Development of a unique system for spatiotemporal and lineage-specific gene expression in mice

Hsiao-Man Ivy Yu*, Bo Liu*, Shang-Yi Chiu*, Frank Costantini†, and Wei Hsu*†‡

*Center for Oral Biology, Department of Biomedical Genetics, Abs Institute of Biomedical Sciences, School of Medicine and Dentistry, University of Rochester, 601 Elmwood Avenue, Box 611, Rochester, NY 14642; and †Department of Genetics and Development, Columbia University Medical Center, 701 West 168th Street, New York, NY 10032

Edited by Kathryn V. Anderson, Sloan–Kettering Institute, New York, NY, and approved May 5, 2005 (received for review January 6, 2005)

We have developed an advanced method for conditional gene expression in mice that integrates the Cre-mediated and tetracycline-dependent expression systems. An rtTA gene, preceded by a loxP-flanked STOP sequence, was inserted into the ROSA26 locus to create a R26STOPrtTA mouse strain. When the STOP sequence is excised by Cre-mediated recombination, the rtTA is expressed in the Cre-expressing cells and all of their derivatives. Therefore, cell type-, tissue-, or lineage-specific expression of rtTA is achieved by the use of an appropriate Cre transgenic strain. In mice also carrying a target gene under the control of the tetracycline response element, inducible expression of the target gene is temporally regulated by administration of doxycycline. Our results demonstrate that this universal system is uniquely suited for spatiotemporal and lineage-specific gene expression in an inducible fashion. Gene expression can be manipulated in specific cell types and lineages with a flexibility that is difficult to achieve with conventional methods.

conditonal gene expression | Cre | ROSA26 locus | rtTA

Development of advanced transgenic systems for conditional gene expression is essential for studying development and modeling human diseases in mice. Two powerful systems that have been used extensively are the Cre-loxP system, which allows genes to be permanently activated or inactivated in specific lineages (1), and the tetracycline-dependent system, which permits temporal as well as cell type- or tissue-specific control of transgene expression (2). The basic strategy for the Cre-loxP system is to delete the DNA sequence flanked by two loxP sites, thereby achieving conditional gene activation and inactivation. Manipulating the expression of Cre in a specific place and at a specific time enables conditional regulation. In the tetracycline-dependent system, three essential elements are required. First is the transcription factor tTA or rtTA, which can be expressed in a spatial specific manner. Second, the tetracycline response element (TRE) is used to control the expression of target genes. The presence of the third element, doxycycline (Dox), which can be administrated in a temporal specific fashion, activates target gene expression. We have developed a mouse strain, R26STOPrtTA, which combines features of these two systems and permits greater flexibility of conditional gene expression in the mouse. An rtTA gene, preceded by a loxP-flanked STOP sequence, was inserted into the ubiquitously active ROSA26 locus. When R26STOPrtTA is crossed with Cre-expressing strains, the STOP sequence is excised, yielding the R26rtTA allele, which expresses rtTA in the Cre-expressing cells and all of their descendants. Thus, the cell type, tissue, or lineage specificity of rtTA expression is dictated by selection of an appropriate Cre transgenic strain. In mice also carrying a target gene under the control of TRE, expression of the target gene is temporally regulated by administration of Dox. Using this approach, we have successfully achieved transgenic expression in a spatially and temporally specific fashion. Our results demonstrate the ability of our system for targeted gene expression in the neuroepithelial and hematopoietic lineages, in the derivatives of neural crest, and in the mammary epithelium. Furthermore, we have achieved conditional gene expression in neural crest-derived osteoblasts and parity-induced mammary epithelial (MEP) progenitors *in vivo* and *in vitro*. In these cases, no promoter/enhancer regulatory elements are currently available to accomplish targeted gene expression by conventional methods. This system is useful not only for spatiotemporal and lineage-specific gene expression in an inducible manner but also for manipulating gene activities in a systematic fashion.

Methods

Creation and Breeding of Transgenic Mice. The SacII-NotI fragment of a modified form of rtTA (rtTA2^S-M2) (3) was inserted into the same enzyme sites of the pBigT plasmid (4). The PacI-AscI fragment, consisting of the adenovirus splice acceptor sequence, loxP flanked pgk-neo expression cassette, triple SV40 polyadenylation sequences, and rtTA with triple bovine growth factor polyadenylation sequences, was then inserted into the same enzyme sites of the pROSA26PA (4) plasmid to create the R26STOPrtTA targeting vector. For targeting the ROSA26 locus in ES cells, the R26STOPrtTA plasmid was linearized with KpnI and electroporated into CSL3 ES cells (5) derived from mouse strain 129SvEv/Tac. Nineteen of 180 G418-ressitant colonies had undergone correct homologous recombination, as determined by Southern blot of the R26STOPrtTA allele. Two of these R26STOPrtTA ES clones were injected into blastocysts to generate chimeras that were bred to obtain mice carrying the R26STOPrtTA allele. Mice were genotyped by PCR analysis by using primers (5′-cgcagtagtccagggtttcct-3′ and 5′-gacgtgctacttccatttgtc-3′) to identify the R26STOPrtTA locus. The PCR reaction was performed by denaturation at 94°C for 5 min and 34 cycles of amplification (94°C for 30 s, 63°C for 60 s, and 72°C for 60 s), followed by a 7-min extension at 72°C. To identify the 3′ junction of the R26STOPrtTA and wild-type loci, primers (5′-agggctgttttggcttttag-3′ and 5′-ttccttgaccctggaaggt-3′) and (5′-5′-agggctgttttggcttttag-3′ and 5′-aagcacgtttccgacttgag-3′) were used, respectively. The PCR reaction was performed by denaturation at 94°C for 5 min and 40 cycles of amplification (94°C for 30 s, 57°C for 30 s, and 72°C for 60 s), followed by a 7-min extension at 72°C. Nestin-Cre (6) and Wnt1-Cre (7) mouse strains were obtained from The Jackson Laboratory. WAP-Cre (8) and Lck-Cre (9) mice were obtained from the National Cancer Institute mouse repository and Taconic Farms, respectively. Dox (2 mg/ml plus 50 mg/ml sucrose) was administrated orally in the drinking water and replaced every 3 days by diluting a freshly prepared 10× Dox/sucrose stock solution. Mice, including pregnant females, were treated with Dox for 2–7 days. For primary osteoprogenitors and MEP cells, Dox (8 μg/ml) was added in culture media for 16 h. Care and use of experimental

