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ORIGINAL PAPER

# Temporally and spatially controlled expression of transgenes in embryonic and adult tissues

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**Abstract** Using ES cell-mediated transgenesis, we generated a novel mouse strain that permits a temporally and spatially controlled expression of responder genes in embryonic and multiple adult tissues. The transgene was constructed in a way that a CMV enhancer linked to the chicken $\beta$-actin promoter (CAG) drives the expression of the tetracycline-controlled transactivator (tTA) in particular tissues upon Cre-mediated excision of a floxed $\beta$geo marker located between the promoter and the tTA. Based on the enzymatic activity of lacZ, the CAG-$\beta$geo-tTA construct exhibits a widespread expression and appears to be very strong in the brain, heart, muscle, pancreas, and skin. Like the embryonic stem cell line that was used to generate this strain, the CAG-$\beta$geo-tTA transgene is already highly active in preimplantation embryos. Using in vivo bioluminescence imaging on MMTV-Cre, CAG-$\beta$geo-tTA, TetO-Luciferase triple transgenic mice and their controls, we demonstrated that the expression of the tTA, which is strictly dependent on the presence of Cre recombinase, induces the activation of the reporter transgene in the absence of any ligands. The tTA-mediated transactivation can be completely ablated through administration of doxycycline, and its subsequent withdrawal lifts the transcriptional block. Based on these characteristics, this novel strain may be useful in experiments that require a sustained expression of transgenes in particular cell types over a prolonged period followed by a rapid downregulation, for example in studies that examine the therapeutic value of cancer-initiating oncogenes during disease progression.

**Keywords** Gene expression regulation · Transgenic · Stem cells · Cre recombinase · Tetracycline · Trans-activators

Qian Zhang and Aleata A. Triplett contributed equally to this work.

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## Introduction

For over two decades, transgenic mice have permitted the study of biological functions of numerous genes during normal organogenesis. In addition, the first generation of transgenic mice that were purposely made to induce neoplasia in a variety of tissues provided valuable insight into cellular processes that are crucial for the initiation of cancer (Brinster et al. 1984; Stewart et al. 1984; Adams et al. 1985). For the development of cancer drugs, in particular for drug target validation, it would be essential to know whether cancer-initiating genetic alterations are still essential for the survival of neoplastic cells within progressing lesions. The scientific challenge of determining whether a multistage cancer process is reversible fueled the development of mouse models that overexpress oncogenes in a temporally and spatially controlled manner. One of the most powerful transgenic approaches that have been developed is the tetracycline (Tet)–dependent system, which permits temporal as well as tissue-specific control of transgene expression (Gossen and Bujard 1992; Furth et al. 1994). The first studies to adapt this technology for the investigation of cancer initiation and progression in mice utilized the MMTV-tTA strain to drive the expression of the simian virus 40 large T-antigen under regulation of the Tet-controlled operator/promoter (TetO; Ewald et al. 1996). This inaugural study provided for the first time experimental evidence that tumorigenesis is reversible at an early stage of neoplastic transformation and that progressing tumor cells can become independent from the tumor-initiating event.

Recent advances in the development of mouse strains that express the reverse tetracycline-responsive transactivator (rtTA, Tet-ON) under the ubiquitously active *Rosa26* locus are very valuable for studying the full spectrum of functions of particular genes in many cell types (Yu et al. 2005; Belteki et al. 2005). A tissue-specific expression of the rtTA as well as the Tet-controlled activation of transgenes can be achieved in these strains through Cre-mediated deletion of a selectable marker cassette that acts as a transcriptional *Stop* sequence located between the *Rosa26* promoter and the *rtTA* coding sequence. The authors suggested that this approach might abrogate the need to generate cell type-specific rtTA transgenes for tissues where well-characterized Cre lines already exist. There are however several shortcomings that limit the applicability of these strains to assess the transforming capabilities of weak oncogenes that require a high expression in adult tissues over an extended period. It is our experience that the activation of the *Rosa26* locus varies greatly between cell-types in adult tissues. In addition, a sustained activation of the TetO-driven transgenes requires a constant administration of doxycycline (Dox, a potent derivative of tetracycline). While this approach is superior for assessing the acute gain-of-function of genes in particular cell types within a short period, the prolonged administration of Dox can be costly or affect the health of an animal depending on the type of administration. Freshly prepared Dox in drinking water appears to be the safest and most effective way to activate transgenes. In contrast, feeding commercially available chow containing Dox is less expensive in the long-term but the transgene activation is not as high (Zhang and Wagner, unpublished). More importantly, high-energy supplements in these foods to mask the bitter taste of Dox can led to a substantial weight gain. In experiments performed in our laboratory, some animals weighed up to 80 g regardless of their genotype after 1 year of administration of Dox-containing food. The histopathological examination revealed that these animals were diabetic and the pancreatic islets of Langerhans of these mice were substantially enlarged and often fused together (Zhang and Wagner, unpublished). Based on these observations, we concluded that the reverse transactivator system might not always be advantageous in experimental settings that require a ligand-inducible activation of genes over a long period. To circumvent some of these problems, we report here the generation of a transgenic strain that expresses the original tetracycline-controlled transactivator (tTA, Tet-OFF) under regulation of a modified chicken $\beta$-actin (CAG) promoter. A detailed analysis demonstrates that this transgene is highly expressed in embryonic stem cells, blastocycts, and selective tissues of adult mice. Similar to the Rosa26-rtTA knockin strains, a tissue-restricted activation of the tTA can be achieved through Cre-mediated recombination. Depending on the cell type, the tTA induces a very strong transactivation of transgenes in the absence of tetracycline. The administration of Dox leads to an instant and highly effective suppression of TetO-regulated target genes, which is a desired feature for modeling a targeted therapy against overexpressed genes to treat a progressing disease such as cancer.

