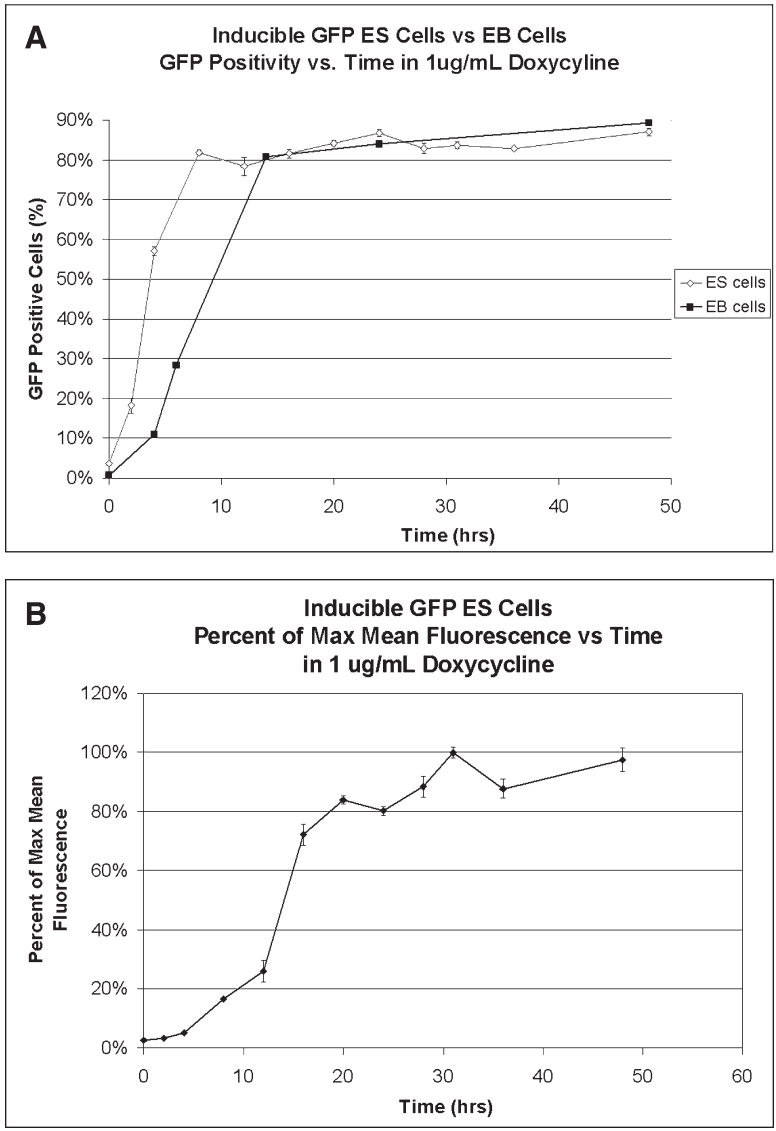

Fig. 5. Induction kinetics of inducible GFP in Ainv15 ES cell line at 1 μg/mL of doxycycline. **(A)** Percent of GFP-positive cells over time. Maximum number of cells with positive GFP expression in both EBs and ES cells is reached by 14 h of incubation; however, GFP positivity increases more rapidly in ES cell culture than in the EBs. 100% GFP positivity is not reached because 10% of the cells are MEFs. The initial lag is caused by 1) the time required for doxycycline to penetrate the cell membrane and increase intracellular concentration above a critical concentration for rtTA binding, 2) the time required to initiate the transcription and translation of GFP, and 3) the time required to synthesize enough GFP to be detectable by FACS.

(Continued on next page)

Fig. 5. *(Continued)* **(B)** Mean GFP fluorescence over time. There is an initial lag in mean fluorescence because of the diffusion of doxycycline into the cell and the time required to initiate GFP synthesis. The following rapid increase of GFP synthesis per cell suggests that either the intracellular doxycycline concentration is rapidly increasing in a non-linear fashion or that the doxycycline–rtTA complex increases the rate of GFP expression through a cooperative mechanism. A steady-state level of GFP in ES culture is reached after approx 24 h.

of the inducible system demonstrates that the timing and level of expression can be accurately controlled, which is essential in the majority of processes during development. This provides a more accurate method of investigating the role of specific genes in development as well as a reproducible and controllable method of engineering a particular tissue or cell type with induced gene expression.