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: TRE, tetracycline response element; Dox, doxycycline; CNC, cranial neural crest; MEP, mammary epithelial; E*n*, embryonic day *n*.

‡To whom correspondence should be addressed. E-mail: wei_hsu@urmc.rochester.edu.

animals described in this work comply with guidelines and policies of the University Committee on Animal Resources at the University of Rochester.

β-Gal Staining and GFP Analyses. Staining for β-gal activity in embryos, cranial skulls, and mammary glands was performed as described (10). In brief, specimens were dissected in PBS and prefixed in PBS containing 1% formaldehyde, 0.2% glutaraldehyde, 2 mM magnesium chloride, 5 mM EGTA, and 0.02% Nonidet P-40 at 4°C for 30–90 min. Samples were washed three times in PBS containing 0.02% Nonidet P-40 at room temperature for 30 min before they were stained in PBS containing 1 mg/ml X-Gal, 5 mM potassium ferricyanide, 2 mM potassium ferrocyanide, 2 mM magnesium chloride, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40 at 30°C for 6–16 h. Primary osteoblast precursors were isolated from the 1-day-old mouse calvaria, as described (11). Isolated calvarial osteoblasts were cultured in αMEM media supplemented with 10% FCS. Isolation and culture of primary MEP cells were performed as described (12). For staining in cultured cells, mouse calvarial osteoblasts were rinsed twice with PBS, fixed in PBS containing 0.25% glutaraldehyde for 5 min, and washed three times with PBS. Cells were then stained in PBS containing 1 mg/ml of X-Gal, 30 mM potassium ferricyanide, 30 mM potassium ferrocyanide, and 1 mM magnesium chloride at 37°C for 1 h. The stained specimens were photographed for whole-mount analyses. For analyses in sections, samples were subsequently fixed in formaldehyde and processed for paraffin or frozen sections. Whole-mount GFP analysis was performed by using fluorescence stereomicroscopy to visualize the thymus (13). To isolate T lymphocytes, thymus was homogenized with frosted ends of glass slides. Cells were suspended in PBS containing 3% FCS after incubation in RBC lysis buffer (Sigma) at 4°C for 4 min. Lymphocytes were then examined for GFP expression by FACS analysis.

Results

Creation of R26STOPrtTA Mouse Strain. Building upon previous successes using the tetracycline-dependent activation system for transgenic expression in mice (13–15), we created a mouse strain that controls gene expression at two levels: first by Cre-mediated recombination and then by rtTA-mediated Dox induction. For this purpose, an improved version of rtTA (rtTA2^S-M2) (3) preceded by a loxP-flanked STOP sequence was targeted to the ROSA26 locus using homologous recombination in ES cells (Fig. 1*a*). We chose the ROSA26 locus because it has been used frequently and reliably to support ubiquitous expression during mouse development (16). These R26STOPrtTA-targeted ES cells were used to derive germline chimeras, which were bred to obtain the R26STOPrtTA strain (Fig. 1*b*). As reported previously for all ROSA26 mutant alleles, the heterozygous mice did not display an overt phenotype. Homozygotes were also found to be viable and fertile, which greatly facilitates the breeding process to obtain mice carrying multiple transgenes.

We next generated an R26rtTA mouse strain by crossing the R26STOPrtTA allele with the β-actin-Cre transgene. The deletion of the STOP sequence permits expression of rtTA under the control of the endogenous ROSA26 promoter. To examine whether this strain supports the uniform and inducible expression of a target gene, embryos carrying the R26rtTA allele and a transgene containing a lacZ reporter under control of TRE (H.-M.I.Y. and W.H., unpublished work) were stained for β-gal. No lacZ activity was detected in untreated embryos, whereas those treated with Dox for 3 days displayed a uniform expression pattern of the lacZ reporter at embryonic day (E)10.5 (Fig. 1 *e* and *f*) or E12.5 (Fig. 1*d*).

