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Cre-mediated recombination in pituitary somatotropes

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Abstract

We report a transgenic line with highly penetrant cre recombinase activity in the somatotrope cells of the anterior pituitary gland. Expression of the cre transgene is under the control of the locus control region of the human growth hormone gene cluster and the rat growth hormone promoter. Cre recombinase activity was assessed with two different lacZ reporter genes that require excision of a floxed stop sequence for expression: a chick β-actin promoter with the CMV enhancer transgene and a ROSA26 knock-in. Cre activity is detectable in the developing pituitary after initiation of *Gh* transcription and persists through adulthood with high penetrance in *Gh* expressing cells and lower penetrance in lactotropes, a cell type that shares a common origin with somatotropes. This Gh-cre transgenic line is suitable for efficient, cell-specific deletion of floxed regions of genomic DNA in differentiated somatotropes and a subset of lactotrope cells of the anterior pituitary gland.

Keywords

anterior pituitary gland; cell specific expression; transgene; growth hormone; prolactin

Introduction

The pituitary gland is the central endocrine organ in vertebrates. Through communication with the hypothalamus, the pituitary regulates a wide range of functions including growth, fertility, lactation, stress response, homeostasis and metabolism. Disruption in normal pituitary gland function may result in failed expansion of pituitary cell types, hormone deficiency, pituitary dwarfism, or pituitary tumors (Cushman and Camper, 2001). Somatotropes are cells responsible for growth hormone (GH) production in the pituitary gland. GH is a key component of the somatotropic axis, stimulating the production of insulin-like growth factor-I (IGF-I) in the liver, which in turn acts on numerous target organs to regulate growth and a variety of other physiological processes including hearing, vision, and glucose homeostasis (Chandrashekar *et al*., 2007; Schneider *et al*., 2003; Walenkamp and Wit, 2007).

Generation of pituitary cell-specific cre transgenic lines has allowed for the study of particular pituitary cell functions and cell lineage relationships (Bingham *et al*., 2006; Charles *et al*., In press; Charles *et al*., 2006; Cushman *et al*., 2000; Jorgez *et al*., 2006; Luque *et al*., 2007; Naik *et al*., 2006; Yin *et al*., 2008). We sought to generate a tool that would be useful for studying the development and function of somatotropes, which represent approximately 40% of the cell population of the adult anterior lobe. Cell specific gene targeting is invaluable for studying the function of genes that have pleiotropic effects on development, function, or carcinogenesis of vital organs causing embryonic lethality or complications due to multi-organ effects. For example, a systemic knockout of the growth hormone receptor (*Ghr)* produces cardiac and

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pulmonary phenotypes, precluding assessment of functions in other organs (Beyea *et al.*, 2006; Egecioglu *et al.*, 2007). Here we present a transgenic cre line, Tg(*Gh-cre*)^{SAC1}, which expresses cre recombinase in pituitary somatotropes, with limited expression in lactotropes, and no detectable expression in other pituitary cell types. We predict this to be a useful tool for targeted gene ablation in committed somatotropes, and therefore a valuable resource for the scientific community.

The *Gh-cre* transgenic construct contains the coding sequences for a nuclear localized cre, 1.6 kb of the human GH locus control region (LCR), 1.77 kb of rat GH promoter, and β-actin polyadenylation sequences (Fig. 1a). The rat GH promoter has produced strong expression in transgenic mice (Akita *et al.*, 1997;Behringer *et al.*, 1988;Burton *et al.*, 1991;Lira *et al.*, 1988;Lira *et al.*, 1993). The hGH LCR fragment (−14.6 kb to −16.2 kb relative to the hGH promoter) contains two pituitary-specific DNase I-hypersensitive sites and is sufficient for restricting expression to somatotropes and somatolactotropes, for appropriately timed induction of hGH transgene expression in mice starting at embryonic day e15.5–e16.5, and for selective extinction of hGH in mature lactotropes (Bennani-Baiti *et al.*, 1998). The human GH LCR improves the penetrance of rat GH promoter-driven transgene expression (Behringer *et al.*, 1988;Bennani-Baiti *et al.*, 1998;Jin *et al.*, 1999;Jones *et al.*, 1995;Nasonkin, 2002).

