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An ES cell system for rapid, spatial and temporal analysis of gene function *in vitro* and *in vivo*

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ABSTRACT

We describe a versatile genetic system for rapid analysis of mammalian gene function. In this, loss of reporter activity in a novel embryonic stem (ES) cell line enables rapid identification of targeting to the ubiquitously expressed Rosa26 locus. Subsequent regulation of gene activity is governed by a dual regulatory strategy utilizing two drugs, Tamoxifen and Doxycycline. To illustrate this approach, a dominant allele of Smoothed was introduced into this cell line, enabling regulated activation of Hedgehog signaling. By coupling Cre-loxP dependent activation with tetracycline dependent transcription in a single allele, we established a conditional method to control Smoothed activity and neural progenitor specification in differentiating ES cells *in vitro* and in chimeric embryos *in vivo*. When crossed to an appropriate Cre driver strain, gene activity can also be temporally regulated within a specific cell lineage. This platform will facilitate rapid analysis of gene function in the mouse.

INTRODUCTION

The sequencing of the human and mouse genomes along with high-throughput genomic analyses (i.e. expression profiling) have produced a wealth of information regarding the repertoire of genes associated with a particular biological process and the hierarchical relationships that exist among these regulatory factors. The challenge now is to elucidate the functions of these genes. Transgenic and gene targeting methodologies have proven to be invaluable approaches for addressing gene function in the mouse. Further, the ability to modulate gene activity using conditional genetic manipulation systems, such as Cre-LoxP mediated DNA recombination and tetracycline based transcriptional transactivation, have greatly facilitated the genetic dissection of complex biological questions (1–3).

However, establishing and maintaining genetically engineered mouse lines are costly, labor intensive and time consuming. To facilitate the study of complex and multi-staged processes, such as embryogenesis and tumor formation, a new generation of genetic systems is required that incorporates spatio-temporal and reversible gene regulation into a platform that enables the rapid analysis of gene function in a relevant biological system.

Here we report an embryonic stem (ES) cell based approach that combines inducible Cre-loxP mediated activation of gene transcription and tetracycline-mediated repression to directly analyze gene function in differentiating ES cells *in vitro* and in chimeric embryos derived directly from these cells. Furthermore, this ES cell based system can be easily tailored to achieve spatio-temporal and reversible gene regulation in the mouse.

MATERIALS AND METHODS

Generation of ES cell lines

We generated ES cell lines from mice carrying four previously published alleles. CAGGS-CreERTM (4) encodes a ubiquitously expressed, 4OH-tamoxifen regulated fusion protein between P1 phage integrase Cre and a modified ligand binding domain of the human estrogen receptor (5). R26R (6), R26-EYFP (7) and R26-ECFP (7) encode LacZ, yellow fluorescent protein (YFP) and CFP insertion at the ubiquitously expressed Rosa26 locus; each allele requires Cre-mediated excision of a Neomycin phosphotransferase cassette to activate reporter expression. Blastocysts were obtained from intercrosses between R26R; CAGGS-CreER males and R26-EYFP or R26-ECFP females and plated onto mouse embryonic fibroblasts (MEFs). These alleles were maintained on a mixed (C57BL6/J, 129 Sv and Swiss Webster) background. ES cell lines were generated using standard protocols (8) with the addition of the MEK1 inhibitor, PD98059 (New England Biolabs), to the culture media. ES cell lines derived from the cultured blastocysts were then expanded and frozen down or genotyped and assayed for specific reporters at the Rosa26

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locus. All genotyping was accomplished by PCR using primers specific for CreER, β -gal, YFP and CFP. YFP or CFP reporters were visualized by epifluorescence microscopy. To assay for the presence of β -galactosidase, a 35 mm plate of cells was fixed in formalin/glutaraldehyde for 2 min, and then subjected to X-gal treatment as described by Whiting *et al.* (9). Of the initial ES cell lines, three (YFP3, YFP7 and CFP3) were tested for their ability to contribute to chimeras. Approximately 50% of embryos recovered from YFP3 cell injection were highly chimeric. Based on this result, we focused on this cell line.

Derivation and genetic analysis of the 3-1 cell line

YFP3 cells were cultured in ES cell media supplemented with 1 μ M 4OH-tamoxifen (Sigma) to induce CreER mediated recombination initially in a single cell suspension, then in culture for 2–5 days until they had formed relatively large colonies. Twelve colonies were picked and incubated in 50 μ l of trypsin at 37°C for 5–8 min in single wells of a 48-well dish. The trypsinized colonies were triturated in 200 μ l of media with 1 μ M 4OH-tamoxifen and then transferred to a single well containing MEFs of a 48-well dish. The cells were then diluted (1:2) serially in a 48-well dish and grown for 3–4 days. Wells were assayed for the presence of the β -gal and YFP reporters and only those colonies that were positive for expression of the two reporters were propagated further. Serially diluted wells for these selected subclones that contained one or at most two colonies were trypsinized and expanded to three 6 cm dishes and one 3.5 cm dish. The former was subsequently frozen down for future use and the latter was subjected to G418 selection. A clonal line, 3–1, that was YFP⁺, β -gal⁺ and completely sensitive to G418, indicating complete recombination at each R26R and R26-EYFP allele, was tested along with the parental YFP3 cells for contribution to chimeras generated by diploid and tetraploid aggregation and by blastocyst injection using standard protocols (8).

Rosa26 targeting

We modified a previously described tetracycline based regulatory unit (10) by removing the PGK-Neo and URA selection cassettes with ClaI and XhoI digestion from the original plasmid. This modified unit was then inserted into the SpeI site 3' of the splice acceptor in the pBigT vector (7), generating a new Rosa26 targeting shuttling vector, pTET-BigT, with multiple cloning sites (NheI, SalI, XhoI and NotI) to enable insertion of cDNAs encoding a gene of interest. cDNAs encoding dominant regulators of signaling pathways were cloned into the pTET-BigT vector. The resulting plasmids were digested with PacI and AscI to release the Tet-off-floxed-neo-gene-of-interest cassettes, which were then inserted into a modified Rosa26 targeting vector, pRosa26PAS. pRosa26PAS was modified from pRosa26PA (6) by replacing the unique KpnI site in the original vector with a unique SwaI site to avoid the likelihood of KpnI sites in the pTET-BigT and gene-of-interests. The final targeting constructs were linearized and electroporated into 3-1 ES cells.

