genesis 46:507–514 (2008)

TECHNOLOGY REPORT

Pitx2 Deletion in Pituitary Gonadotropes is Compatible With Gonadal Development, Puberty, and Fertility

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Received 28 November 2007; Revised 11 April 2008; Accepted 20 April 2008

Summary: This report introduces a gonadotrope-specific cre transgenic mouse capable of ablating floxed genes in mature pituitary gonadotropes. Initial analysis of this transgenic line, *Tg(Lhb-cre)1Sac*, reveals that expression is limited to the pituitary cells that produce luteinizing hormone beta, beginning appropriately at e17.5. Cre activity is detectable by a reporter gene in nearly every LHβ-producing cell, but the remaining hormone-producing cell types and other organs exhibit little to no activity. We used the *Tg(Lhb-cre)1Sac* strain to assess the role *Pitx2* in gonadotrope function. The gonadotrope-specific *Pitx2* knockout mice exhibit normal expression of LHβ, sexual maturation, and fertility, suggesting that *Pitx2* is not required for gonadotrope maintenance or for regulated production of gonadotropins. genesis 46:507–514, 2008. **© 2008 Wiley-Liss, Inc.**

Key words: Cre recombinase; homeobox gene; luteinizing hormone beta subunit; transgenic mice; floxed allele; reproduction; conditional knockout

Gonadotropes are one of the five mature hormone-producing cell types of the anterior pituitary gland. Upon release of GnRH from the hypothalamus, gonadotropes secrete heterodimeric hormones composed of a β-subunit of luteinizing hormone (LHβ) and follicle-stimulating hormone (FSHβ), in combination with a common α-subunit (CGA). These glycoprotein hormones act on the gonads to promote sexual maturity and fertility (Kendall *et al*., 1995). Transgene ablation of gonadotropes results in near-elimination of the gonadotrope population, hypogonadism, infertility, reduced circulating LH, and reduced levels of PRL (Kendall *et al*., 1991; Seuntjens *et al*., 1999).

Identifying the factors that regulate gonadotrope differentiation is a critical step toward understanding fertility at the molecular level. Transcription factors and signaling pathways important for gonadotrope specification and function include *Gata2, Pitx2, Nr5a1* (SF1), *Egr1, Inhibin, ActRII*, and *ActivRI* (Burns and Matzuk, 2002; Charles *et al*., 2005, 2006; Kumar *et al*., 2003; Suh *et al*., 2002; Vesper *et al*., 2006; Yoshikawa *et al*., 2000). Examining the roles of some individual genes can be difficult because they are important in many physiological systems essential for embryonic development.

To overcome this problem, *cre* transgenic lines have been developed with activity in the pituitary gland such as *Cga-cre*, *Nr5a1-cre*, and a tetracycline inducible *Cga-cre*. The *Cga-cre* transgene targets anterior pituitary cells effectively, although it also exhibits activity in muscle (Charles *et al*., 2006; Cushman *et al*., 2000; Zhao *et al*., 2001). *Nr5a1-cre* drives *cre* expression in pre-gonadotropes early in development of the anterior pituitary. *Nr5a1* is also expressed in the gonads, adrenal cortex, spleen, and hypothalamus, making it difficult to distinguish between the loss of gene function in gonadotropes and the other steroidogenic tissues (Bingham *et al*., 2006). A transgenic line with tetracycline-inducible *Cga-cre* activity is effective for gonadotrope-specific excision, but the requirement for drug administration is not compatible with all applications (Naik *et al*., 2006).

Pitx2 is expressed in the developing and adult anterior pituitary gland (Charles *et al*., 2005; Gage and Camper, 1997; Semina *et al*., 1996). Analysis of *Pitx2* null mice established the role of *Pitx2* in the development of many organs including the pituitary gland, but mutants die at e14.5 due to severe heart defects (Gage *et al*., 1999a; Kitamura *et al*., 1999; Lin *et al*., 1999; Lu *et al*., 1999). To study the role of *Pitx2* in later pituitary development, a hypomorphic allele ($Pitx2^{neo}$) was generated. Mice homozygous for the hypomorphic allele live until postnatal day 1 (P1) allowing for pituitary cell specification studies, (Gage *et al*., 1999a; Suh *et al*., 2002). $Pitx2^{neo/neo}$ pituitaries lack gonadotropes and have a decrease in somatotropes and thyrotropes. There is little or no detectable expression of important gonadotrope transcription factors *Gata2, Egr1*, and *Nr5a1* in the hypomorphic pituitaries, demonstrating that the dosage of *Pitx2* is critical for the differentiation of the gonadotropes (Suh *et al*., 2002).