Twelve transgenic founder mice were initially surveyed for transgene expression by mating with the chick β-actin-LacZ cre reporter strain, known as Tg(flox-lacZ)^{J7Sac} (Cushman *et al.*, 2000), and analysis of X-gal staining in doubly transgenic progeny (Nasonkin, 2002). Five of those lines exhibited robust staining in the adult pituitary gland, and the best line, Tg(*Gh-cre*)^{SAC1}, was chosen for detailed analysis with two cre reporter strains: chick β-actin-LacZ and Rosa26-LacZ, officially known as B6;129S4-*Gt(ROSA)26Sor*^{tm1Sor}/J (Soriano, 1999).

Strong X-gal staining is evident in the anterior lobe of pituitaries from mice carrying both the transgene Tg(*Gh-cre*)^{SAC1} and the Rosa26-LacZ reporter allele, and there is no significant background activity in mice with the Rosa-LacZ allele but no cre transgene (Figure 1b). The cell-specificity of cre-excision was assessed in mice carrying Tg(*Gh-cre*)^{SAC1} and the chick β-actin-LacZ cre reporter by immunohistochemical staining for pituitary hormones and X-gal staining. Nearly all X-gal stained cells also immunostained for GH, and the majority of GH cells were X-gal stained (Figure 1d). Despite this 1:1 correspondence between X-gal staining and GH immunostaining, a subset of the prolactin (PRL) expressing cells stain for X-gal (Figure 1e). These PRL, X-gal double positive cells are likely to represent somatomammotropes, which express both PRL and GH (Frawley and Boockfor, 1991).

To facilitate comparison of the Tg(*Gh-cre*)^{SAC1} transgene with other cre strains, an analysis of cell specificity and developmental activation was performed using the popular Rosa26-LacZ allele. X-gal staining was observed in the majority of GH immunopositive cells and a subset of the PRL immunopositive cells (Figure 2a, b). Quantitation of cre-mediated excision revealed 61 ± 11% of the somatotropes are X-gal positive and 9 ± 5% of the lactotropes. These results are similar to those obtained with the chick β-actin-LacZ reporter. X-gal stained cells expressing the Rosa26-LacZ reporter have a very small blue precipitate in a restricted cytosolic region, while the entire nucleus turns blue using the chick β-actin-LacZ reporter. This could result in underestimated penetrance with Rosa26-LacZ. No cre-mediated excision is observed in adult thyrotropes, gonadotropes, or corticotropes (Figure 2c–e, respectively). Although it is not uncommon for transgenes to exhibit variable activity, even within the same line, the majority of Tg(Gh-cre)^{SAC1};Rosa-LacZ double transgenic mice exhibited similar X-gal staining in the pituitary gland (17/18), suggesting that Tg(*Gh-cre*)^{SAC1} is a reliable resource for deletion in somatotropes. *Gh* expression is normally detected in the mouse pituitary by embryonic day 15.5 (e15.5) (Japon *et al.*, 1994). Tg(*Gh-cre*)^{SAC1} transgene activity is not

detectable at e14.5 (Fig. 2f), but it becomes readily apparent between e15.5 and e18.5 (Fig. 2g).

Recombination in non-pituitary tissues was assessed in Tg(*Gh-cre*)^{SAC1}, Rosa-LacZ reporter mice. X-gal staining was observed in the skin surrounding the forearm of e16.5-e17.5 embryos. Low level X-gal staining is detected in the kidney, ovary and testis of Tg(*Gh-cre*)^{SAC1};Rosa-LacZ mice (Figure 3). The recombination in nonpituitary tissues may not represent ectopic expression because *GH* is expressed human skin, and *Gh* transcripts are detectable in the brain and testis of wild type mice (Slominski *et al.*, 2000), (http://www.informatics.jax.org/searches/reference.cgi?46734). No X-gal staining was observed in the adult liver, skeletal muscle, adipose tissue, lung, spleen, or heart, and in one occurrence minimal staining was observed in pancreas (data not shown). No X-gal staining was seen in the placentas of double transgenic embryos at e16.5. In addition, we detected no evidence of leaky cre activity in the germline (data not shown).