After selection in media containing 100 μ g/ml G418 for 7–8 days, resistant colonies were picked and transferred to 96-well plates. After 2–3 days when the majority of ES cells in 96 wells had reached confluence, each well was split into two

96-well plates, one a master plate and the other for 'color' screening to identify probable targeting events at the Rosa26 locus. On the screening plate, positive targeting events were first scored for loss of YFP under the fluorescence microscope, followed by β -galactosidase staining to identify *lacZ* negative colonies. Once the positive clones were identified (either YFP⁻;LacZ⁺ or LacZ⁻;YFP⁺ depending on which Rosa26 allele underwent homologous recombination), these colonies were expanded on the master plate and frozen for future analysis.

In vitro ES cell analysis, immunocytochemistry and western blot

The targeted 3-1 ES cells were grown on a gelatin-coated 4-well chamber slide. 4OH-tamoxifen (1 μ g/ml) (Sigma) and Doxycycline (1 μ g/ml) (Sigma) were added into culture media at different time points. After 2–5 days, the cells were fixed in 4% paraformaldehyde for 30 min at 4°C for subsequent immunostaining, or harvested, lysed, and proteins were separated by SDS-PAGE prior to western blot analysis using an anti-GFP antibody (AB293, Abcam), 1:5000.

The embryoid body (EB) differentiation assay was performed as described previously (11). Two days after EB formation, medium was replaced and supplemented with retinoic acid (RA) (500 nM) (Sigma) and 4OH-tamoxifen (1 μ g/ml) (Sigma), and EBs were cultured for an additional 3–5 days. EBs were then fixed, sectioned and processed for immunofluorescence staining as described previously (11). The following antibodies were used in this study: rabbit anti-GFP 1:1000 (AB293, Abcam), mouse anti-Nkx2.2 1:30 (Developmental Studies Hybridoma Bank), mouse anti-FoxA2 1:20 (Developmental Studies Hybridoma Bank), mouse anti-MNR2 1:300 (Developmental Studies Hybridoma Bank) and rat anti β -galactosidase 1:500 (Biogenesis).

Generation of R26T-SmoM2 chimeras

The targeted R26T-SmoM2 ES cells (YFP⁻;LacZ⁺) were injected into host (C57BL6J, Jackson Laboratories) blastocysts. The blastocysts were transferred into the uterus of pseudopregnant hosts (Swiss Webster, Taconic) at E2.5. Embryos were collected at E10.5 and E13.5 after administration of Tamoxifen (Sigma) at a single dose of either 4 mg/40 g or 2 mg/40 g body weight by intraperitoneal injection into the pregnant dam at E8.5. Chimerism was assessed by whole mount β -galactosidase staining as described (9). For tetracycline-mediated regulation, one day prior to embryo transfer pseudopregnant dams were administered 200 μ l of Doxycycline (10 mg/ml) by oral gavage. In addition, the drinking water was supplemented with 1 mg/ml of Doxycycline to maintain silencing of tTA driven regulatory cassettes.

Generation of a R26T-SmoM2 line and neural crest-specific activation of Hh signaling

The targeted R26T-SmoM2 3-1 ES cells (YFP⁻;LacZ⁺) were injected into C57BL6J blastocysts to generate chimeras. β -Galactosidase staining performed on tail and ear biopsies taken from chimeras was used to assess chimerism. The CAGGS-CreER transgene was segregated from the R26T-SmoM2 line after germline transmission. Wnt1-Cre has

been described before (5). Wnt1-Cre and R26T-SmoM2 embryos were obtained from the crosses of Wnt1-Cre females and R26T-SmoM2 males.

RESULTS

Generation of the 3-1 ES cell line

The general strategy we have adopted is outlined in Figure 1. Since its identification as a ubiquitously expressed locus that enables position independent expression of a gene of interest, targeting at the Rosa26 locus has become a common strategy for the general widespread tissue independent expression of a gene under study. To simplify and speed up the analysis of successful targeting events at this locus, we envisioned a scheme where each Rosa26 allele ubiquitously expressed a reporter whose loss as a result of homologous recombination at that locus could be readily assayed. For example, if one allele expresses the *Escherichia coli lacZ* gene, and the other a YFP encoding gene, a simple histochemical staining procedure for β -galactosidase production or direct visualization of YFP fluorescence enables the ready identification of targeting at either allele. Further, the remaining constitutively expressed marker enables ES cells and their derivatives to be visualized in chimeric progeny.

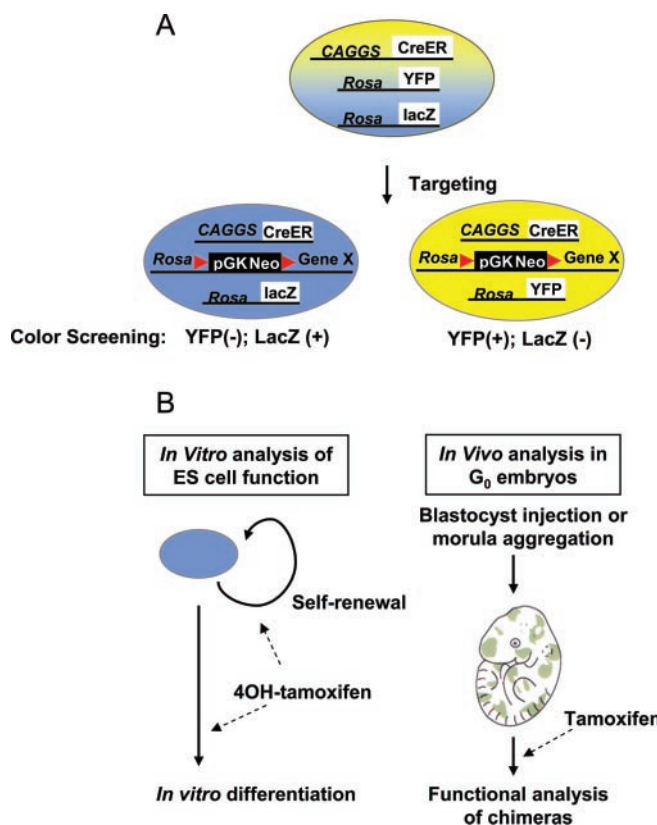


Figure 1. 3-1 ES cells and rapid functional analysis *in vitro* and *in vivo*. (A) Schematic representation of targeting the Rosa26 locus in 3-1 ES cells. YFP and β -gal are expressed under the endogenous Rosa26 promoter in 3-1 ES cells that also possess a CAGGS-CreER transgene. When a gene of interest is targeted into Rosa26 locus, positive colonies can be screened by loss of either YFP or β -gal expression. (B) Functional analysis on ES cells or G₀ chimeric embryos derived from 3-1 ES cells.