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Published online 18 September 2008 in
Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/dvg.20398

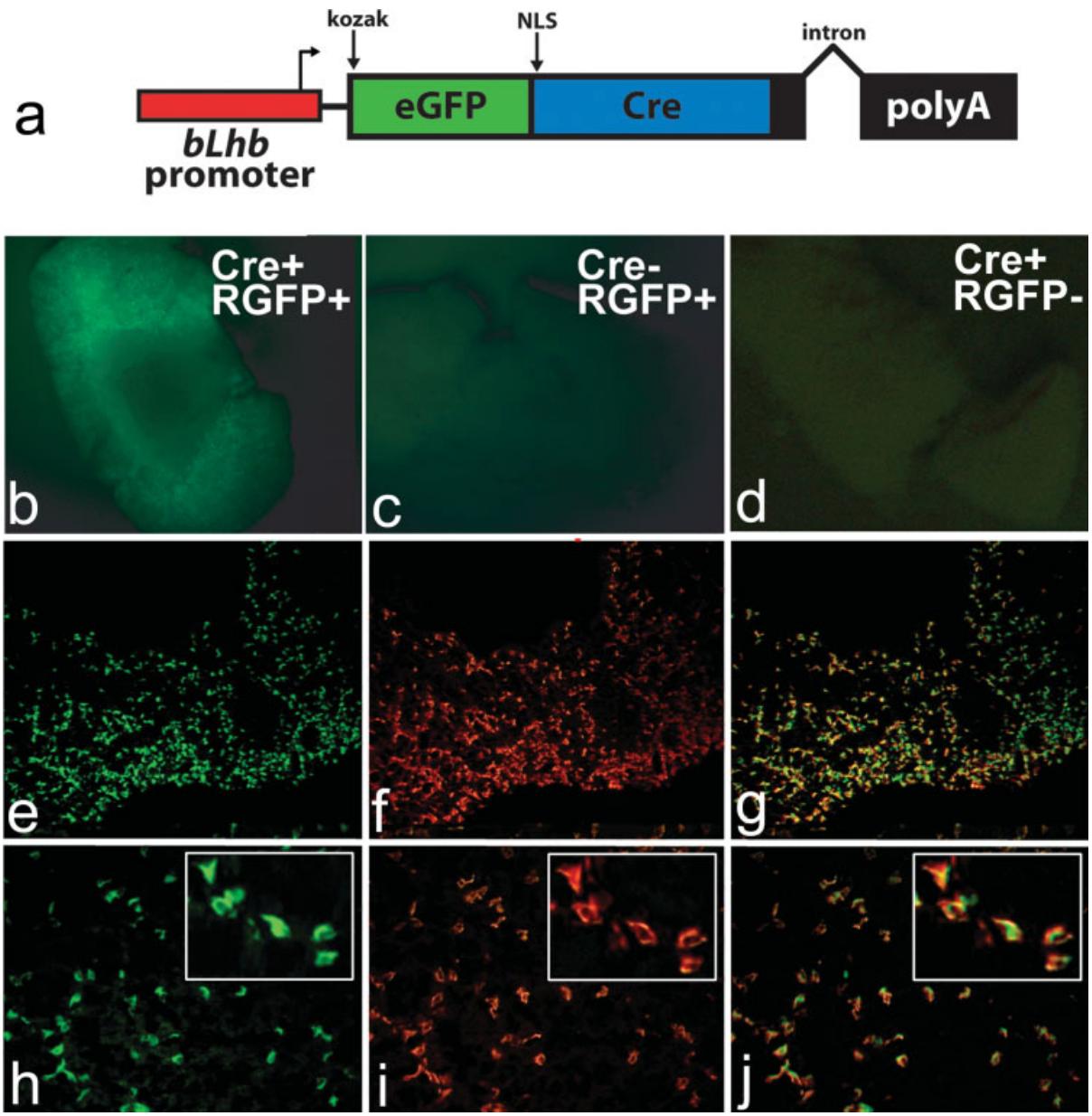

FIG. 1. Gonadotrope-specific *cre* transgenic construct and expression. The *Tg(Lhb-cre)1Sac* transgene (**a**) is controlled by the bovine *Lhb* promoter, containing a KOZAK translation initiation site, *eGFP* and *cre* recombinase fusion gene with a nuclear localization signal (NLS), and a poly adenylation signal and intron from the rabbit β-globin gene. Pituitaries from mice with the *Tg(Lhb-cre)1Sac* transgene (Cre+) and the *Rosa26-GFP* reporter allele (RGFP+) (**b**, **e–j**) were compared with pituitaries from Cre-, RGFP+ (**c**) and Cre+, RGFP- (**d**). GFP fluorescence is detected in Cre+, RGFP+ mice (b), but not in mice carrying only the reporter (cre-, RGFP+) (c) or only the transgene (Cre+, RGFP-) (d). Frozen pituitary sections of a mouse carrying the *Tg(Lhb-cre)1Sac* transgene and the *Rosa26-GFP* reporter allele (e–j) at ×10 (e–g) and ×20 (h–j) power objective lenses, with high magnification insets. Colabeling with antibody for GFP (e, h) and LHβ (f, i) is detected by yellow color in merged picture (g, j) in over 80% of anterior pituitary cells.

In adult mouse pituitaries, the majority of Pitx2-positive cells coexpress the gonadotropins or thyrotropin, suggesting that *Pitx2* also plays a role in the maintenance of these cell types (Charles *et al.*, 2005). To test this hypothesis, we have developed a gonadotrope-specific *cre* transgenic line to ablate *Pitx2* from mature pituitary gonadotropes.

The *Tg(Lhb-cre)1Sac* transgenic construct (Fig. 1a) was generated using a 776-bp sequence from the bovine *Lhb* promoter (Virgin *et al.*, 1985) fused to *eGFP* (Kaspar *et al.*, 2002); however, we were unable to detect *eGFP* in transgenic mice. Thus, founder mice were mated to the *RosaGFP cre*-reporter mice (B6;129-*Gt(ROSA)26-Sor*^{tm2Sbo}/J), generating *Tg(Lhb-cre)1Sac*;*RosaGFP* progeny. *Cre* activity was estimated from GFP fluorescence in whole pituitaries (Fig. 1b). In the highest expressing line, only the anterior lobe had detectable fluorescent