## Materials and methods

### Cloning and verification of functionality of the CAG-βgeo-tTA transgene in 293T cells

The tTA coding sequence was cloned by proofreading PCR using the following primer sets: 5′-CGC GAA TTC GCC ACC ATG TCT AGA TTA GAT AAA AGT AAA GTG-3′ and 5′-CGC GGA TCC CTA CCC ACC GTA CTC GTC AAT TCC-3′. The PCR fragment was digested with *Eco*RI and *Bam*HI and cloned into the corresponding sites of the pMSCV-IRES-GFP vector. The tTA-IRES-GFP cassette was released as an *Eco*RI/*Not*I blunted fragment and transferred into the blunted *Eco*RI site of the pCAGGS vector (kindly provided by Dr. Miyazaki, Osaka University Medical School). Next, the tTA-IRES-GFP-polyA cassette was directionally cloned as an *Ase*I(blunt)-*Not*I(sticky) fragment into the *Bgl*II (blunt) and *Not*I (sticky) sites of vector pCCALL2 to generate the final CAG-βgeo-tTA transgene.

The functionality of the CAG-βgeo-tTA construct was tested by transfecting this vector along with a TetO-Luciferase reporter construct and a pCAGGS-Cre plasmid into 293T cell using SuperFect (Qiagen, Inc.) according to the manufacture's protocol. The cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, and viable cells were analyzed 48 h later for expression of GFP under an inverted fluorescence microscope (Axiovert 35, Carl Zeiss, Inc., Germany). Subsequently, luciferin (150 μg/ml) was administered to these cells, and the expression of luciferase (i.e. emitted photons) was quantified using a bioluminescence imaging machine (IVIS200, Caliper Life Sciences, Alameda, CA).

### *Embryonic stem cell culture and ES cell-based transgenesis*

The CAG-βgeo-tTA vector was linearized using *Not*I, phenol-chlorophorm extracted, and electroporated into E14 (129/Ola) ES cells (30 μg DNA per $5 \times 10^6$ cells). After selecting neomycin-resistant ES cells (300 μg/ml Geneticin), individual clones were isolated and grown on duplicate plates. Based on the uniformity and intensity of lacZ expression, four clones (2A1, 2A5, 2A10, and 2A11) were expanded and used for the production of chimeric mice. The injection of ES cells into C57/Bl6 blastocytes was carried out at the UNMC Mouse Genome Engineering Core Facility. Chimeric males were mated with C57/Bl6 females, and the transgene of two ES cell clones (2A5 and 2A11) was transmitted though the germline.

To assess whether the tTA was expressed in these transgenic stem cells upon Cre-mediated recombination, we co-electroporated ES cells from a duplicate plate with the CAG-Cre construct and the TetO-puromycin responder gene (5 μg CAG-Cre and 18 μg TetO-puro per $7.8 \times 10^4$ cells). Drug-resistant clones (1.5 μg/ml puromycin) were examined under an inverted fluorescence microscope for co-expression of GFP from the recombined CAG-tTA-IRES-GFP transgene.

Subcultures of ES cell clones 2A5 and 2A11 were grown at low density in the presence of LIF (10 ng/ml; Millipore Corp) on gelatin-coated plates and differentiated in vitro using 5 μM retinoic acid (Sigma) for 48 h (Kelly and Rizzino 2000). To induce differentiation of these ES cells in vivo, less than $1 \times 10^5$ cells of each clone were injected subcutaneously into Athymic nude mice. Approximately 40 days post injection, teratomas were collected and processed for sectioning on a cryostat.

### *Genotyping protocols*

Protocols to genotype MMTV-Cre and TetO-Luc transgenic mice were described previously (Wagner et al. 1997; Creamer et al. 2009). The presence of the CAG-βgeo-tTA transgene [MGI:3849835; Tg(CAG-bGeo,-tTA,-EGFP)2A11Kuw] was detected by PCR using a primer pair that amplifies about 330 bps of cloning junction between the CAG promoter and the *lacZ* coding sequence (primer 2004 5′-GGC TCT AGA GCC TCT GCT AAC C-3′; primer 418 5′-CTT CGC TAT TAC GCC AGC TGG-3′). For verification purposes, we also used generic primer sets that recognize the *tTA* and *GFP* coding sequences (provided upon request). All animals used in this study were treated humanely and in accordance with institutional guidelines and federal regulations.

### *Southern blot analysis*

We used the entire coding region of GFP from the transgene as an internal probe to determine the copy number (i.e. single integration) of the CAG-βgeo-tTA transgene and to verify germline transmission.

Genomic DNA from tail biopsies was prepared using standard phenol/chloroform extraction. Fifteen μg of DNA was digested with *Asp*718 at 37°C overnight and separated on a 0.8% agarose gel. The DNA was denatured and blotted onto a nylon membrane (Genescreen plus, NEN), and hybridized overnight with a $^{32}$P-labeled probe at 65°C using QuickHyb (Stratagene). Membranes were washed in 0.5× SSC buffer containing SDS and exposed for 16–24 h to a KODAK XOMAT-AR film.