In the next step, we wanted to control the expression of a gene introduced into the Rosa26 locus. Previous studies from our group have described a transgenic mouse line, CAGGS-CreER, which ubiquitously expresses a CreER fusion protein (4). In this transgene line, recombination catalyzed by the P1 integrase, Cre, at its target *LoxP* recognition sites, is Tamoxifen dependent, enabling drug-dependent control of Cre-mediated DNA recombination. In an ES cell line that expresses the CreER transgene, this enables a simple genetic switch to control gene expression at the Rosa26 locus in ES cells and their differentiated descendants (Figure 1). When a gene, X, is targeted into the Rosa26 locus with a *LoxP*-flanked gene cassette (pGKNEO in Figure 1) positioned to block transcription of gene X, addition of 4OH-tamoxifen *in vitro* or Tamoxifen *in vivo* will result in the excision of this cassette initiating gene X transcription thereby enabling the function of this gene to be explored in ES cells and their differentiated derivatives in culture, in chimeric embryos and in adult mice that are derived directly from targeted ES cells (Figure 1).

In the first step towards the generation of the desired ES cell system, we generated a male ES cell line, YFP3, which carried the CAGGS-CreER transgene and previously described LacZ and YFP reporter alleles at each Rosa26 locus (6,7). These reporter alleles are themselves silent until Cre-mediated removal of a *LoxP*-flanked neomycin phosphotransferase positive selection cassette introduced in the original gene targeting events. Only occasional, sporadic expression of the reporters was observed either *in vitro* in ES cell culture or *in vivo* in ES cell derived chimeras, in the absence of drug (data not shown). In contrast, the addition of 4OH-tamoxifen to ES cell culture and Tamoxifen injection into pregnant dams at E8.5 lead to a robust induction of reporters in the ES cell line (Figure 2A and B) and in chimeric embryos assayed at E10.5 (Figure 2C and D). These analyses, and others, verified the genetic identity of the YFP3 cell line, validated the drug induced genetic switch and demonstrated the ability of the ES cell line to contribute widely to embryonic and adult tissues including the male germline (data not shown).

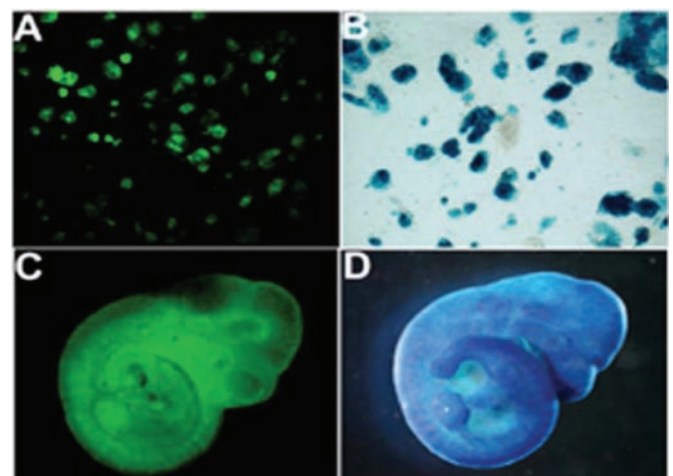


Figure 2. Reporter analysis of YFP3 cells in culture and in chimeras. YFP3 cells induced in 1 μ M 4OH-tamoxifen and then subjected to fluorescence (A) or X-gal (B). YFP3 chimera induced at E8.5 and allowed to develop to E10.25. The embryo was visualized under fluorescence (C) and later stained with X-gal (D).

In the next step, we plated out YFP3 cells at limiting dilution in the presence of 4OH-tamoxifen (Materials and Methods) to isolate a clonal line that excised the neomycin phosphotransferase cassettes at both the R26-EYFP and R26R alleles; such a line would be G418 sensitive, YFP⁺ and β -galactosidase⁺. We identified a clonal line, 3-1, which met all three criteria. Chimeric analysis demonstrated that 3-1 cells show similar high-level chimerism and germline competence as the parental YFP3 cells. Further, analysis in chimeras generated with tetraploid embryos demonstrated that 3-1 cells are able to generate late gestational stage embryos that are wholly 3-1 ES cell derived (data not shown).

Cre-*LoxP* and tetracycline-mediated reversible gene expression in 3-1 ES cells

While the 3-1 cell system in principle provides a rapid strategy for introducing genes into the *Rosa26* locus and enable expression at the locus to be drug regulated, once a gene is activated by a Cre-mediated recombination event, expression is permanent. To improve the spatio-temporal regulation, we next combined the Cre-*LoxP* system with tetracycline regulation (Figure 3). To this end, we engineered a modified *Rosa26* knock-in vector. This contains a *LoxP*-flanked selection and transcriptional stop cassette that enables positive selection for vector integration and transcriptional silencing of a downstream gene of interest, preceded by a tetracycline regulatory unit. In this, a cDNA sequence encoding the Doxycycline (Dox) controlled transactivator (tTA) is coupled with a minimal CMV promoter and the *tet* operator sequence (*TetO*) (10).

In theory, when integrated into the *Rosa26* locus, the tTA proteins are expressed under the ubiquitously expressed, constitutive *Rosa26* promoter, but the *LoxP*-flanked stop cassette will render a gene of interest transcriptionally silent (Figure 3A). However, on Cre-mediated recombination, tTA-mediated transcriptional activation is initiated (Figure 3B). This situation is also reversible; on addition of Doxycycline, tTA binds to the drug, and the gene of interest is transcriptionally silenced (Figure 3C).