Mouse strains that confer gene deletion in somatotropes have been developed using the promoter from the rat GH releasing hormone receptor (*Ghrhr*) (Yin *et al.*, 2008) and the rat *Gh* promoter, similar to our strategy, except without the human growth hormone locus control region (Luque *et al.*, 2007). In contrast to the *Ghrhr-cre* strain, we find no evidence of cre-activity in thyrotropes. The Tg(*Gh-cre*)^{SAC1} strain is the first to be fully characterized for ectopic excision activity during development and in adult tissues. Thus, the thorough histological information we provide using a well-characterized cre-reporter gene is an advantage of the Tg(*Gh-cre*)^{SAC1} strain.

We expect that these transgenic mice will have many uses. Permanently marked somatotropes could be dispersed and sorted for gene expression profiling (Muzumdar *et al.*, 2007) or used in intact tissue to study the role of the interconnected cellular network in producing sex-specific pulsatile patterns of GH secretion (Bonnefont *et al.*, 2005). Another potentially intriguing application would be somatotrope-specific deletion of *Ghr* to discover the role of GH feedback on the anterior pituitary, given the many metabolic effects of systemic *Ghr* deletion on skeletal and cardiac muscle function, adiposity, liver function, carcinogenesis and aging (reviewed in (Clark *et al.*, 2006).

METHODS

Generation of Transgene Construct

The *Gh-cre* transgene construct was generated by PCR amplification of 1.77 kb of sequence from the rat GH (rGH) promoter (accession #X12967) using primers F 5′-GGGTACCTCTAGAAGCTTAGTTTCTAGTAGG and R, 5′-CCTGAGCAGTTTGGAATCTGG. The PCR product was amplified using the Expand High Fidelity polymerase mix (Roche, Indianapolis, IN) under the following conditions: 95°C for 2 minutes, followed by 5 cycles of 94°C for 45 seconds, 58°C for 1 minute and 72°C for 2 minutes, then 25 cycles of 94°C for 45 seconds, 61°C for 1 minute and 72°C for 2 minutes, and a final 10 minute extension at 72°C. The rGH promoter was cloned upstream of the cassette containing the nuclear localized Cre and βactin-polyadenylation sequences, in the pML78 plasmid at the XhoI site (Meyers *et al.*, 1998). To minimize the possibility of position effect variegation (PEV) of the transgene insertion site, a 1.6 kb BglII fragment of sequence from the human growth hormone (hGH) locus control region (LCR) was cloned upstream of the 1.77 kb rGH promoter (Bennani-Baiti *et al.*, 1998; Jin *et al.*, 1999; Jones *et al.*, 1995). hGH LCR sequences (accession # AF039413) were obtained by PCR amplification of human DNA using hGH LCR primers F, 5 ′-CGGGGTACCTCTAGAGATCTTGTCTCAGAAAAACCC, (KpnI and XbaI cloning sites underlined) and R, 5′-GGGGTACCTCTAGAGATCTTGGCCTAGGCCTCG with Expand High Fidelity

polymerase under the following conditions: 95°C for 3 minutes, followed by 5 cycles of 94° C for 45 seconds, 60°C for 1 minute and 72°C for 2 minutes, then 25 cycles of 94°C for 40 seconds, 65°C for 1 minute and 72°C for 1 minute 45 seconds, and a final extension at 72°C for 10 minutes.