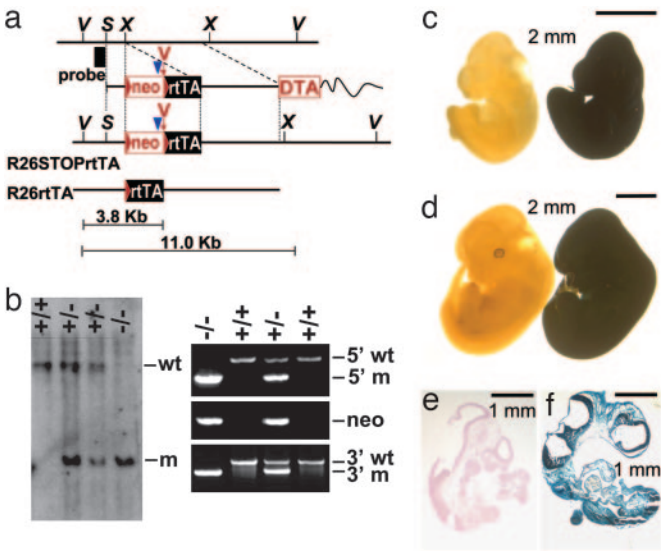

Fig. 1. Development of an inducible expression system in mice. (*a*) A schematic representation of our strategy to generate the R26STOPrtTA allele. The targeted locus contains a pgk-neo gene (neo) for positive selection and a triple polyadenylation site (a STOP sequence indicated by blue arrowhead), both flanked by loxP sites, preceding the rtTA2^S-M2 gene, which is followed by a triple polyadenylation sequences. The STOP sequence is removed by Cre-mediated recombination between loxP sites (red triangles) to activate the expression of rtTA. Diphtheria toxin (DTA) also was used for negative selection (16). *V*, EcoRV; *S*, SacII; *X*, XbaI. (*b*) Southern and PCR analysis. Using a 5′-external probe, the EcoRV-digested wild-type band (wt, 11.0 kb) or a smaller-sized mutant band (m, 3.8 kb) was detected by Southern blot analysis. Mice heterozygous and homozygous for the R26STOPrtTA allele were analyzed by PCR for the 5′ and 3′ junction of the wt and mutant alleles, as indicated. The presence of neo in the R26STOPrtTA mice also was confirmed by PCR analysis. (*c–f*) Expression of the lacZ reporter in triple transgenic embryos (β-actin-Cre, R26STOPrtTA and TRE-lacZ) at E10.5 (*c*) or E12.5 (*d*) was analyzed by whole-mount β-gal staining at 37°C for 16 h. (*Right*) Dox induction for 3 days or (*Left*) without Dox induction. (*e* and *f*) Sections of the stained embryos shown in *c*, counter-stained with nuclear fast red, revealed a uniform expression of the reporter in the presence (*f*) but not in the absence (*e*) of Dox.

Inducible Gene Expression in a Spatial-, Temporal-, and Lineage-Specific Fashion. To test whether our system permits conditional gene activation, the R26STOPrtTA mouse strain was crossed with a Nestin-Cre transgenic line expressing Cre in neuroepithelial precursors (Fig. 2 *a* and *b*). In embryos that also carry the TRE-lacZ reporter transgene (17), the lacZ gene was expressed in the developing brain and neural tube upon treatment with Dox for 5 days (Fig. 2 *a* and *b*). Embryos carrying only two of the three transgenes, either R26STOPrtTA and TRE-lacZ or R26STOPrtTA and Nestin-Cre, showed no detectable β-gal staining at E13.5 (Fig. 2*a*). Sections of the Nestin-Cre/R26STOPrtTA/TRE-lacZ triple transgenic embryo revealed expression of the target gene in dorsal neural tube (Fig. 2*b*). The lacZ expression pattern of the E13.5 triple transgenic embryos matches that of embryos carrying Nestin-Cre and R26RlacZ transgenes (the two-component genetic cell-labeling system) (data not shown).

We next studied whether our strategy permits gene expression in hematopoietic lineages. The Lck-Cre mouse strain (9), where Cre is expressed in immature T lymphocytes, was crossed with the R26STOPrtTA and TRE-Axin-GFP mice. The TRE-Axin-GFP transgene (13) expresses Axin1 and a GFP reporter simultaneously, because GFP was included under the control of an internal ribosomal entry sequence (IRES). The Dox inducible expression of TRE-Axin-GFP was observed in the triple transgenic thymus (heterozygous for each of the three transgenes), as