### *X-gal staining*

293T and ES cells as well as blastocysts were fixed for 10–15 min in 0.08% glutaraldehyde in 1× PBS and washed at least three times in 1× PBS prior to the X-gal staining procedure. Tissues of adult mice and teratomas were fixed for 1–2 h in 2% paraformaldehyde, 0.25% glutaraldehyde, 0.01% NP-40 in 1× PBS. Following fixation, cells, embryos, and tissue specimen were washed repeatedly in 1× PBS. Normal tissues and teratomas were transferred into a 20% sucrose solution prior to embedding in OCT and cryosectioning. Alternatively, tissue fragments of various sizes were stained directly in X-gal buffer as described previously (Wagner et al. 1997). After staining the specimens overnight at 30°C cells in a hybridization oven, cells, embryos, and tissues were washed again repeatedly in 1× PBS. Only stained tissue fragments were postfixed in 10% formalin, dehydrated to 100% ethanol, and placed overnight in xylene before being processed for paraffin sectioning. Paraffin sections and X-gal-stained frozen sections were counterstained with Nuclear Fast Red. Images of histological slides were taken on a Zeiss AxioImager microscope (Carl Zeiss, Inc., Germany) equipped with a SPOT FLEX camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

### *Bioluminescence imaging of luciferase expression*

The expression and activity of the luciferase reporter gene was determined using in vivo bioluminescence imaging (IVIS200, Caliper Life Sciences, Alameda, CA). According to the manufacture's recommendations, luciferin (1 mg D-luciferin potassium salt in 0.2 ml 1× PBS) was injected intraperitoneally 10 min prior to the imaging procedure. The mice were kept under anesthesia (isoflurane) during the acquisition of the images that were collected at intervals ranging from 10 s to 4 min. To determine reporter gene activation in embryonic tissues, E12.5 embryos were isolated from a CAG-βgeo-tTA, TetO-Luc double transgenic female that was mated with a MMTV-Cre male. Prior to the imaging procedure, embryos were transferred into media containing 150 μg/ml luciferin.

## Results

### Cre-mediated expression of the tetracycline-controlled transactivator (tTA) and TetO-driven reporter genes in embryonic stem cells

To construct a transgene that allows a widespread expression of the Tet-regulatible transactivator (tTA) protein in embryonic and adult tissues, we inserted the coding sequence of the tTA along with an internal ribosomal entry site (IRES), a GFP reporter, and a polyA site into the pCCALL2 vector (Fig. 1a). The transgene carries a CMV enhancer linked to the chicken β-actin promoter (CAG) that induces a constitutive expression of the βgeo (lacZ/neoR fusion) selectable marker gene. The *βgeo* coding sequence and the following three polyadenylation sites are surrounded by *loxP* sites and can be excised upon Cre-mediated recombination (Lobe et al. 1999). After determining the correct inducible expression of all its components in 239T cells (Suppl. Fig. 1), the CAG-*loxP*-βgeo-*loxP*-tTA-IRES-GFP (referred to as CAG-βgeo-tTA) construct was linearized with *Not*I and electroporated into mouse embryonic stem (ES) cells (Fig. 1b). Following selection with G418, resistant ES cell clones were isolated, split, and grown on duplicate plates. ES cells on one plate were fixed and stained with X-gal to evaluate the uniformity and strength of lacZ expression (Fig. 1c, d). To assess whether transgenic ES cells were capable of expressing the transactivator upon Cre-mediated activation, we co-electroporated the CAG-Cre construct along with a Tet operator-driven puromycin resistance gene (TetO-puro) into an ES cell line that exhibited a high and uniform X-gal staining. Although this co-transfection procedure was inefficient and yielded only few puromycin-resistant colonies, many of the surviving subclones expressed GFP, suggesting that these ES cells underwent a Cre-mediated recombination event and activation of the tTA-IRES-GFP cassette (Fig. 1e, f).