As a proof of principle of this approach, we targeted a dominant activator of the Hh pathway, SmoM2, into the *Rosa26* locus of 3-1 ES cells with dual Cre-*LoxP* and tetracycline-mediated regulation (Figure 3D). Smoothened is a 7 pass membrane protein and key component of the Hh pathway essential for the transmission of a Hh signal in all Hh responsive cells. Smo-M2 was originally identified as a point mutation, W539L, within the seventh transmembrane domain that leads to constitutive, high-level, ligand independent activation of Hedgehog signaling in responsive cells (12). To directly visualize SmoM2 in targeted cells, we generated a fusion protein, SmoM2-YFP, with YFP fused to the C-terminus of SmoM2 such that SmoM2 activity was not modified.

We electroporated the drug regulated SmoM2 targeting construct into the 3-1 ES cells and screened for colonies that had lost YFP or LacZ expression (see strategy in Figure 1). In general, 10–15% of the G418 resistant colonies were identified by this color screen. Since the color screen can be readily completed in the first passage after picking G418 resistant colonies, it avoids the frequent freezing and thawing of ES cells and Southern analysis normally required for

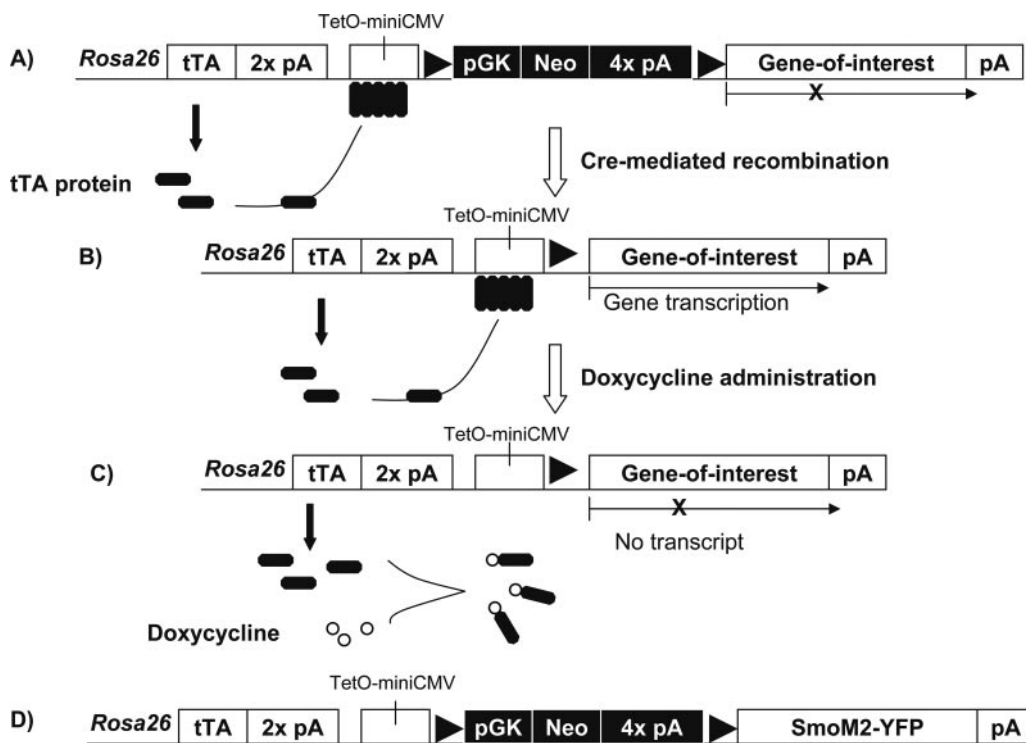


Figure 3. Cre-*LoxP* and tetracycline dependent gene expression in *Rosa26* locus. The native *Rosa26* promoter drives expression of the tetracycline transactivator (tTA) that induces transcription of the gene of interest after Cre-mediated removal of the PGK-Neo-4XpA cassette (A and B). (C) Doxycycline (open circles) binds to tTA proteins (black bar), which undergo conformational change and dissociate from *tetO*, resulting in termination of transcription. (D) Schematic representation of the R26T-SmoM2 allele.

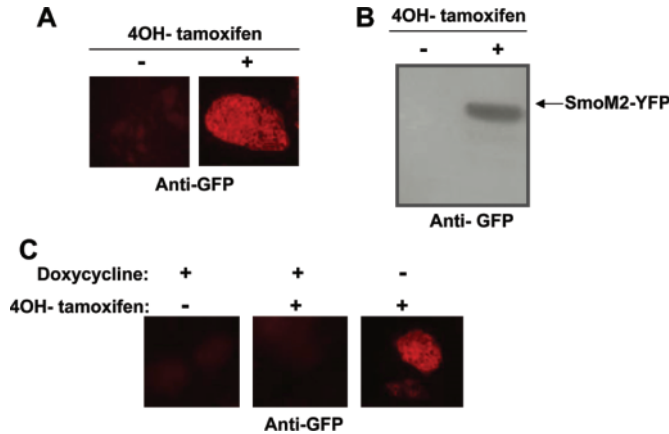


Figure 4. Regulation of SmoM2-YFP expression in 3-1 ES cells. The expression of SmoM2-YFP in targeted 3-1 ES cells was induced by 1 $\mu\text{g/ml}$ of 4OH-tamoxifen, as shown by immunostaining (A) and western blot (B). (C) Doxycycline controls SmoM2-YFP expression in ES cells. In the presence of 1 $\mu\text{g/ml}$ of Doxycycline, 4OH-tamoxifen (1 $\mu\text{g/ml}$) failed to induce SmoM2-YFP expression. SmoM2-YFP was expressed after removal of Doxycycline.

identifying recombinant clones, and hence these laborious and time-consuming steps can be removed from the procedure.