Generation, Characterization and Maintenance of Mice

The GH-cre transgene was injected into (C57BL/6J × SJL/J) $F_1 \times F_1$ zygotes, and the fertilized eggs were then transferred into pseudopregnant foster mothers. Transgene positive progeny were genotyped using PCR amplification of genomic DNA for the presence of cre using the following primers: 5′-GCATAACCAGTGAAACAGCATTGCTG-3′ and 5′-GGACATGTTCAGGGATCGCCAGGCG-3′, under the following conditions: 94°C for 3 minutes, followed by 32 cycles of 94°C for 30 seconds, 60°C for 60 seconds and 72°C for 90 seconds, and a final 10 minute extension at 72°C.

To determine the number of copies of the transgene inserted into the genome, a Southern analysis was performed using 10μg of KpnI digested genomic tail DNA. The copy number of $Tg(GH\text{-}cre)^{SAC1}$ was estimated at 30–50 copies per haploid genome (data not shown).

Dissected pituitaries from adult progeny were assayed for X-gal activity, as previously described (Brinkmeier *et al.*, 1998). The pituitaries of the doubly transgenic progeny (n=8) were stained for X-gal activity as previously described (Cushman *et al.*, 2000). Tissues were embedded in paraffin and sectioned at 5μm thickness. Immunohistochemistry was performed for each of the pituitary hormones as previously described (Kendall *et al.*, 1991).

Quantitation of cre-mediated excision in somatotropes and lactotropes was accomplished using three doubly transgenic mice positive for Tg(GH-cre)SAC1 and the Rosa26-LacZ reporter. Three separate regions of the pituitary were photographed after histochemical staining for PRL or GH and X-gal. The average percentage of cells positive for GH and X-gal was calculated by dividing the total number of double positive cells by the total number of GH positive cells. The same calculations were done for PRL.

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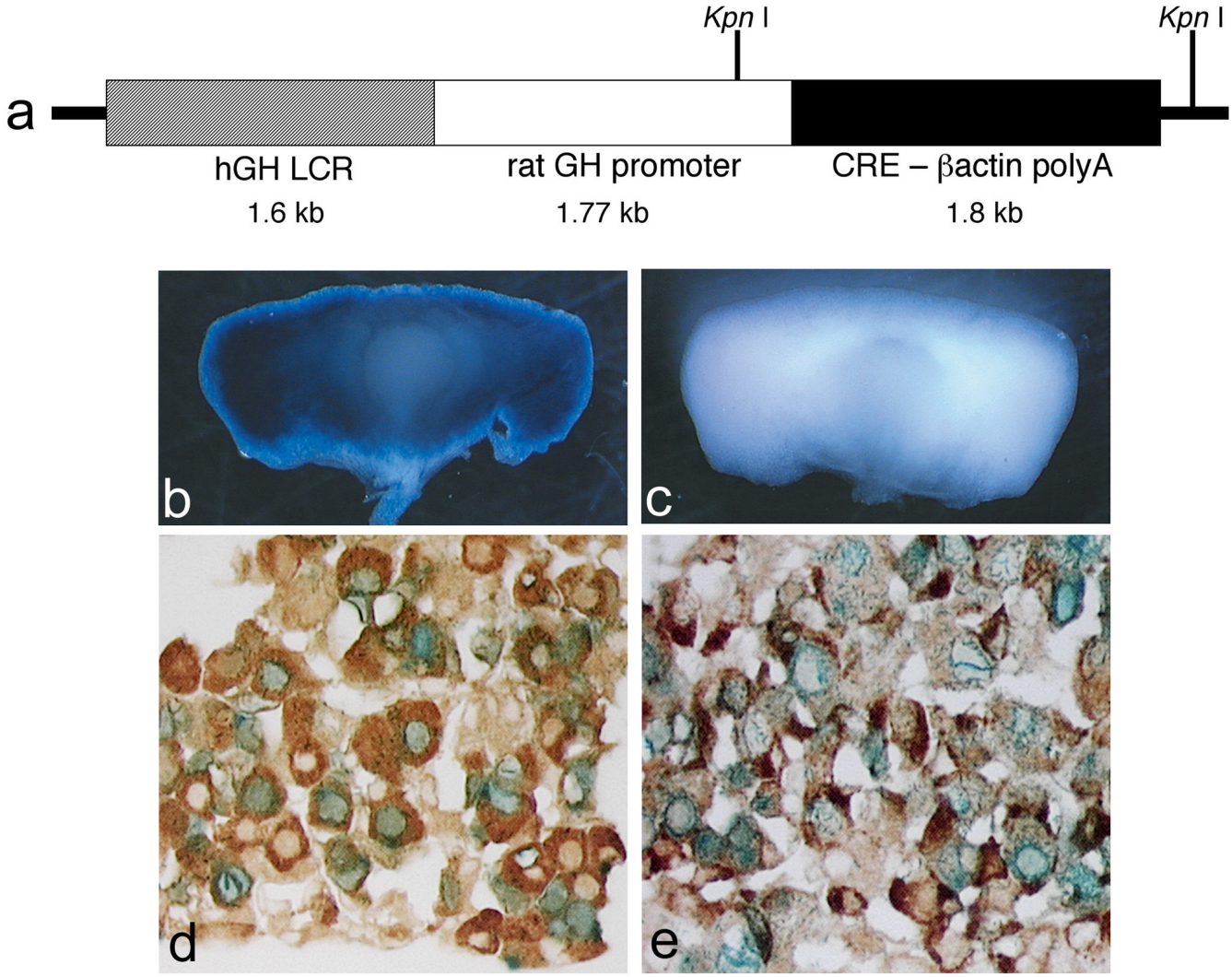