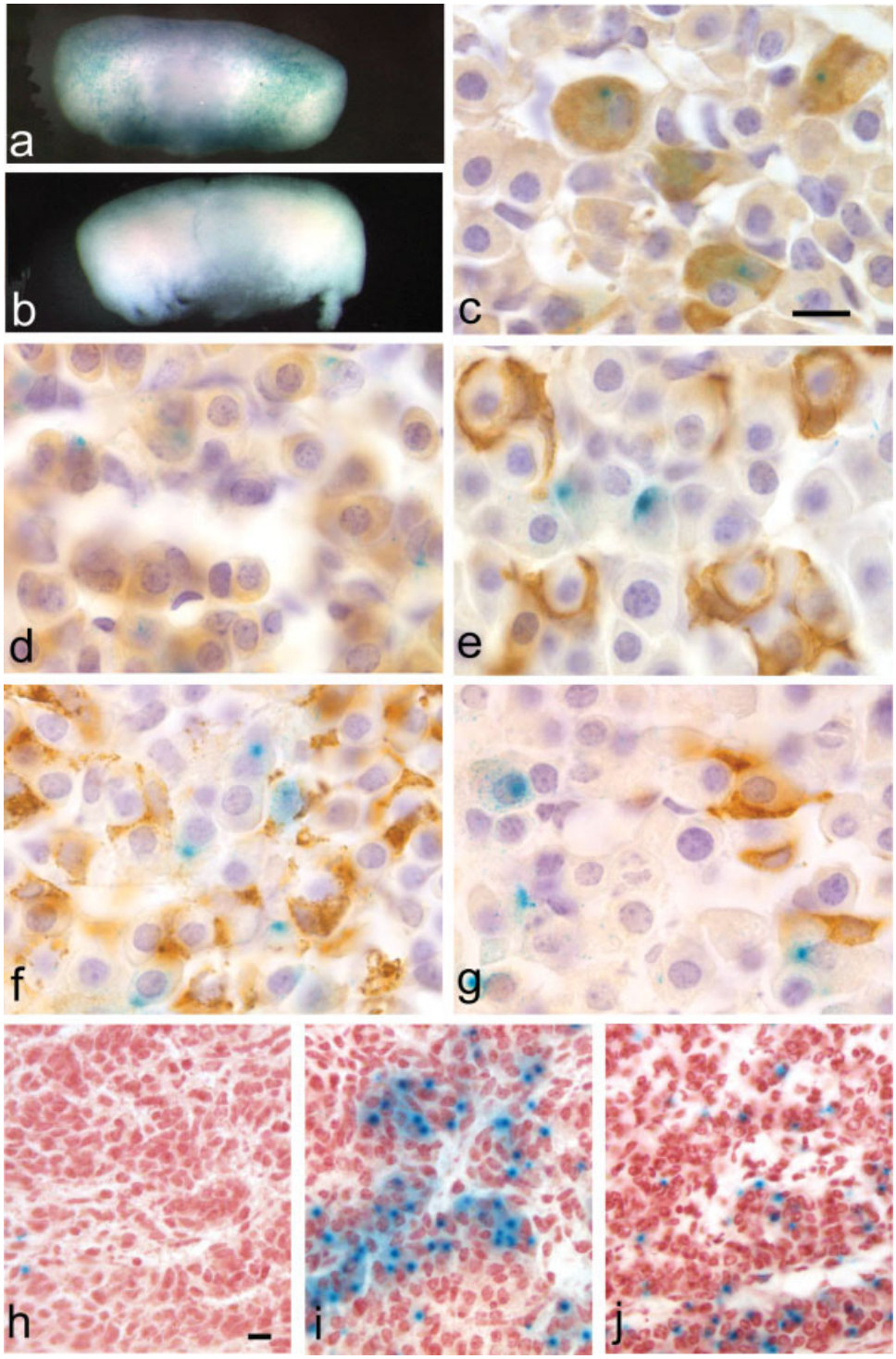

FIG. 2. *Tg(Lhb-cre)1Sac* transgene is active in late gestation and is specific to pituitary gonadotropes. Adult pituitaries of *Tg(Lhb-cre)1Sac*; *Rosa26LacZ* reporter progeny (**a**) and nontransgenic (**b**) stained with X-gal. Staining is detectable in the adult anterior lobe of the *Tg(Lhb-cre)1Sac*; *Rosa26LacZ* reporter progeny (a, **c–g**) but not in the nontransgenic (b). Immunohistochemical staining of pituitary hormones shows colocalization of the X-gal staining with LHβ immunoreactivity (c), but very little or no colocalization with GH (d), POMC (e), PRL (f), or TSHβ immunoreactivity (g). *Tg(Lhb-cre)1Sac*; *Rosa26LacZ* embryos reveal the onset of transgene activity by X-gal staining in only one or two cells at e16.5 (**h**), and increased penetrance of X-gal staining comparable to normal LHβ expression at e17.5 (**i**) and e18.5 (**j**). Scale bars represent 10 μm for panels c–g (×100 magnification) and panels h–j (×40 magnification).