3.3. Induction of Target Gene HoxB4 and Culture of EB-Derived Cells on OP9 Stroma to Develop Definitive HSCs

The characterization of the inducible system demonstrates the ability to have consistent control of gene expression in ES and ES-derived cells. The use of this system will vary depending on the genes and developmental processes investigated. We will discuss an example of using this system in the context of developing ES-derived HSCs. This was achieved through inducible expression of HoxB4 and a combination of culturing methods. The culturing method we used involved EB formation ***(95–97)*** followed by co-culture on the OP9 stromal cell line ***(89)***. Induction of HoxB4 is performed at the time when the hemangioblast commits to the primitive HSC, which is from d 4 to 6 of EB development. The EBs were then dissociated and plated on OP9 stromal cells in the presence of a unique set of cytokines and various doxycycline concentrations. Experiments to determine whether doxycycline concentration variation correlated with HoxB4 expression levels and, consequently, phenotypic changes in OP9 cultured cells are summarized in **Fig. 8**. As shown, HoxB4 levels can be carefully controlled with variations in doxycycline concentration (**Fig. 9A**). The number of ES-derived cells growing on OP9 increased with increasing HoxB4 levels (**Fig. 9**). This demonstrates the ability of HoxB4 to drive proliferation of these ES-derived cells. Moreover, previous experiments have shown that induced HoxB4 expression in these ES-derived cells results in the development of the definitive HSC ***(61)***. These conclusions were drawn from in vitro data showing a shift from embryonic (β-H1) to adult (β-major) globin and the increase in the known homing genes CXCR-4 and TEL, which is consistent with definitive hematopoiesis. Furthermore, colony-forming cell (CFC) assays showed an increase in the numbers of the multipotential progenitor colony-forming unit-granulocyte erythroid megakaryocyte macrophage (CFU-GEMM), which produces myeloid and erythroid lineage cells. This was further supported by data delineating a HoxB4-dependent increase in the surface marker phenotypes Sca-1 ***(98–107)*** and CD41/c-kit ***(108,109)***, which have been implicated as possible surface phenotypes for the definitive HSC (**Fig. 9C**). These results are suggestive of the successful development and propagation of ES-derived definitive HSCs.