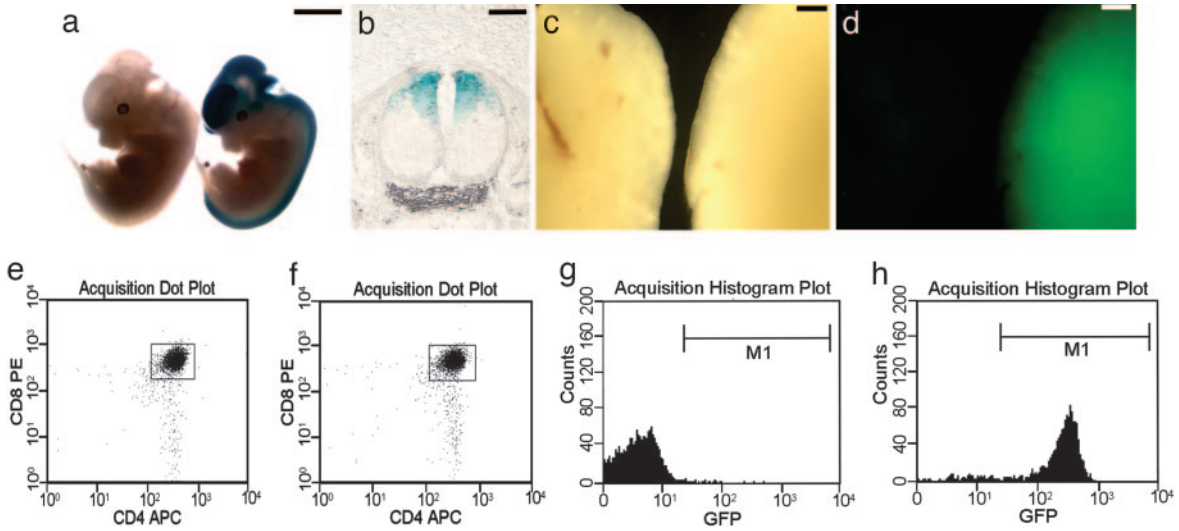

Fig. 2. Conditional gene expression in a spatial- and temporal-specific fashion. (*a*) Nestin-Cre/R26STOPrtTA/TRE-lacZ triple transgenic E13.5 embryo (*Right*) showing expression of the lacZ reporter in the neuroepithelial lineage and control double transgenic (R26STOPrtTA and TRE-lacZ) E13.5 embryos (*Left*). Both embryos were analyzed by β-gal staining at 37°C for 2 h, after 5 days of Dox induction. (*b*) Section of the β-gal-stained triple transgenic embryo from *a* showing targeted gene expression in the dorsal neural epithelium. A similar pattern of expression, in only the dorsal neural tube, was also observed in Nestin-Cre/R26RlacZ embryos (data not shown). (*c* and *d*) Whole-mount GFP analysis (exposure time, 2 sec) of thymus of a triple transgenic mouse carrying Lck-Cre/R26STOPrtTA/TRE-Axin-GFP revealed that the target gene was conditionally activated upon Dox treatment for 7 days (*d*, right), but not the control without Dox (*d*, left). A phase-contrast image of the thymuses is shown in *c*. (*e–h*) FACS staining analysis, further demonstrating the expression of GFP (*g* and *h*) in the CD4 and CD8 double-positive T cells (*e* and *f*) isolated from the Lck-Cre/R26STOPrtTA/TRE-Axin-GFP thymus after Dox induction for 3 days (*f* and *h*) but not the control thymus (*e* and *g*). M1, 0.35% (e); 90.11% (*f*). [Scale bars, 2 mm (*a*), 200 μm (*b*), and 500 μm (*c* and *d*).]

indicated by whole-mount fluorescence microcopy (Fig. 2 *c* and *d*). The expression of GFP in the T lymphocytes was further studied by FACS analysis. Only ≈0.35% of the CD4/CD8 double-positive lymphocytes (Fig. 2*e*) isolated from the control thymus without Dox treatment expressed GFP above the basal level (Fig. 2*g*). In contrast, >90% of the CD4/CD8 double-positive lymphocytes (Fig. 2*f*) from the Dox-induced thymus were positive for GFP (Fig. 2*h*). The apparent lack of GFP expression in <10% of CD4/CD8 double-positive thymocytes may be due to inefficient function of the IRES or to impaired survival of thymocytes overexpressing Axin1, as shown (13). Overall, these data show that our transgenic expression system permits gene expression in a temporally specific as well as a tissue-specific fashion. One copy of the R26STOPrtTA allele apparently is sufficient to enable Dox-dependent gene activation. These results clearly demonstrate the efficacy of our system.

Targeted Gene Expression in Neural Crest Derivatives. Another advantage of this system is that it can be used for conditional gene expression in places difficult to target using conventional tissue-specific promoters. For instance, the emigrant cranial neural crest (CNC) cells that give rise to a variety of facial tissues and structures regulate craniofacial morphogenesis. In lineage tracing/fate mapping analysis using mice carrying both a Wnt1-Cre transgene (7) and the Cre-dependent R26RlacZ reporter allele (16), the lacZ gene was expressed in all CNC derivatives in addition to the dorsal neural tube where Wnt1 is normally expressed (18, 19). It was further shown, using the Wnt1-Cre/R26RlacZ genetic cell-labeling system, that the CNC-derived mesenchyme contributes to formation of the mammalian skull (20). Therefore, Wnt1 is expressed in the neural tube precursors of the CNC but not in the migrating CNC cells or their derivatives. Using the Wnt1-Cre transgene in our expression system, it should be possible to achieve conditional gene expression in all CNC descendants (Fig. 3*a*).

To test this possibility, the Wnt1-Cre mouse strain (6) was bred into the R26STOPrtTA and TRE-lacZ background. Indeed, the lacZ reporter gene was specifically expressed in all derivatives of the CNC cells, including maxilla, mandible, cranial nerves, skull, eyes, skin, and salivary glands, at E11.5 and E16.5 (Fig. 3 *b–k* and *o–p*). In addition, the target gene was expressed in the CNC-derived anterior skull structures (nasal cartilage and nasal and frontal bones, as well as metopic, coronal, and sagittal sutures and Jugum Limitans) and parts of the interparietal bones, which are located at the posterior skull (Fig. 3 *l–n*). However, parietal bone, which is mesodermal in origin, had no detectable β-gal staining. These expression patterns resemble the ones obtained using the Wnt1-Cre/R26RlacZ genetic cell-labeling system (18–20).