Figure 1. Structure and function of Tg(*Gh-cre*)^{SAC1} transgene

A total of 1.6 kb of the human growth hormone locus control region was cloned upstream of a 1.77 kb fragment containing the rat growth hormone promoter, the cre recombinase coding sequences, and the beta-globin poly adenylation and terminator sequences (a). Pituitaries of adult mice doubly transgenic for Tg (*Gh-cre*)^{SAC1} and the Rosa26 floxed stop lacZ reporter mouse strain stained blue with X-gal indicating cre-mediated recombination (b), while cre transgene negative mice carrying the reporter have no X-gal staining (c). Tg(*Gh-cre*)^{SAC1} mice were mated to the cβ-actin-CMV-floxed stop NLS-LacZ reporter and tissues from doubly transgenic progeny stained with X-gal (Cushman *et al.*, 2000). Nuclear localized blue staining, evidence of cre activity, is detected in cells immunostained for GH (d) and PRL (e). Black arrows identify hormone-positive cells with cre activity, and white arrows identify hormone-positive cells without cre activity.

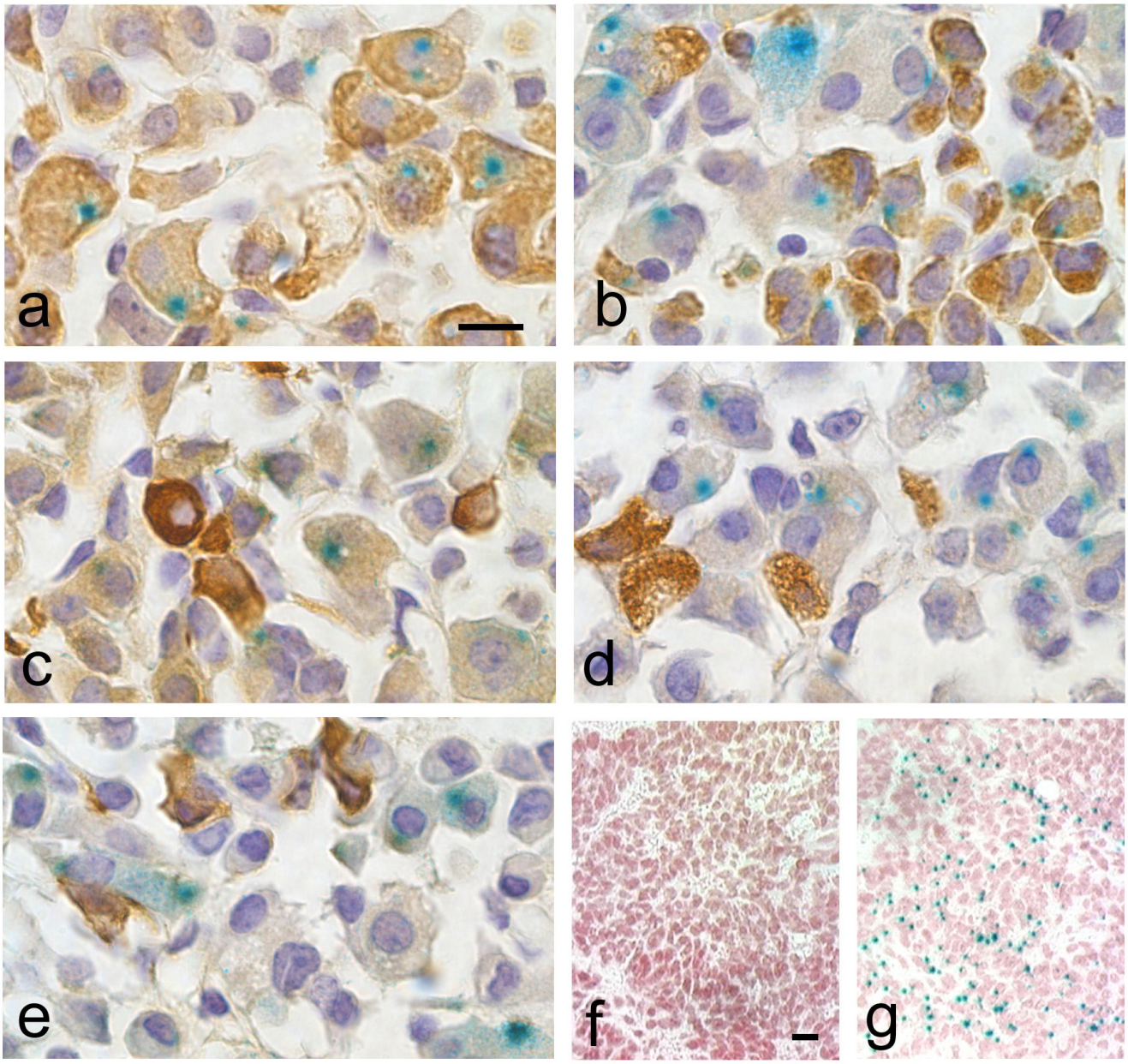

Figure 2. Tg(*Gh-cre*)^{SAC1} is specific for the somatotrope, lactotrope lineages

Adult pituitaries from mice doubly transgenic for Tg(*Gh-cre*)^{SAC1} and the Rosa26 lacZ reporter strain were stained with X-gal and antibodies specific for GH (a), prolactin (b), TSHβ (c), LHβ (d), or POMC (e), developed with DAB to produce a brown color, and photographed at 100x oil immersion magnification. Although doubly transgenic embryos stained for X-gal show no blue staining at e14.5 (f), it is detected at e15.5 (g) when endogenous growth hormone expression begins. Magnification bars (a, f) represent 10μm (a–e, f–g, respectively).