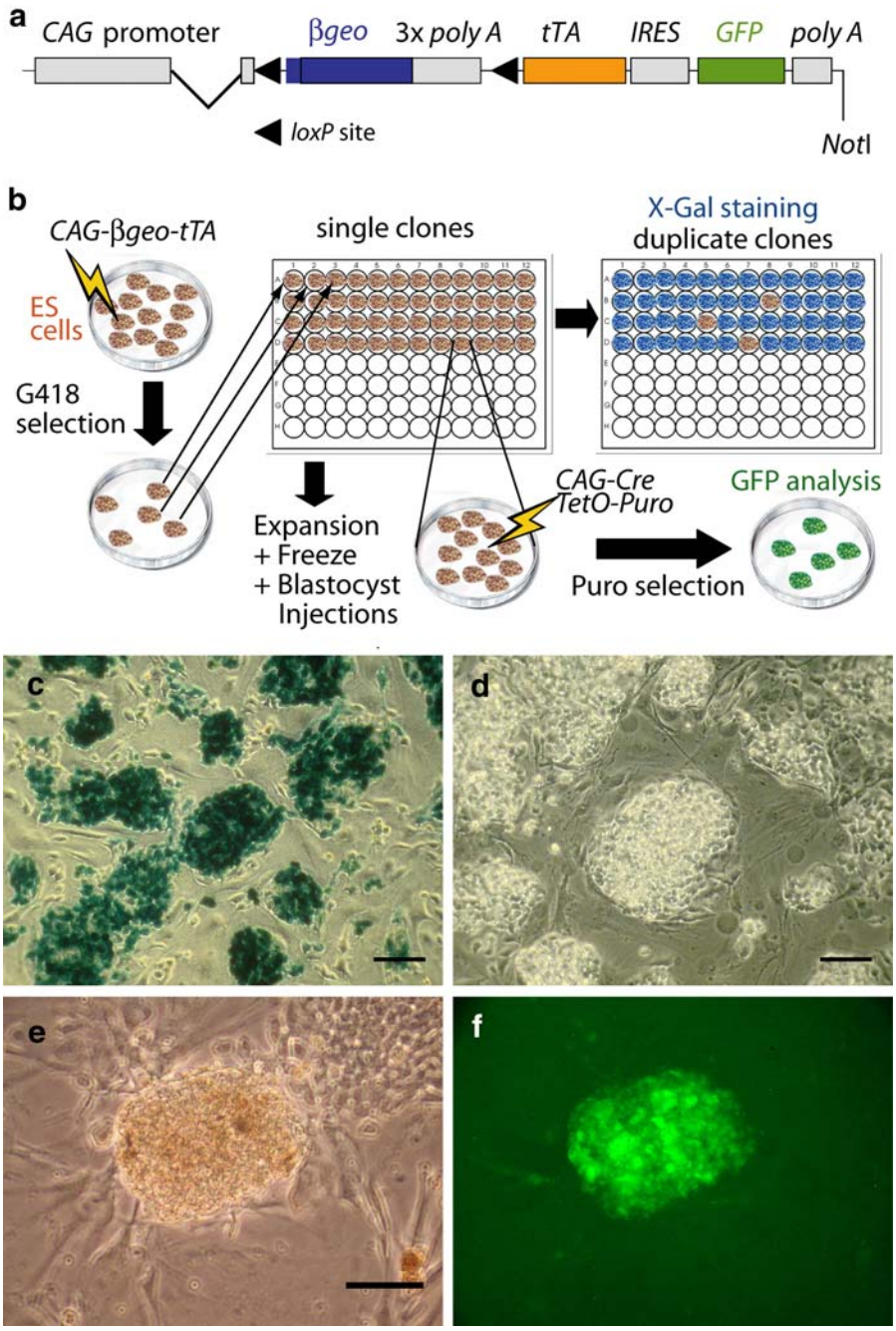


**Fig. 1** Construction of the CAG-$\beta$geo-tTA transgene and generation of transgenic embryonic stem (ES) cells. **a** Structural organization of the transgene. **b** Experimental design. **c**, **d** X-gal staining of G418-resistant, transgenic ES cells (**c**) and their unmodified controls (**d**). **e**, **f** Brightfield (**e**) and GFP fluorescence (**f**) images of a puromycin-resistant CAG-$\beta$geo-tTA transgenic ES cell clone, which expresses Cre recombinase (CAG-Cre) and a tTA-driven puromycin reporter construct (TetO-puro). *Bars* represent 100 μm

## Expression of the CAG-$\beta$geo-tTA transgene in differentiated cell types is integration site-dependent

Based on the uniformity of lacZ expression, we selected four ES cell clones for the production of chimeric mice. Although the injection of these ES cell lines into blastocysts generated a number of highly chimeric males per clone, only two of the four lines were germline competent (lines 2A5 and 2A11). Using Southern blot analysis, we confirmed that the resulting hemizygous progeny carried a single copy of the CAG-$\beta$geo-tTA transgene in both lines at distinctly different locations (not shown). Next, we performed an X-gal assay on embryos of both strains at mid-gestation to assess whether the CAG-$\beta$geo-

tTA transgene was ubiquitously expressed. Surprisingly, only the line 2A11 exhibited a widespread lacZ expression pattern (Fig. 2a). The transgene appeared to be completely silent in the 2A5 line, and a retinoic acid mediated ES cell differentiation assay revealed that the transgene was inactivated during very early steps of differentiation (data not shown). We therefore cryopreserved and excluded this line from further investigation. In contrast, the vast majority of germline-competent ES cells of line 2A11 retained high expression of the transgene in the differentiated state using retinoic acid (Fig. 2b). After injecting these ES cells into immunocompromised animals (Fig. 2c), we however noticed that the expression of the transgene was not uniform and absent in a subset of cells. Collectively, the results of this line of investigations clearly indicate that CAG-driven transgenes are not ubiquitously expressed and that, despite high activation in ES cells, their expression in adult tissues is integration site-dependent.