We next examined whether this conditional expression system could be used to manipulate gene activity in the calvarial osteoblast culture system. Primary osteoblast precursors were isolated from either nasal/frontal or parietal bones of the 1-day-old mouse calvaria carrying the Wnt1-Cre, R26STOPrtTA and TRE-lacZ transgenes. Only calvarial osteoblasts (≈95%) isolated from the nasal/frontal bone allow Dox-inducible gene activation, because they are derived from the Wnt1-expressing CNC cells (Fig. 3 *q* and *r*). Osteoblasts isolated from the parietal bone did not express rtTA, nor did they permit Dox-inducible gene expression (data not shown). Examination of calvarial osteoblasts isolated from the Wnt1-Cre/R26RlacZ nasal/frontal bones showed that ≈95% of the cells come from CNC (Fig. 3 *s* and *t*). Therefore, the lack of expression in ≈5% of the cells is not due to insufficient gene activation in our system. These results also demonstrated our ability to isolate a pure population of osteoblasts from either nasal/frontal or parietal bones. Thus allows us to examine properties of the osteoblasts from different skull regions *in vitro*. We have successfully manipulated gene expression in the neural crest lineage at embryonic and postnatal stages using this system. Most importantly, our conditional gene expression system works effectively with no leaky expression without Dox in the primary osteoblasts *in vitro* (Fig. 3 *q* and *r*). Therefore, this system is ideal for