The presence of CAGGS-CreER in 3-1 ES cells also enabled rapid *in vitro* analysis of the induction of SmoM2-YFP, and importantly the testing of activation and Doxycycline-mediated repression of SmoM2-YFP in targeted ES cells and their derivatives. We selected a YFP⁻, LacZ⁺ cell line and examined induction and repression of SmoM2-YFP by immunocytochemistry and western blot following 4OH-tamoxifen and Doxycycline treatment. In the absence of Doxycycline and 4OH-tamoxifen, expression of SmoM2-YFP was rarely observed (Figure 4A). In contrast, supplementing the culture media with 1 $\mu\text{g/ml}$ of 4OH-tamoxifen lead to synthesis of a membrane localized SmoM2-YFP fusion protein in most ES cells with 2 days of culture (Figure 4A). Western blot analysis confirmed the 4OH-tamoxifen dependent induction of SmoM2-YFP (Figure 4B). On addition of 1 $\mu\text{g/ml}$ of Doxycycline, expression was inhibited by Doxycycline and inhibition was reversible following Doxycycline removal (Figure 4C). These results demonstrate that gene expression at the Rosa26 locus is, as expected, dependent on an initial 4OH-tamoxifen triggered recombination event. However, as the resulting expression is itself tTA dependent, the transcription of the targeted gene at the Rosa26 locus can be controlled after Cre recombination by Doxycycline administration.

***In vitro* functional analyses of R26T-SmoM2 3-1 ES cells**

To evaluate the utility of the 3-1 ES cell line as a tool to study gene function *in vitro*, we generated neural precursors from EBs derived from R26T-SmoM2 3-1 ES cells (11). The EBs were treated with RA to caudalize neural progenitor cells, and 4OH-tamoxifen to activate expression of SmoM2 thereby initiating Hh signaling. Induction of FoxA2 in the floor plate and Nkx2.2 in pV3 interneuron progenitors occurs as a direct

response to Shh signaling and requires Smo activity (13,14). Joint exposure of EBs to RA and 4OH-tamoxifen induced a large number of Nkx2.2⁺ (pV3 interneuron progenitor marker) and FoxA2⁺ (floor plate marker) cells neither of which were present in untreated controls (Figure 5A and B). The robust induction of the two most ventral progenitor markers indicates high-level Hh signaling in SmoM2 expressing cells as these two populations require the highest levels of Hh signaling input for their normal specification in the developing neural tube (13).

To further determine if motor neurons are also generated in the R26T-SmoM2 3-1 ES cell derived EBs, we analyzed the expression of MNR2, a transcription factor specifically expressed by mature motor neurons (15). RA exposure alone failed to efficiently induce MNR2 expression, but exposure of RA-treated EBs to 4OH-tamoxifen for 5 days generated MNR2⁺ neurons in a subset of SmoM2 expressing cells and neighboring cells, most probably the result of induction of Shh itself in response to SmoM2-YFP activation (Figure 5C). MNR2⁺ cells that were SmoM2-YFP⁺ generally exhibited low levels of SmoM2-YFP expression, in contrast to the high levels of SmoM2-YFP routinely present in FoxA2⁺ or Nkx2.2⁺ cells, presumably a reflection of the variable onset of recombination during the extended culture period.

We next examined Doxycycline-mediated repression of SmoM2-YFP in EBs generated from R26T-SmoM2 3-1 ES cells. In culture media containing 4OH-tamoxifen, RA and Doxycycline (1 $\mu\text{g/ml}$), no evidence of motor neuron, pV3 or floor plate specification was observed (Figure 5A–C). Thus, SmoM2 expression was tightly regulated by Doxycycline during the *in vitro* differentiation of EB derived neural progenitors.

G₀ chimeric functional analysis using 3-1 ES cells

To test the utility of the R26T-SmoM2 3-1 ES cells for rapid functional analysis *in vivo*, we generated G₀ chimeric embryos by either blastocyst injection or morula aggregation, and directly analyzed G₀ progeny. Tamoxifen (4 mg/40 g body weight) was injected intraperitoneally at E8.5 into pregnant recipients, and embryos were collected 48 h post-injection. In the neural tube of high contribution chimeras, assessed by the production of *E. coli* β -galactosidase from the Rosa26 allele that was not targeted by the SmoM2-YFP insertion (Figure 6A), we observed the dorsal expansion of Nkx2.2⁺, FoxA2⁺ and MNR2⁺ ventral cell fates following Tamoxifen injection (Figure 6A and data not shown). In contrast, highly chimeric embryos isolated from recipients treated with 200 μl of 10 mg/ml Doxycycline by oral gavage the day before surgical transplantation and maintained on Doxycycline supplemented drinking water (1 mg/ml) thereafter, showed an entirely normal neural tube pattern following Tamoxifen injection (Figure 6A).

When chimeric embryos were examined at E13.5, following a lower dose Tamoxifen injection at E8.5 (2 mg/40 g body weight), embryos displayed phenotypes typical of gain-of-function in Hh signaling, such as dorsal CNS hyperproliferation and polydactyly, that were not observed in chimeras from recipients maintained on Doxycycline (Figure 6B). Thus, the dual regulatory system allows drug-mediated control of gene activity *in vivo* as predicted.