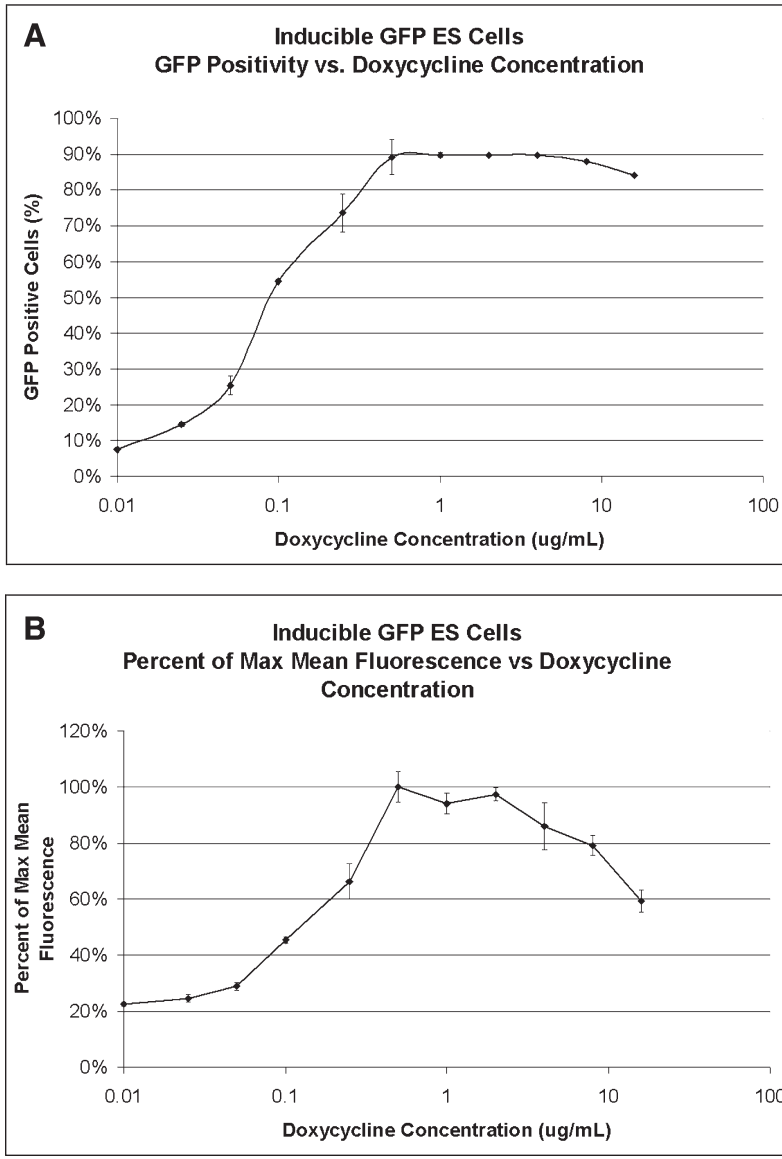

Fig. 6. Induced GFP expression dependence on doxycycline concentration. **(A)** Percentage positivity vs concentration shows that the kinetics of doxycycline penetration into ES cells is concentration dependent. After 24 h, all of the ES cells are induced at a concentration of 0.5 mg/mL. 100% GFP positivity is not reached because 10% of the cells are MEFs. **(B)** The sigmoidal curve is consistent with a cooperative mechanism for GFP expression. With increasing doxycycline concentration, more rtTAs bind the promoter unit and work synergistically to increase the rate of GFP expression in a nonlinear fashion. Above 2 mg/mL of doxycycline, there is toxicity from either doxycycline or rtTA as indicated by the dropping fluorescence levels.

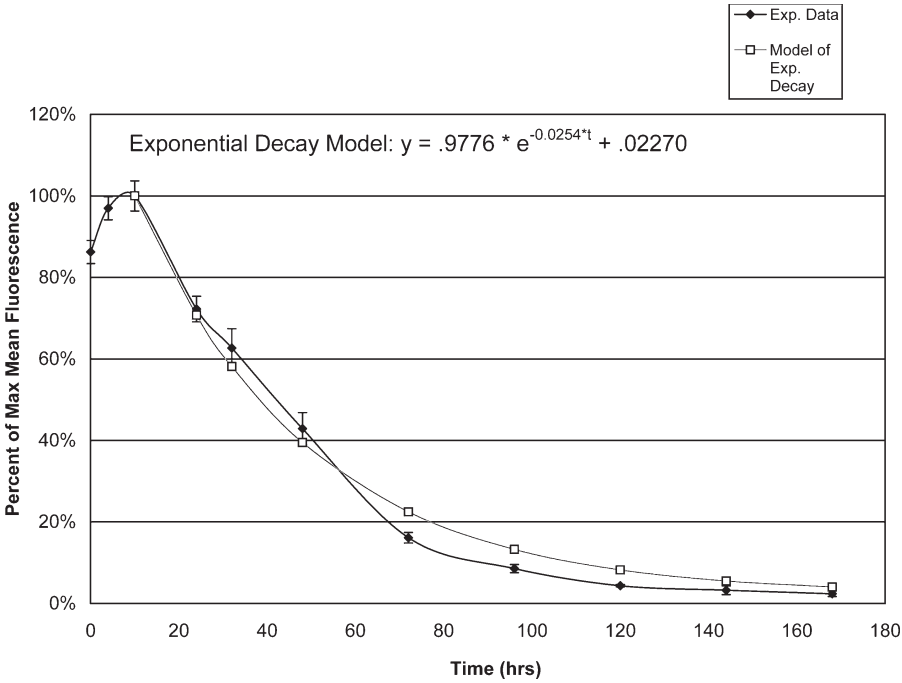

Fig. 7. Off kinetics of GFP expression after incubation with 1 μg/mL of doxycycline over 24 h. The decay kinetics will be unique to each transgene because proteins will have differing half-lives. GFP is a relatively long-lived protein and persists for over 5 d. In the graph, it is notable that there is an initial rise in GFP expression in the first 10 h as the result of a combination of untranslated RNA, delay of rtTA unbinding from the promoter unit, and the time for intracellular doxycycline concentrations to drop. A model of exponential decay of GFP after the initial 10-h rise in fluorescence is graphed. The decay model fits relatively well with a time constant of 0.0254 1/h and a half-life for GFP of around 27 h.