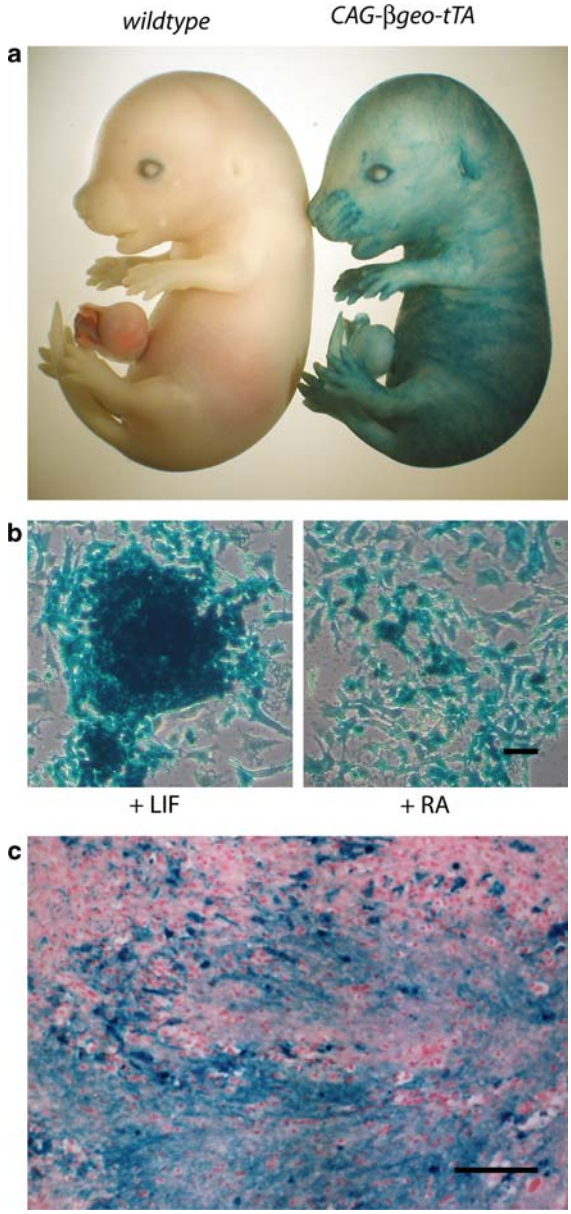


**Fig. 2** Widespread expression pattern of the CAG-*β*geo-tTA transgene in embryos and differentiated ES cells in vitro and in vivo. **a** X-gal staining of a transgenic embryo of line 2A11 and a wildtype littermate control. **b** CAG-driven expression of lacZ in ES cells of line 2A11 and their differentiated descendants following administration of retinoic acid (*RA*). *Bar* represents 50 μm. **c** X-gal staining on frozen sections of teratomas derived from this ES cell line. *Bar* represents 100 μm

## Strong, widespread but not uniform expression of the CAG-*β*geo-tTA transgene in preimplantation embryos and in tissues of adult mice

Since the CAG-*β*geo-tTA construct was highly expressed in ES cells in culture, we reasoned that the transgene might be active at all stages of development including preimplantation embryos. To verify this assumption, we collected blastocysts from super-ovulated wildtype females that were mated with CAG-*β*geo-tTA males. Approximately half of all preimplantation embryos were clearly X-gal-positive (Fig. 3a). After culturing blastocysts on feeder cells for three to 6 days, we observed that the majority but not all embryo-derived cells retained high levels of transgene activation (Fig. 3b). This finding suggests that, similar to our observation in teratomas, a mosaic expression pattern of the CAG-driven transgene appears to emerge early during normal embryonic development.

To determine the tissues-specific expression pattern of the CAG-*β*geo-tTA transgene in adult animals, we collected a panel of organs from five sexually mature male and female mice and a similar number of wildtype littermate control animals and performed X-gal staining on tissue fragments as well as frozen sections. As illustrated in Fig. 4, muscle, heart, and pancreatic tissues (panels A–C) exhibited a very strong expression of lacZ under regulation of the CAG promoter. In addition, the entire brain was X-gal positive in all transgenic mice with the most

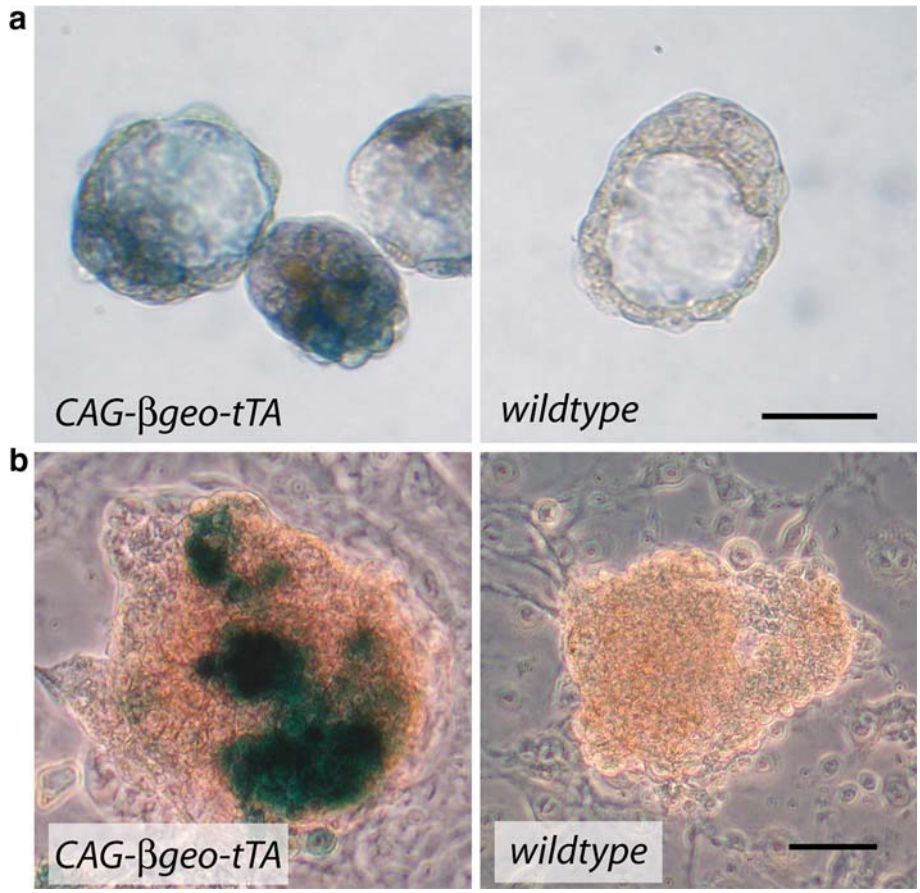


**Fig. 3** CAG-$\beta$geo-tTA transgene expression in preimplantation embryos. X-gal staining of blastocysts (**a**) as well as cultured embryos (**b**) from CAG-$\beta$geo-tTA transgenic line 2A11 and their wildtype controls. *Bars* represent 50 μm