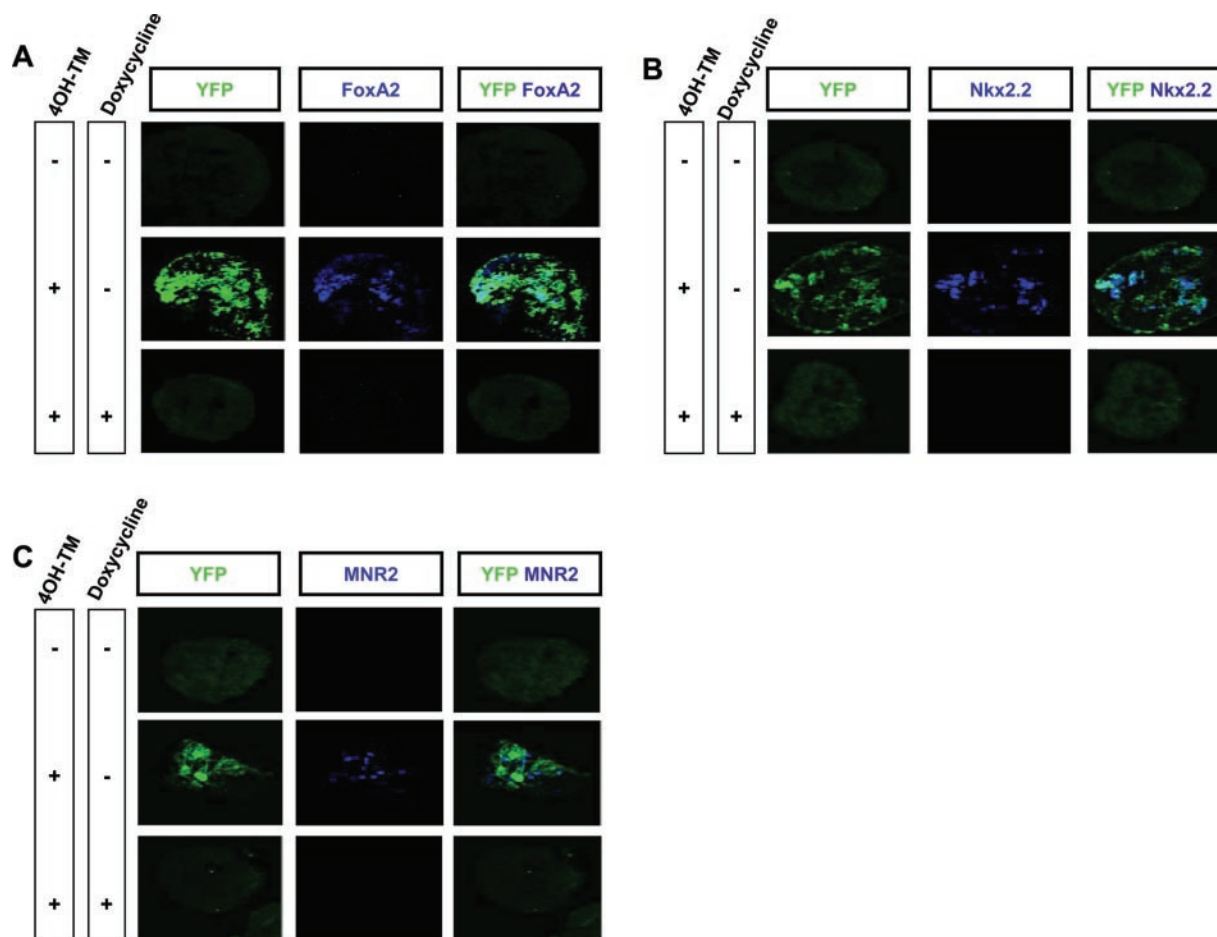


Figure 5. Directed neural differentiation in EBs derived from 3-1 ES cells. (A) EBs grown for 3 days with RA but not tamoxifen did not contain FoxA2 progenitor cells. EBs grown for 3 days with both RA and Tamoxifen contained many FoxA2 cells, which also expressed high levels of SmoM2-YFP. EBs grown for 3 days with RA, 4OH-tamoxifen and Doxycycline did not express SmoM2-YFP and did not contain FoxA2 cells. (B) Coexpression of SmoM2-YFP and Nkx2.2 in the EBs grown for 3 days with RA and 4OH-tamoxifen, but not Doxycycline. (C) Coexpression of SmoM2-YFP and MNR2 in a subset of SmoM2-YFP expressing cells in the EBs grown for 5 days with RA and 4OH-tamoxifen.

Lineage-specific control of the Hh pathway through a R26T-SmoM2 allele

In addition to enabling rapid functional G_0 analysis, the regulatory scheme we describe can be coupled with any existing Cre line for a cell-type specific G_1 study. Here, Doxycycline control enables expression of the gene of interest to be temporally regulated within a particular cell lineage following the initial recombination event. To demonstrate the utility of the 3-1 ES cell system with cell-type specific Cre lines, we established a germline R26T-SmoM2 mouse strain. These mice were crossed with a Wnt1-Cre transgene, a cross that is expected to drive widespread expression of SmoM2 throughout the neural crest lineage as well as in much of the dorsal neural tube (5). Consistent with a previous report (16), ectopic Hh signaling in the Wnt1-Cre and R26T-SmoM2 embryos resulted in a severe dorsal CNS overgrowth and defective development of neural crest cells arising from this region, one result of which was a gross dysmorphology within the frontal-nasal processes. These phenotypes were completely suppressed in embryos isolated from dams maintained on Doxycycline supplemented drinking water, validating the temporal suppression of SmoM2-YFP activity (Figure 7).

Spatio-temporal regulation of major signaling pathways

Having demonstrated the utility of the 3-1 ES cells in providing a rapid and robust means to target the Rosa26 locus and having tested the feasibility of dual regulatory drug control in regulation of targeted allele by manipulating Hh signaling with a SmoM2-YFP allele, we envisioned the establishment of a suite of similarly designed ES cells that enable the manipulation of each of five major developmental signaling pathways (Hedgehog, Wnt, BMP, FGF and Notch) to aid in the genetic dissection of complex processes *in vitro* and *in vivo*. Towards this long term goal we have established Cre-*LoxP* and Doxycycline regulated ES cell lines with ROSA26 knock-ins of a dominant active allele of β -catenin, a dominant negative mutant of FGFR2 and a Noggin-CD4-GFP fusion to modulate canonical Wnt, FGF or BMP2/4 signaling, respectively. The construction of other cell lines are in progress.