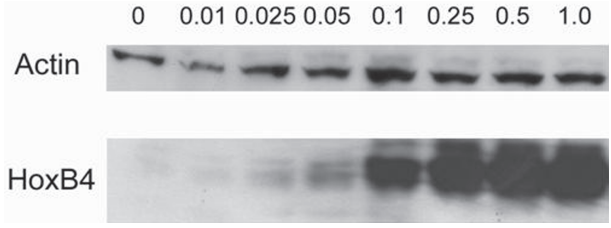

Fig. 8. Titration of HoxB4 levels with increasing concentrations of doxycycline in OP9 culture. Cell lysates of inducible HoxB4 d 6 EB-derived cells growing on OP9 at different doxycycline concentrations were subjected to immunoblot for HoxB4 (27 kDa) with second probing for actin (42 kDa) There is no detectable basal level of HoxB4 expression in the system without doxycycline, and the expression levels of HoxB4 can be titrated with doxycycline up to an apparent maximum of 0.5 μg/mL of doxycycline.

1. Starting from a confluent ES culture in a 25-cm^2 T-flask, trypsinize culture with 0.25% trypsin/EDTA for 10 to 15 min at 37°C.
2. Add 5 mL of EB differentiation media to the culture and disrupt any cell clumps with a pipettor. Incubate cells for 45 min at 37°C to allow MEFs to re-attach, while the majority of the ES cells will be retained in suspension.
3. Count cells and make suspension of 10,000 cells/mL in EB differentiation medium.
4. Make as many hanging drops as possible with the multichannel pipettor of 10 μL (100 cells/drop) on 15-cm nontissue culture-treated dishes (*see* **Note 3**).
5. Incubate for 2 d at 37°C (d 0–2) inverted so that drop is hanging.
6. On d 2, flood Petri dishes with 10 to 15 mL of PBS, collect in a 15-mL tube, and allow the EBs to sediment by gravity for 3 min.
7. Aspirate PBS, resuspend EBs in 10 mL of EB differentiation medium, and put into 10-cm bacterial Petri dish bottom with 10-cm tissue culture dish top.
8. Place on rotator at 50 rpm in a dedicated 37°C/5% CO_2 incubator.
9. Feed the cultures every 2 d with a half media exchange.
10. On d 4, add 1 μL of doxycycline (1 mg/mL) directly to the EB media in the rotating dish to make a final concentration of 1 μg/mL (*see* **Note 4**).
11. On d 6, collect the EBs in a 15-mL tube and dissociate EBs by washing with PBS, incubating with 900 μL of PBS and with 100 μL of 10X collagenase/DNase I mixture at 37°C for 15 to 20 min, and then pipetting the EBs against the wall of the tube.
12. Add 4 mL of cell dissociation buffer (Gibco 13151-014), mix cells, and then pellet the cells in a centrifuge.
13. Resuspend in PBS and repeat procedure from **step 2** until EB is sufficiently dissociated (usually requires two treatments total).
14. Resuspend in OP9 culture VFTS media and plate cells on 80% confluent OP9 stroma cells in appropriate sized flask (*see* **Note 5**).
15. Add appropriate amount of doxycycline (1 mg/mL) to make final concentration 1 μg/mL.
16. Discrete semiadherent colonies should begin to form within 3 to 4 d.
17. Split cells onto fresh OP9 when confluent through trypsinization with 0.04% trypsin/EDTA for 5 to 10 min.