extensive staining in the cerebellum (panel H). Despite very high transgene activation in these tissues with little variation among individual mice, we always observed some degree of mosaic expression pattern, in particular in the pancreas. As shown in panel B of Fig. 4, X-gal positive and negative cells with similar overall morphology were adjacent to each other within pancreatic acini. Besides cells of the exocrine pancreas, many islets of Langerhans also expressed lacZ (not shown). The expression of the CAG-$\beta$geo-tTA transgene in other tissues was more restricted to specific cell types. While spermatogonial stem cells appeared to be X-gal negative, most spermatids were intensively stained (panel D). X-gal positive cells were observed in the renal medulla and the bronchial epithelium of the lung (panels E and F). The activation of the CAG-driven transgene in the skin was particularly high in hair follicles and parts of the epidermis (panel G). Although the majority of hematopoietic cells in the thymus and spleen did not appear to express the CAG-$\beta$geo-tTA construct (panel I), many megakaryocytes were stained intensely in transgenic mice. Significant levels of transgene expression were never detected in the salivary gland, prostate, as well as the virgin and lactating mammary gland (not shown). The corresponding tissues from wildtype littermate controls did not exhibit detectable levels of endogenous $\beta$-galactosidase activity (not shown). In summary, based solely on the expression profile of lacZ, the newly generated CAG-$\beta$geo-tTA transgenic strain should be quite useful to target a strong, ligand-regulatable expression of responder genes to the brain, heart, muscle, and pancreas in adult animals. The applicability of this strain to activate transgenes in defined cell types of the kidney, lung, skin, and hematopoietic system