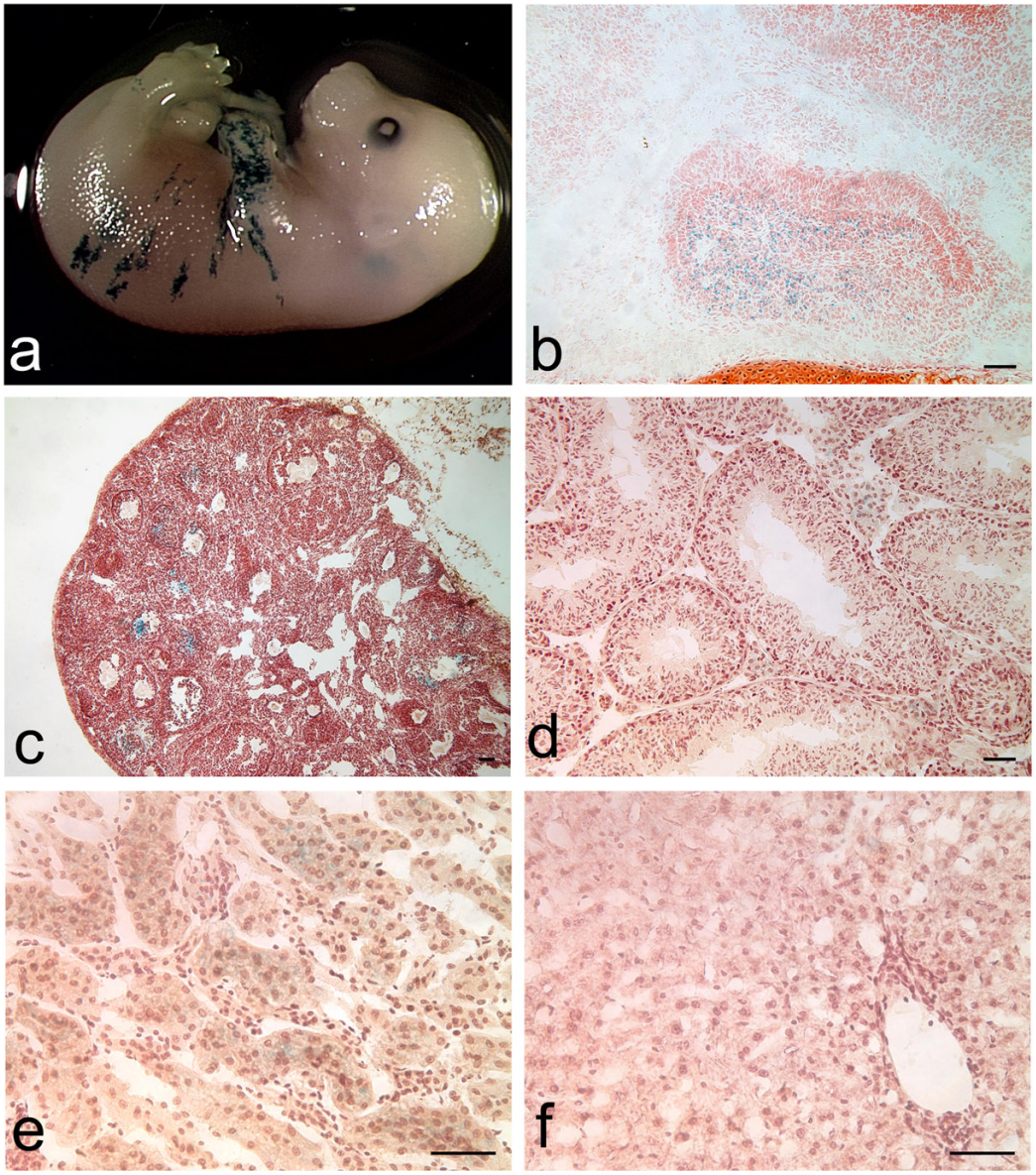

Figure 3. Minimal ectopic Tg(*Gh-cre*)^{SAC1} transgene expression

Very limited X-gal staining is detected in non-pituitary tissues such as the skin surrounding the limbs and trunk of developing embryos (a). At e18.5, X-gal staining is appropriately restricted to the developing anterior lobe (A) and is absent from the intermediate (I) and posterior (P) lobes (b). In adult tissues, X-gal staining is seen in the developing follicles of some ovaries (c). Sporadic but very faint X-gal staining appears in the Leydig cells of the testes (d) and in the renal cortex (e). No staining is detected in the adult liver (f). All magnification bars represent 50μm.