3.4. Therapeutic Transplantation of ES-Derived Cells In Vivo

The in vitro data indicating that induced HoxB4 expression develops the definitive HSC was confirmed in vivo with bone marrow transplantation studies ***(61)***. Long-term engraftment studies were performed in 2- to 3-mo-old isogenic 129 Ola/Hsd mice. To follow the ES-derived iHox cells in vivo, the cells were infected with a GFP-expressing retrovirus, sorted, and recultured on OP9 cells before transplantation. Transplants were performed in lethally irradiated mice (1000 cGy of γ- irradiation) with approx 2×10^6 cells of OP9 cultured cells injected via lateral tail vein. GFP expression was seen in primary mice up to 15 wk after transplant and in secondary mice more than 5 mo after transplant. Interestingly, induction after transplant was not necessary for long-term engraftment, which suggests that HoxB4 is required at a specific time in development to guide a cell towards the definitive HSC fate. This finding demonstrates the value and power of the inducible system as a developmental research tool and a reliable method for modeling cell therapies.

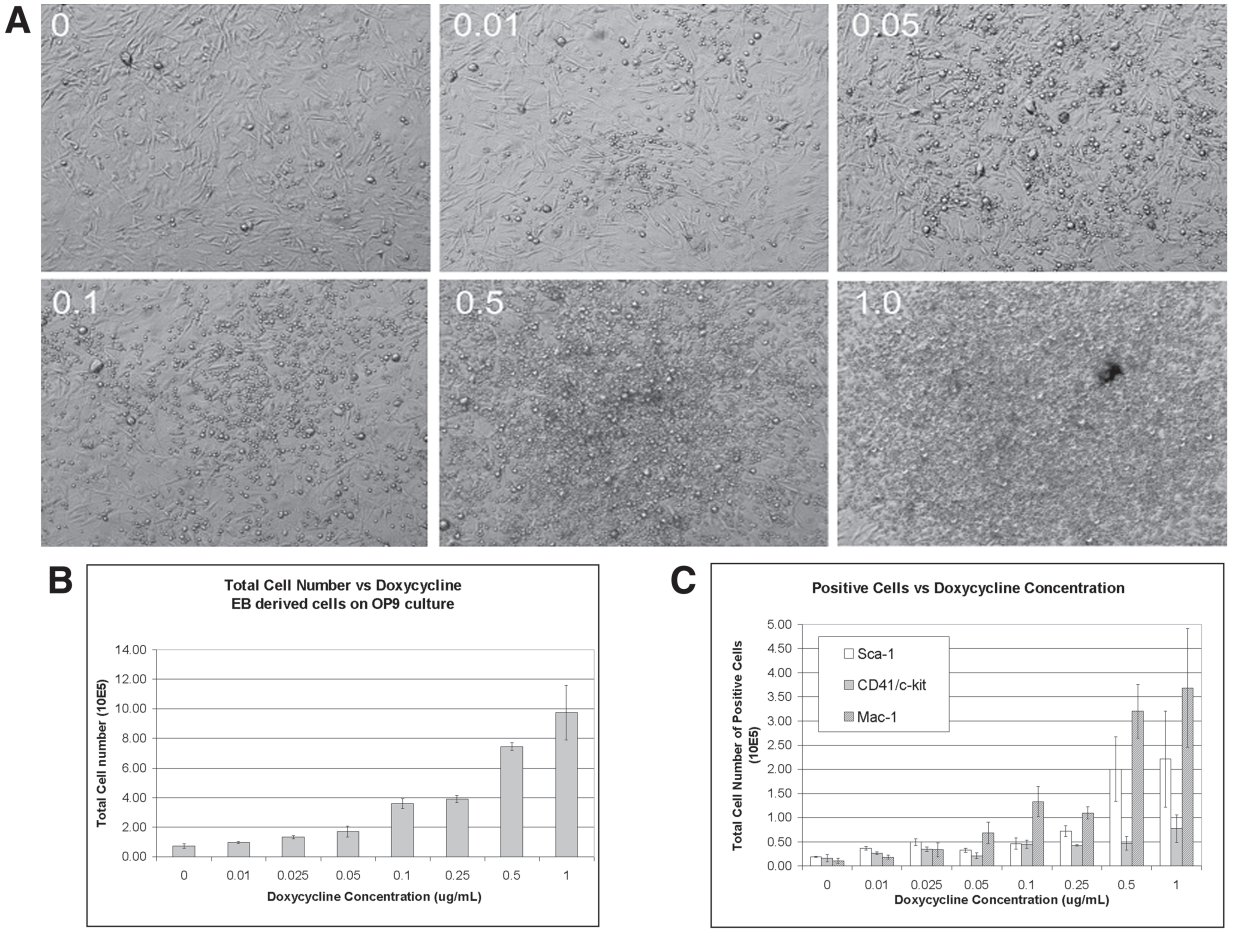
A
0
0.01
0.05
0.1
0.5
1.0
B
Total Cell Number vs Doxycycline
EB derived cells on OP9 culture
Total Cell number (10E5)
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
0
0.01
0.025
0.05
0.1
0.25
0.5
1
Doxycycline Concentration (ug/mL)
C
Positive Cells vs Doxycycline Concentration
Total Cell Number of Positive Cells (10E5)
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Sca-1
CD41/c-kit
Mac-1
0
0.01
0.025
0.05
0.1
0.25
0.5
1
Doxycycline Concentration (ug/mL)