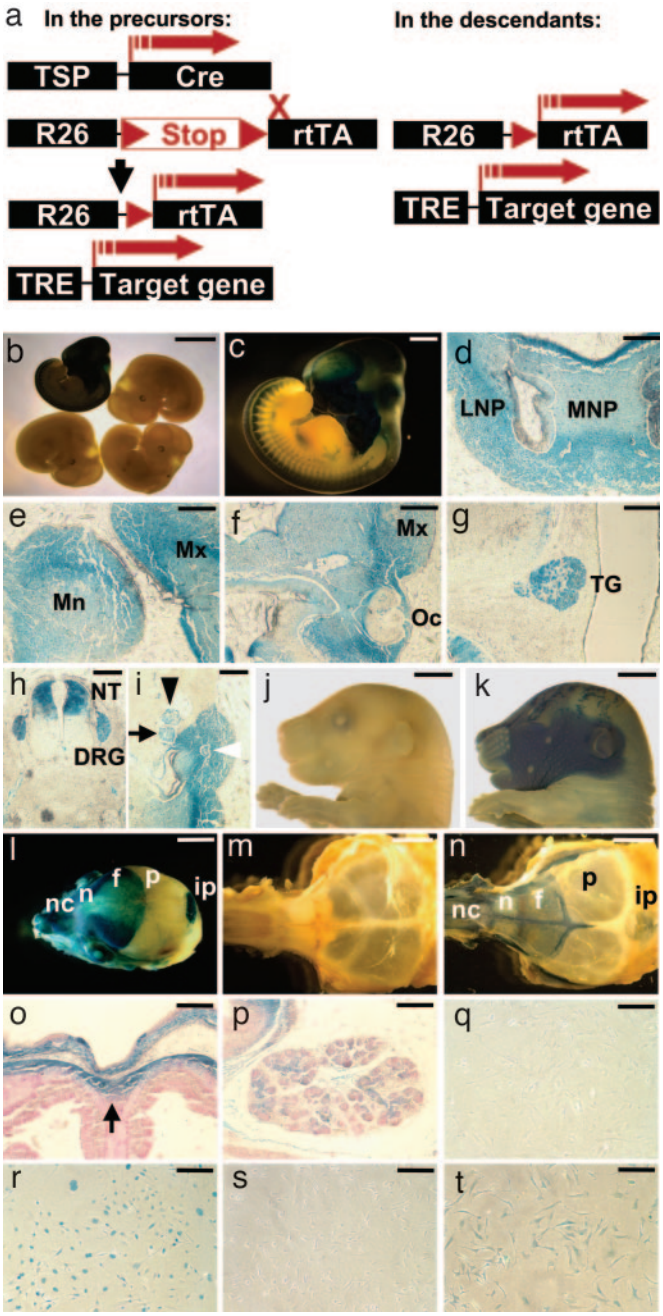

Fig. 3. Lineage-specific gene expression in CNC derivatives *in vivo* and *in vitro*. (*a*) Our strategy for conditional expression of a target gene using the dual-level expression system. Tissue or lineage specificity is provided by Cre-mediated excision of the loxP-flanked STOP sequence, and further temporal activation is controlled by Dox administration. The expression of rtTA in this system becomes independent of regulation by the tissue-specific promoter (TSP) after the excision of the loxP-flanked STOP sequence. The R26STOPrtTA gene activated by Cre remains active in all of the daughter cells even after Cre expression has been turned off. (*b*) E11.5 Wnt1-Cre/R26STOPrtTA/TRE-lacZ triple transgenic embryos, induced with Dox for 5 days (embryo, *Upper Left*) or without Dox induction (other three embryos) (β-gal staining for 2 h). (*c*) Enlargement of Dox-induced embryo from *a*, showing expression in derivatives of the Wnt1-expressing neural crest cells, including maxilla, mandible, cranial nerves, skull, eyes, skin, and salivary glands. (*d–i*) Sections of the β-gal-stained E11.5 embryo showing targeted gene expression in neural crest derivatives, including the lateral (LNP) and medial (MNP) nasal processes (*d*), the mandibular (Mn) and maxillary (Mx) components of branchial arch (*e* and *f*), the optic cup (*f*, Oc), the trigeminal (*g*, TG) and dorsal root ganglia (*h*, DRG), the dorsal neural tube (*h*, NT), the united ganglion of XI and X nerves (black arrowhead), the inferior ganglion of XI nerve (black arrow), and the branches of VII nerve (white arrowhead) (*i*). β-Gal staining of a triple transgenic E16.5 embryo (*k* and *l*) and 21-day-old mouse (*n*) showed that conditional gene expression could be manipulated in the CNC-derived skeletal elements of the developing mouse skull vault. Control mice without Dox induction revealed no expression of lacZ (*j* and *m*). f, frontal bone; ip, interparietal bone; n, nasal bone; nc, nasal cartilage; p, parietal bone. Sections of the β-gal-stained E16.5 embryos revealed that the lacZ reporter is expressed in the cranial sutures (*o*, arrow) and salivary glands (*p*). The neural crest-derived osteoblasts isolated from nasal and frontal bones were cultured in medium containing (*r*) or lacking (*q*) Dox for 16 h and analyzed by β-gal staining (at 37°C for 1 h) for inducible expression of the target gene. Absolutely no activation of the TRE-lacZ reporter (0%) was observed in cells without Dox. In contrast, 95% of the neural crest-derived osteoblasts exhibited nuclear β-gal staining upon Dox treatment. A similar expression pattern was observed in calvarial osteoblasts isolated from the newborn carrying Wnt1-Cre/R26RlacZ transgenes. No lacZ expression was detected in osteoblasts isolated from the parietal bone (*s*), whereas ≈95% of the cells were positive for β-gal staining (at 37°C for 2 h) in the nasal/frontal bone-derived osteoblasts (*t*). [Scale bars: 3 mm (*b*, *m*, and *n*); 1 mm (*c*); 100 μm (*d–g* and *i*); 200 μm (*h*, and *o–t*); and 2 mm (*j–l*).]

alterations of gene expression in the CNC-derived osteoblasts *in vivo* and *in vitro*.

Manipulating Gene Expression in the Parity-Induced Mammary Progenitors. The parous mammary gland differs from the nulliparous gland despite a close morphological resemblance (21). A recent study suggested that parity-induced MEP progenitors contribute to differences in cellular composition between the nulliparous and parous glands (22). These recently identified MEP cells might provide the key to understanding cancer refractoriness of the parous gland (23). The descendants of differentiated alveoli have previously been traced during mammary gland remodeling by using a genetic cell-labeling system similar to the one described for the CNC derivatives (22). In mice carrying the R26RlacZ reporter allele, the alveolar cells were labeled upon activation of lacZ by the WAP-Cre transgene (8). A small group of the MEP cells, which originate from differentiated cells during pregnancy, survived the massive apoptosis during mammary involution.

Our conditional expression system is ideal for systematically analyzing gene functions in this recently identified MEP cell population (Fig. 4*a*). Female mice carrying the WAP-Cre, R26STOPrtTA, and TRE-lacZ transgenes were obtained to study the inducible gene expression during mammary morphogenesis. Indeed, expression of the lacZ target gene in differentiated cells was successfully activated by Dox induction in late pregnancy (data not shown). Furthermore, the target gene could be conditionally expressed in these recently identified MEP cells that survived through involution in parous glands of the triple transgenic females for 2–4 weeks after weaning (Fig. 4 *b–d*), as well as in primary cultures of the parous mammary epithelium (Fig. 4*e*). The results demonstrate that gene activity can be manipulated in these MEP progenitors *in vivo* and *in vitro*.

Discussion

We have created an advanced system for conditional gene expression in mice, and our data have demonstrated the effectiveness of this system for spatiotemporal and lineage-specific gene expression. Using several different Cre mouse strains in our system, we have successfully targeted gene expression in the neuroepithelial, hematopoietic, neural crest, and MEP lineages. One copy of the R26STOPrtTA allele is sufficient for Dox-dependent gene activation. The ability to generate fertile R26STOPrtTA homozygotes greatly facilitates the breeding process for obtaining mice carrying multiple transgenes. Furthermore, there was no toxicity associated with the Dox treat-