DISCUSSION

We report and validate a novel ES cell based system that couples the advantages of position independent transcriptional regulation with drug-mediated control of gene activity. This

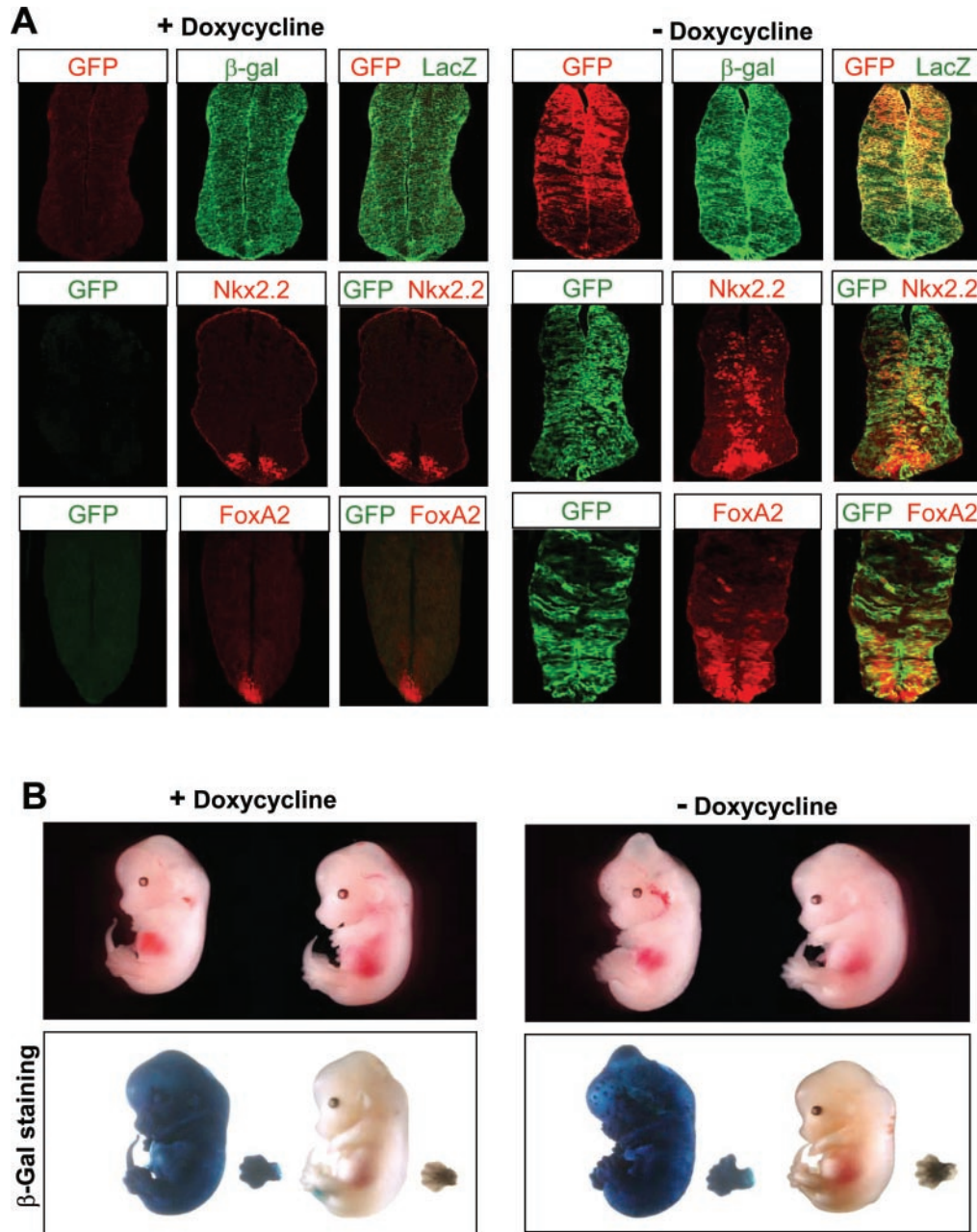


Figure 6. Functional analysis of G_0 chimeric embryos derived from R26T-SmoM2 3-1 ES cells. (A) Ectopic dorsal expansion of ventral markers Nkx2.2 and FoxA2 in the neural tube of chimeras with high ES cell contribution at E10.5 after tamoxifen injection at E8.5. (B) Hh signaling gain-of-function phenotypes such as dorsal CNS hyperproliferation and polydactyly on highly chimeric embryos at E13.5 after tamoxifen injection at E8.5. (A and B) No phenotype was observed on the high contribution embryos with Doxycycline treatment.

genetic system itself, and the ES cell lines we have generated for the dissection of signaling pathway activities in ES cells *in vitro* and in the developing and adult mouse, complement the growing genetic resources for the mouse model. Given the current interest in directing differentiation of ES cells *in vitro* towards specific cell types of clinical relevance, the 3-1 ES cell system will facilitate the rapid dissection of regulatory mechanisms and enable the potential genetic screening of regulatory components. Further, the ability to move from tissue culture to direct analysis of G_0 animals is a significant advantage offered by the strategy we describe, especially

considering two key drawbacks of the mouse system; time in breeding strains and cost in their subsequent maintenance.

To date, there have been several reports of inducible Cre-mediated recombination systems in ES cells that enable the temporal induction of gene activity (17,18). Here we further optimized gene expression at the Rosa26 locus with a reversible spatio-temporal regulation. Several tetracycline (tTA or rtTA) based ES cell systems have also been developed (19–23). The requirement for multiple components and/or the lack of spatial and lineage-specific regulation limit their general utility for certain *in vivo* studies. By coupling Cre

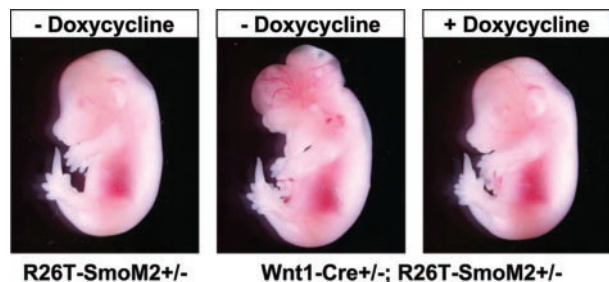


Figure 7. Temporal controlled activation of the Hh signaling in neural crest lineage. Dorsal CNS hyperproliferation and frontal-nasal defects in the *Wnt1-Cre⁺* and *R26T-SmoM2⁺* embryos without Doxycycline treatment, in contrast to the embryos with Doxycycline treatment or the wild-type (*R26T-SmoM2⁺*) embryos.

regulation with Doxycycline-mediated gene control at a single locus, we are able to utilize the growing resources of cell-type specific Cre strains for functional analyses within specific cell or tissue types with the added advantage of temporal control of gene activity within descendants of a specific lineage. While this paper was in preparation, a similar dual regulatory strategy based on rtTA dependent, Doxycycline-mediated activation of a gene of interest following a Cre-excision event was reported (24). Our Doxycycline based repression system with the advantage of the 3-1 ES cell line provides a complementary approach. The utility of this system has been demonstrated here using the example of a Hh signaling component having well-understood and predictable phenotypes to provide a rigorous validation of the model. The cell autonomous manipulation of dominant active or dominant negative forms of key members of the signaling pathways is probable to be widely applicable to the analysis of development and the understanding and treatment of diseases. For example, increasing evidence links Hedgehog, Wnt, Notch and BMP signaling pathways to the regulation of stem cell biology and tumor formation (25,26). The cellular and genetic tools we describe will facilitate the analysis of their regulatory actions in normal and pathological situations.