Fig. 9. *(Opposite)* Effect of varying induced levels of HoxB4 gene expression on ES-derived cells co-cultured on OP9. **(A)** Images at 40X of OP9 cultures at different doxycycline concentrations. As shown, the density of ES-derived cells (small round cells) increases dramatically with increases in doxycycline concentration. **(B)** Total cell number vs doxycycline concentration. With increasing doxycycline, cell number increases rapidly up to 1 mg/mL. **(C)** Total cell number of positive cells stained for either Sca-1, CD41 + c-kit, or Mac-1. Sca-1 and CD41 + c-kit are two surface phenotypes that have recently been suggested to indicate the definitive HSC. As doxycycline concentration increases, the detectable level of both sets of markers also increases. Increasing HoxB4 expression appears to affect Sca-1 positive population more than the CD41/c-kit double positive population. Mac-1 was included as a marker of myeloid lineage cells because it had been previously shown that expression of this marker increases with induction of HoxB4.

4. Notes

1. In this protocol, the rtTA-nls is used as the transactivator and a bidirectional minimal promoter derived from CMV is used to express the target gene. The nls stands for nuclear localization signal, which was created to target the rtTA to the nucleus and improve transactivation. This variation was shown to provide enrichment of rtTA in the nucleus. One problem of the first rtTA and tTA was the toxicity of constitutive expression of these transactivators. This has been improved by diminishing the VP16 portion to 12 amino acids, which was shown to be better tolerated at higher intracellular concentrations ***(45)***. Others have generated an autoregulatory system with the transactivators to reduce toxicity ***(110)***. In addition, work has been performed to modify the rtTA to be more sensitive to doxycycline and to have lower background activity. Urlinger et al. have demonstrated new rtTA mutants that operate at doxycycline concentrations 10 times less than the original rtTA and have undetectable background expression ***(111)***. Other groups have fused TetR to another transcriptional activator E2F4 as an alternative to tTA and rtTA ***(112)***. There have been many other modifications and refinements to the system, including variations in promoter systems ***(113)*** and alterations in transactivator DNA binding specificities ***(114)***.
2. The lox-targeting plasmid we generated was derived from pPGK-loxP-Xist ***(90,91)***, which was generously provided by Anton Wutz and Rudolf Jaenisch. This was modified using pNEO-EGFP, which is no longer available from Clontech. The Cre-expression plasmid pSalk-Cre was generously provided by Stephen O'Gormon. There are a number of other plasmids that are commercially available to generate a lox-targeting plasmid and a Cre-expression plasmid. It is only important to maintain the location of the loxP site in relation to the transgene and the PGK1 promoter-ATG.
3. EBs can be made with more than 100 cells/drop or with larger drop sizes if EBs fail to properly form or grow to a desirable size.
4. Doxycycline induction concentration and times will vary depending on quantitative, temporal, and spatial characteristics desired in expressing a gene of interest.
5. Optional FACS sorting of blast populations with surface markers such as CD41, c-kit, CD34, or Sca-1 can be done before plating on OP9 culture to enrich the cultures for hematopoietic blast cells.

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