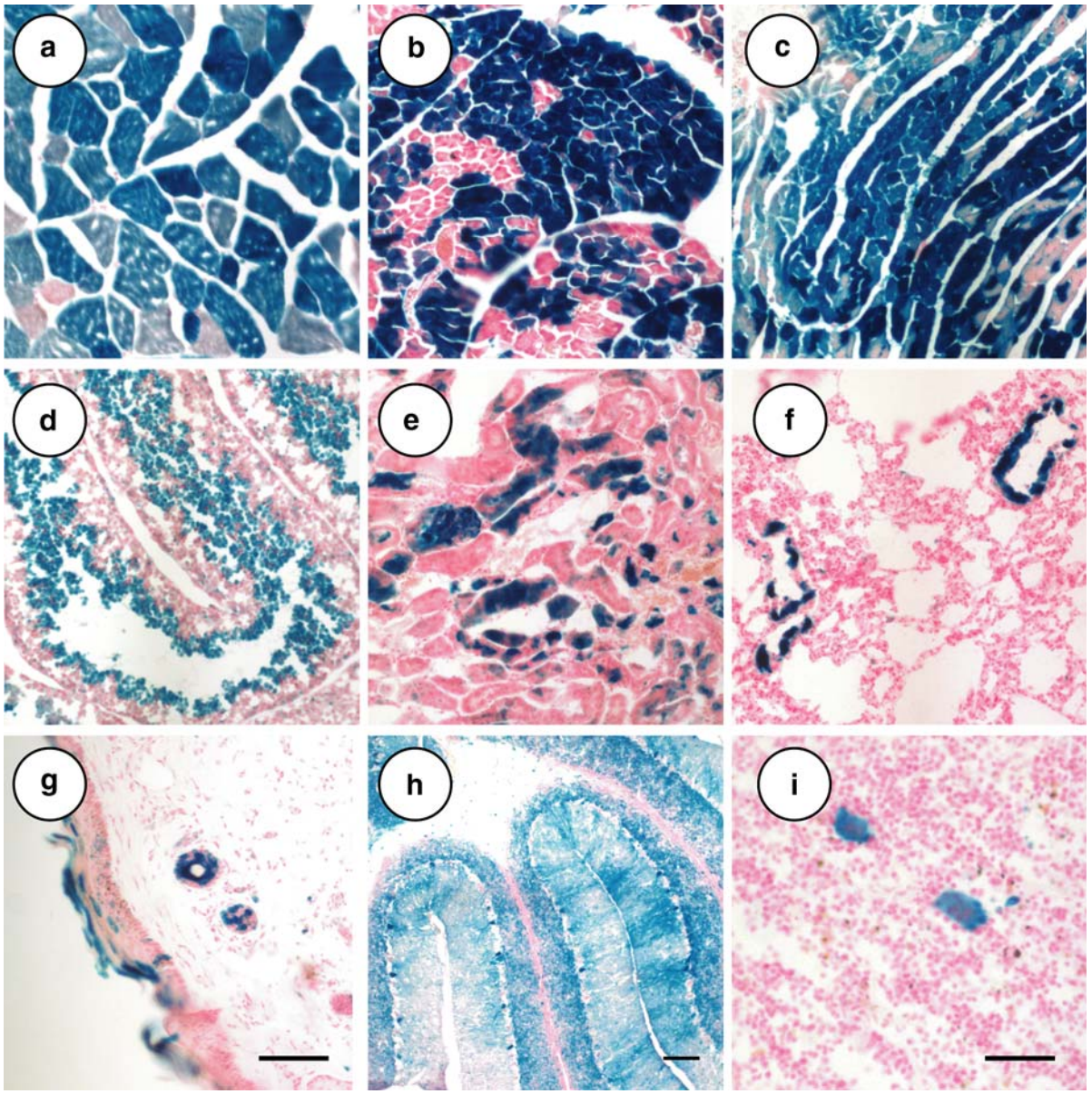


**Fig. 4** Expression pattern of the CAG-$\beta$geo-tTA transgene in tissues of adult mice. X-gal staining on frozen sections of **a** muscle, **b** pancreas, **c** heart, **d** testis, **e** kidney, **f** lung, **g** skin, **h** cerebellum, and **i**. spleen. *Bars* represent 100 μm (A–H) and 50 μm (**i**), respectively

depends on the availability of particular Cre lines that effectively activate the tTA in these adult tissues.

## Efficient doxycycline-controlled repression of tTA-induced target genes

We generated triple transgenic mice that carry the MMTV-Cre (line D) and a reporter gene (a firefly luciferase under the Tet operon; TetO-Luc) in addition to the CAG-$\beta$geo-tTA construct to assess the proper Cre-mediated activation of the tTA. For this initial study, we had chosen the MMTV-Cre strain, which was generated in our laboratory and examined extensively in combination with various floxed genes and reporter transgenes (Wagner et al. 2000, 2001, 2004). The MMTV-LTR-driven expression of the Cre

recombinase occurs in a mosaic expression pattern in a variety of tissues, in particular in secretory organs such as the salivary and mammary gland, the hematopoietic system, and the skin. It is also important to note that the MMTV-Cre transgene is active in some of these organs (e.g. skin) during embryogenesis. Among these MMTV-Cre target tissues, only the skin exhibited a strong expression of the CAG-$\beta$geo-tTA transgene. We therefore expected that the luciferase reporter gene should be easily detectable throughout the entire surface area of MMTV-Cre, CAG-$\beta$geo-tTA, TetO-Luc triple transgenic embryos and adult mice. The expression of the tTA-induced luciferase reporter gene was analyzed in these animals and their double transgenic littermate controls (CAG-$\beta$geo-tTA, TetO-Luc) using in vivo bioluminescence imaging (IVIS200). Initially, we examined the activation of the reporter transgene in embryos at day 12.5 of gestation from a CAG-$\beta$geo-tTA, TetO-Luc double transgenic female that was mated with a MMTV-Cre male. Expression of luciferase was only detected in MMTV-Cre, CAG-$\beta$geo-tTA, TetO-Luc triple transgenic embryos (Fig. 5a). This initial experiment demonstrated that the CAG promoter-driven expression of the tTA is strictly dependent on the presence of Cre recombinase. The un-activated CAG-$\beta$geo-tTA construct did not exhibit any measurable levels of background activity. Next, we wanted to determine whether the tTA-induced reporter gene can be efficiently repressed through administration of doxycycline (Dox). As shown in the left panel of Fig. 5b, the reporter transgene was strongly activated in triple transgenic adult mice whereas luciferase activity was completely absent in animals that did not possess a Cre transgene. These animals were given Dox in their drinking water for 48 h and examined again using the bioluminescence imaging tool (Fig. 5b, middle panel). The results of this experiment revealed that the expression of the transactivator–driven reporter transgene was effectively and completely silenced in response to a short-term administration of the ligand. Subsequently, doxycycline was withdrawn from these animals to assess the re-expression of the tTA-induced luciferase reporter. It is evident from the results shown in the right panel of Fig. 5b, that the tTA-mediated transactivation of the luciferase reporter had resumed after 2 weeks of ligand withdrawal, but it had not yet reached the expression level equivalent to prior treatment with Dox (Fig. 5b, left panel). Subsequently, we analyzed more than six MMTV-Cre, CAG-$\beta$geo-tTA, TetO-Luc triple transgenic mice of both genders prior to treatment with Dox. We observed some degree