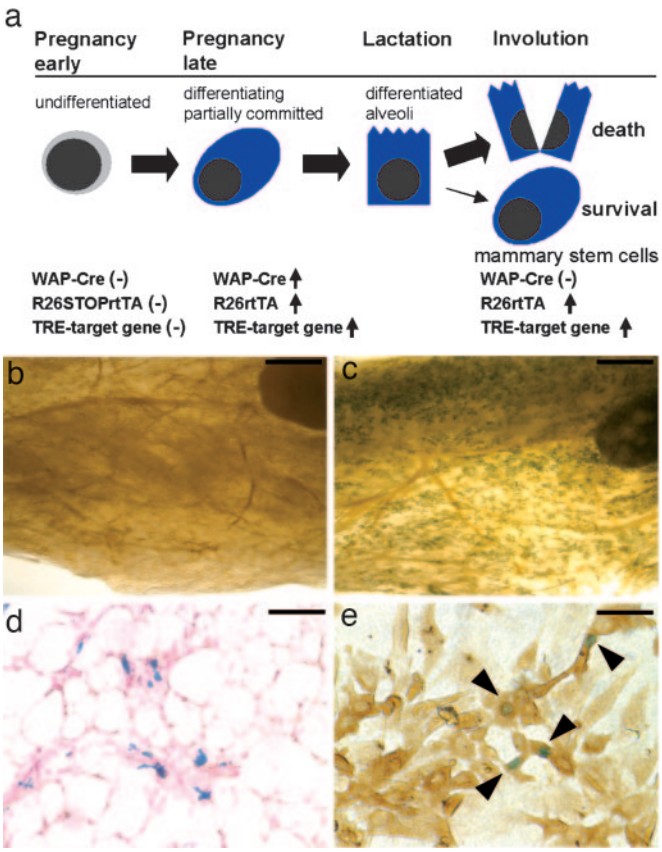

Fig. 4. Manipulating gene expression in the mammary gland. (*a*) A schematic diagram illustrates the strategy for targeted expression. The WAP-Cre transgene, which is active in late pregnancy, turns on the expression of rtTA in differentiating epithelia and differentiated alveoli. The rtTA remains expressed in derivatives of the WAP-expressing cells (MEP progenitors and their progeny), even though Cre is no longer present in the involved gland. Diagram modified from Wagner *et al.* (22). β-Gal staining (37°C for 5 h) of the WAP-Cre/R26STOPrtTA/TRE-lacZ parous gland revealed no expression of the target gene without Dox (*b*) and induction of expression with Dox for 7 days (*c*). The TRE-lacZ transgene was conditionally activated in the recently identified MEP progenitors of the triple transgenic female 2 weeks after weaning/mammary gland remodeling. A section of the β-gal-stained parous gland was counterstained with nuclear fast red (*d*). β-Gal staining (37°C for 1 h) of the primary mammary epithelia, which were isolated from the WAP-Cre/R26STOPrtTA/TRE-lacZ parous gland 4 weeks after weaning, revealed nuclear staining of the MEP cells (*e*). The cultured cells were subsequently stained by immunostaining with anti-keratin8/18 antibody (brown) to confirm identify of the MEP cells. This system enables manipulation of gene expression in a subpopulation of the MEP cells *in vivo* and *in vitro*. [Scale bars: 1 mm (*b* and *c*) and 50 μm (*d* and *e*).]

ment under our experimental conditions. Our conditional gene expression system works effectively without leaky expression in the absence of Dox *in vivo* and *in vitro*.

It has been shown that the ROSA26 allele permits expression of an inserted gene at a significant level. For instance, the Cre-mediated lacZ expression from the R26RlacZ allele can be easily detected (16, 24). We have directly compared our three-component system with the two-component system (tissue-specific Cre transgene and R26RlacZ allele) with several different Cre transgenes. The results suggest that, in most cases, they work in a similar fashion, whereas sometimes our system provides even stronger expression of the target gene. In the cases of primary cultured osteoblasts and MEP cells, it takes ≈2–4 h to detect the β-gal activity in the two-component system but only 30 min to 1 h in our system. This is probably due to an amplification of the signals by the tetracycline-dependent activation system.

This method allows the expression of target genes in a spatially and temporally specific manner. Although similar results can be achieved by the expression of rtTA under the control of a tissue-specific promoter/enhancer (14, 25, 26), our system provides two important advantages. First, it takes advantage of the many well characterized Cre mouse lines (6–9, 27–30) that are available. Therefore, one does not need to generate and characterize a new tissue-specific rtTA transgenic line to express the rtTA in the desired spatial or temporal pattern. Another advantage is that our system is best suited for targeting gene expression in a lineage-specific fashion. Compared with a mouse strain that expresses rtTA directly under the control of a tissue-specific promoter, in our system, the expression of rtTA from the ROSA26 locus, once activated by Cre recombination, becomes independent of the continued expression of Cre. The rtTA encoded by the R26rtTA allele will remain active in all of the daughter cells, regardless of whether Cre continues to be expressed in those cells. This is particularly useful for manipulating gene expression in certain cell types, such as CNC-derived osteoblasts (Fig. 3) and adult mammary stem cells (Fig. 4). In these cases, expression of the target gene cannot be achieved by conventional methods due to a lack of the promoter/enhancer regulatory element. However, gene expression can be manipulated in the CNC-derived osteoblasts and the parity-induced MEP progenitors, with our experimental strategy. Although we did not directly analyze this recently identified subpopulation of MEP cells present in the parous gland, their stem cell-like activities have been addressed in a previous report (22). Furthermore, our study revealed that the CNC- and mesoderm-derived osteoblasts possess distinct developmental properties in mice with targeted disruption of Axin2, a negative regulator of the Wnt pathway (10). The ability of our system to express target genes in a lineage-specific fashion provides a systematic way to study the CNC-derived osteoblasts. Finally, we have demonstrated that conditional gene expression can be accomplished in the parity-induced MEP progenitors. Using this advanced system to manipulate gene activity, thereby altering cellular signaling or gene transcription, might provide new insights into the role of these parity-induced MEP progenitors in normal development and neoplastic transformation. Therefore, this system has outstanding potential for analyzing gene functions in mammalian development and modeling human diseases in mice.