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REFERENCES

- Lewandoski, M. (2001) Conditional control of gene expression in the mouse. *Nature Rev. Genet.*, **2**, 743–755.
- Gossen, M. and Bujard, H. (2002) Studying gene function in eukaryotes by conditional gene inactivation. *Annu. Rev. Genet.*, **36**, 153–173.

- Jonkers, J. and Berns, A. (2002) Conditional mouse models of sporadic cancer. *Nature Rev. Cancer*, **2**, 251–265.
- Hayashi, S. and McMahon, A.P. (2002) Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. *Dev. Biol.*, **244**, 305–318.
- Danielian, P.S., Muccino, D., Rowitch, D.H., Michael, S.K. and McMahon, A.P. (1998) Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. *Curr. Biol.*, **8**, 1323–1326.
- Soriano, P. (1999) Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nature Genet.*, **21**, 70–71.
- Srinivas, S., Watanabe, T., Lin, C.S., Williams, C.M., Tanabe, Y., Jessell, T.M. and Costantini, F. (2001) Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev. Biol.*, **1**, 4.
- Hogan, B., Beddington, R., Costantini, F. and Lacy, E. (1994) *Manipulating the Mouse Embryo; A Laboratory Manual*. Cold Spring Harbor Laboratory Press, .
- Whiting, J., Marshall, H., Cook, M., Krumlauf, R., Rigby, P.W., Stott, D. and Alleman, R.K. (1991) Multiple spatially specific enhancers are required to reconstruct the pattern of Hox-2.6 gene expression. *Genes Dev.*, **5**, 2048–2059.
- Bond, C.T., Sprengel, R., Bissonnette, J.M., Kaufmann, W.A., Pribnow, D., Neelands, T., Storck, T., Baetscher, M., Jerecic, J., Maylie, J. *et al.* (2000) Respiration and parturition affected by conditional overexpression of the Ca²⁺-activated K⁺ channel subunit, SK3. *Science*, **289**, 1942–1946.
- Wichterle, H., Lieberam, I., Porter, J.A. and Jessell, T.M. (2002) Directed differentiation of embryonic stem cells into motor neurons. *Cell*, **110**, 385–397.
- Xie, J., Murone, M., Luoh, S.M., Ryan, A., Gu, Q., Zhang, C., Bonifas, J.M., Lam, C.W., Hynes, M., Goddard, A. *et al.* (1998) Activating Smoothed mutations in sporadic basal-cell carcinoma. *Nature*, **391**, 90–92.
- Briscoe, J. and Ericson, J. (2001) Specification of neuronal fates in the ventral neural tube. *Curr. Opin. Neurobiol.*, **11**, 43–49.
- Wijgerde, M., McMahon, J.A., Rule, M. and McMahon, A.P. (2002) A direct requirement for Hedgehog signaling for normal specification of all ventral progenitor domains in the presumptive mammalian spinal cord. *Genes Dev.*, **16**, 2849–2864.
- Tanabe, Y., Williams, C. and Jessell, T.M. (1998) Specification of motor neuron identity by the MNR2 homeodomain protein. *Cell*, **95**, 67–80.
- Jeong, J., Mao, J., Tenzen, T., Kottmann, A.H. and McMahon, A.P. (2004) Hedgehog signaling in the neural crest cells regulates the patterning and growth of facial primordia. *Genes Dev.*, **18**, 937–951.
- Vooijs, M., Jonkers, J. and Berns, A. (2001) A highly efficient ligand-regulated Cre recombinase mouse line shows that LoxP recombination is position dependent. *EMBO Rep.*, **2**, 292–297.
- Seibler, J., Zevnik, B., Kuter-Luks, B., Andreas, S., Kern, H., Hennek, T., Rode, A., Heimann, C., Faust, N., Kauselmann, G. *et al.* (2003) Rapid generation of inducible mouse mutants. *Nucleic Acids Res.*, **31**, e12.
- Niwa, H., Burdon, T., Chambers, I. and Smith, A. (1998) Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes Dev.*, **12**, 2048–2060.
- Wutz, A. and Jaenisch, R. (2000) A shift from reversible to irreversible X inactivation is triggered during ES cell differentiation. *Mol. Cell*, **5**, 695–705.
- Niwa, H., Miyazaki, J. and Smith, A.G. (2000) Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nature Genet.*, **24**, 372–376.
- Kyba, M., Perlingeiro, R.C. and Daley, G.Q. (2002) HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. *Cell*, **109**, 29–37.
- Miyazaki, S., Yamato, E. and Miyazaki, J. (2004) Regulated expression of pdx-1 promotes *in vitro* differentiation of insulin-producing cells from embryonic stem cells. *Diabetes*, **53**, 1030–1037.
- Belteki, G., Haigh, J., Kabacs, N., Haigh, K., Sison, K., Costantini, F., Whitsett, J., Quaggin, S.E. and Nagy, A. (2005) Conditional and inducible transgene expression in mice through the combinatorial use of Cre-mediated recombination and tetracycline induction. *Nucleic Acids Res.*, **33**, e51.
- Beachy, P.A., Karhadkar, S.S. and Berman, D.M. (2004) Tissue repair and stem cell renewal in carcinogenesis. *Nature*, **432**, 324–331.
- Sancho, E., Battle, E. and Clevers, H. (2004) Signaling pathways in intestinal development and cancer. *Annu. Rev. Cell Dev. Biol.*, **20**, 695–723.