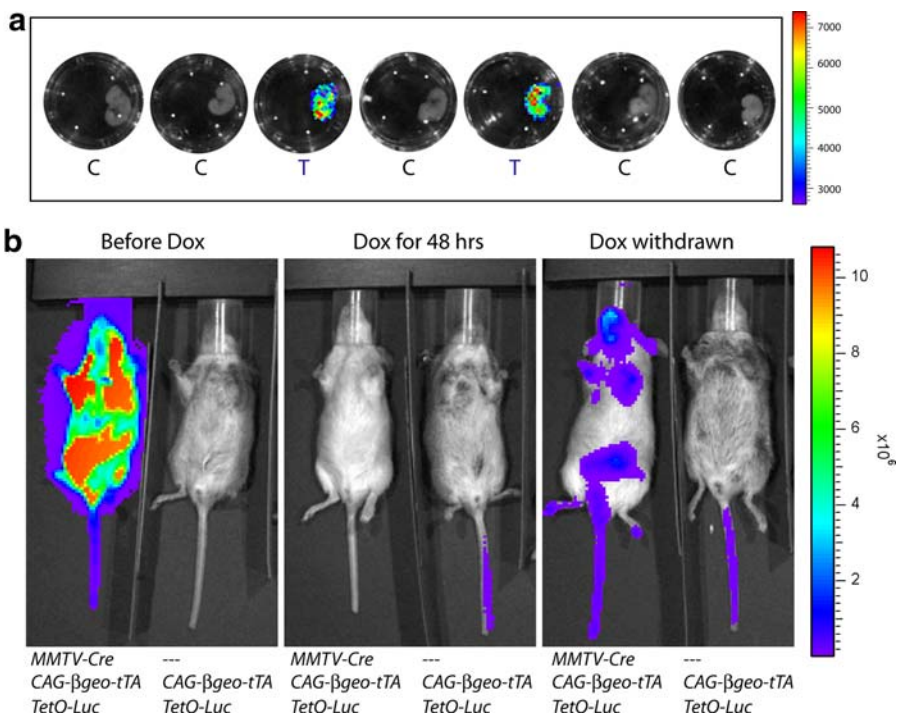


**Fig. 5** Effective doxycycline-controlled repression of CAG-tTA-induced target genes. **a** Bioluminescence imaging (IVIS200, Caliper Life Sciences) of MMTV-Cre, CAG-$\beta$geo-tTA, TetO-Luc triple transgenic embryos (T) and their littermate controls (C). **b** In vivo imaging of a triple transgenic mouse and a double transgenic littermate control lacking Cre recombinase. Both mice were injected with luciferin and imaged prior to and during doxycycline (Dox) administration for 48 h (*left* and *center*) as well as 2 weeks after Dox withdrawal (*right*)

of variation in luciferase expression among these untreated animals, which is expected since the MMTV-Cre line D exhibits a mosaic expression pattern in the skin. More importantly, differences in the coat color (i.e. white vs. yellow, agouti, and black) have a significant impact on the emission of photons. Expression of luciferase, however, was never detected in mice treated with doxycycline.

In summary, the in vivo bioluminescence study of triple transgenic mice and their controls demonstrated clearly that the expression of a tTA-driven reporter transgene can be efficiently activated in specific tissues using Cre recombinase in the absence of any ligands. The tTA-mediated transactivation of Tet operator-driven responder genes can be rapidly and completely blocked through administration of doxycycline, and withdrawing the ligand lifts the transcriptional block from the Tet-regulatible promoter.

## Discussion

Using ES cell-based transgenesis, we generated a CAG-$\beta$geo-tTA transgenic strain that allows a temporally and spatially controlled expression of transgenes in multiple adult tissues. The cell type-specific excision of the $\beta$geo marker from the transgene using Cre recombinase leads to the expression of the tTA (Tet-OFF) transactivator protein, which, in turn, mediates the activation of responder genes driven by the Tet-regulatible operon. Unlike strains that express the rtTA (Tet-ON) under other ubiquitously active regulatory elements such as the endogenous *Rosa26* locus (Yu et al. 2005; Belteki et al. 2005), this novel tTA-based strain does not require the administration of doxycycline for the transactivation of responder genes. Besides reducing experiment-related expenses for the antibiotic and the need to frequently replace the drug in the drinking water, this feature is advantageous in experimental settings that require a sustained expression of a transgene over a long period, for example studies that examine tumorigenic properties of weak oncogenes in sporadic cancer models.

The expression of the randomly integrated CAG-$\beta$geo-tTA construct is widespread but not ubiquitous. The pattern of expression among tissues of an adult animal is quite similar to that reported for the CAG-$\beta$geo-hPLAP transgene (Lobe et al. 1999). It should be noted that the expression of the tTA and its effector genes can be very strong in multiple tissues depending on the activity of Cre recombinase. For example, using the MMTV-Cre line A, which is more uniformly expressed in the skin (Wagner et al. 2001), we achieved a more uniform activation of the tTA (data not shown), and the expression level of the luciferase reporter was 60–100 times higher than the one shown in the left panel of Fig. 5b. We never observed such a high transactivation of the same responder gene using Rosa26-rtTA knockin mice in which the reverse transactivator was ubiquitously expressed following Cre-mediated recombination in the female germline. Despite ubiquitous activation of the Rosa26-rtTA and ad libitum administration of Dox, the ligand-inducible transactivation varied among animals depending on the route of administration (food, water) and the effective amount of Dox that had been consumed. Based on these observations, we propose that the CAG-$\beta$geo-tTA strain might be beneficial in studies that require a very high activation of transgenes in selected adult tissues. Moreover, since the CAG-$\beta$geo-tTA transgene is active in preimplantation embryos (Fig. 3) and an expression of a tTA-responder gene could be activated during embryogenesis (Fig. 5a), this transactivator strain might be useful for studying the effects of a deregulated expression of genes during early stages of development and differentiation.

Despite the rapid and complete suppression of responder gene expression upon administration of Dox, we noticed that it took several weeks after withdrawing the ligand until the tTA-mediated transactivation was completely restored. This kinetics, which is very different from the quick activation of reporter genes in mice expressing the reverse transactivator, for example the WAP-rtTA (Creamer et al. 2009), seems not to be unique for the CAG-$\beta$geo-tTA transgene. This phenomenon was also observed in all other tissue-specific tTA strains that we examined over the past 4 years in our laboratory such as CaMKIIa-tTA (Mayford et al. 1996) and MMTV-tTA (Hennighausen et al. 1995) transgenic mice (Zhang, Lin, Creamer, and Wagner, unpublished). Consequently, strains expressing the Tet-OFF transactivator might be less suitable for a swift upregulation of responder genes after withdrawal of Dox. Although tTA lines are superior for mediating a sustained expression of transgenes, this limitation

should be considered in experimental designs that require a re-activation of transgenes from the repressed state.

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