We thank Zaiqi Wu for excellent technical assistance, Peter Keng for advice on FACS analysis, and Wolfgang Hillen (Universität Erlangen, Erlangen, Germany) for reagents. This work was supported in part by grants from the National Institutes of Health [Grants CA106308 (to W.H.) and HD44265 and DK55388 (to F.C.)].

1. Abremski, K., Hoess, R. & Sternberg, N. (1983) *Cell* **32,** 1301–1311.
2. Gossen, M. & Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 5547–5551.
3. Urlinger, S., Baron, U., Thellmann, M., Hasan, M. T., Bujard, H. & Hillen, W. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 7963–7968.
4. Srinivas, S., Watanabe, T., Lin, C. S., William, C. M., Tanabe, Y., Jessell, T. M. & Costantini, F. (2001) *BMC Dev. Biol.* **1,** 4.
5. Bourc'his, D., Xu, G. L., Lin, C. S., Bollman, B. & Bestor, T. H. (2001) *Science* **294,** 2536–2539.
6. Tronche, F., Kellendonk, C., Kretz, O., Gass, P., Anlag, K., Orban, P. C., Bock, R., Klein, R. & Schutz, G. (1999) *Nat. Genet.* **23,** 99–103.
7. Danielian, P. S., Muccino, D., Rowitch, D. H., Michael, S. K. & McMahon, A. P. (1998) *Curr. Biol.* **8,** 1323–1326.
8. Wagner, K. U., Wall, R. J., St-Onge, L., Gruss, P., Wynshaw-Boris, A., Garrett, L., Li, M., Furth, P. A. & Hennighausen, L. (1997) *Nucleic Acids Res.* **25,** 4323–4330.
9. Lee, P. P., Fitzpatrick, D. R., Beard, C., Jessup, H. K., Lehar, S., Makar, K. W., Perez-Melgosa, M., Sweetser, M. T., Schlissel, M. S., Nguyen, S., *et al.* (2001) *Immunity* **15,** 763–774.

10. Yu, H. M., Jerchow, B., Sheu, T. J., Liu, B., Costantini, F., Puzas, J. E., Birchmeier, W. & Hsu, W. (2005) *Development (Cambridge, U.K.)* **132,** 1995–2005.
11. Mansukhani, A., Bellosta, P., Sahni, M. & Basilico, C. (2000) *J. Cell Biol.* **149,** 1297–1308.
12. Seagroves, T. N., Krnacik, S., Raught, B., Gay, J., Burgess-Beusse, B., Darlington, G. J. & Rosen, J. M. (1998) *Genes Dev.* **12,** 1917–1928.
13. Hsu, W., Shakya, R. & Costantini, F. (2001) *J. Cell Biol.* **155,** 1055–1064.
14. Shin, M. K., Levorse, J. M., Ingram, R. S. & Tilghman, S. M. (1999) *Nature* **402,** 496–501.
15. Yamamoto, A., Lucas, J. J. & Hen, R. (2000) *Cell* **101,** 57–66.
16. Soriano, P. (1999) *Nat. Genet.* **21,** 70–71.
17. Hennighausen, L., Wall, R. J., Tillmann, U., Li, M. & Furth, P. A. (1995) *J. Cell Biochem.* **59,** 463–472.
18. Chai, Y., Jiang, X., Ito, Y., Bringas, P., Jr., Han, J., Rowitch, D. H., Soriano, P., McMahon, A. P. & Sucov, H. M. (2000) *Development (Cambridge, U.K.)* **127,** 1671–1679.
19. Jiang, X., Rowitch, D. H., Soriano, P., McMahon, A. P. & Sucov, H. M. (2000) *Development (Cambridge, U.K.)* **127,** 1607–1616.
20. Jiang, X., Iseki, S., Maxson, R. E., Sucov, H. M. & Morriss-Kay, G. M. (2002) *Dev. Biol.* **241,** 106–116.
21. Smith, G. H. (1987) *J. Cell Physiol.* **131,** 190–199.
22. Wagner, K. U., Boulanger, C. A., Henry, M. D., Sgagias, M., Hennighausen, L. & Smith, G. H. (2002) *Development (Cambridge, U.K.)* **129,** 1377–1386.
23. Vonderhaar, B. K., Smith, G. H., Pauley, R. J., Rosen, J. M. & Topper, Y. J. (1978) *Cancer Res.* **38,** 4059–4065.
24. Mao, X., Fujiwara, Y. & Orkin, S. H. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 5037–5042.
25. Mansuy, I. M., Winder, D. G., Moallem, T. M., Osman, M., Mayford, M., Hawkins, R. D. & Kandel, E. R. (1998) *Neuron* **21,** 257–265.
26. Tichelaar, J. W., Lu, W. & Whitsett, J. A. (2000) *J. Biol. Chem.* **275,** 11858–11864.
27. Ovchinnikov, D. A., Deng, J. M., Ogunrinu, G. & Behringer, R. R. (2000) *Genesis* **26,** 145–146.
28. Jiao, K., Kulessa, H., Tompkins, K., Zhou, Y., Batts, L., Baldwin, H. S. & Hogan, B. L. (2003) *Genes Dev.* **17,** 2362–2367.
29. Vincent, S. D. & Robertson, E. J. (2003) *Genesis* **37,** 54–56.
30. Kimmel, R. A., Turnbull, D. H., Blanquet, V., Wurst, W., Loomis, C. A. & Joyner, A. L. (2000) *Genes Dev.* **14,** 1377–1389.