signals. Furthermore, the proportion of discrete GFP positive cells in the anterior lobe is consistent with the gonadotrope population size (Fig. 1e,h). We assessed cell specificity by examining colocalization of GFP with LHβ using immunohistochemistry (Fig. 1f,i). We detected colocalization of GFP with over 80% of LHβ immunoreactive cells and with little or no LHβ negative cells, indicating that the *Tg(Lhb-cre)1Sac* line is gonadotrope-specific in the pituitary (Fig. 1g,j).

We utilized a separate *cre*-reporter strain to analyze the expression of the transgene in other pituitary cell types to avoid co-immunohistochemistry with antibodies generated in the same species. Transgenic mice were mated to the *Rosa26LacZ cre* reporter mouse strain

(officially B6;129S4-*Gt(ROSA)26Sor*^{tm1Sor}/J) (Friedrich and Soriano, 1991; Zambrowicz *et al*., 1997). Progeny of the cross, *Tg(Lhb-cre)1Sac*;*Rosa26LacZ*, yielded varying degrees of *lacZ* expression, although the penetrance of expression in the majority of pituitaries (n = 14) was comparable to expectations for gonadotropes (Fig. 2a,c). For each pituitary, 3–6 slides were examined for each hormone. Little to no colocalization is detectable between the X-gal-positive cells and GH, ACTH, PRL, or TSHβ in most pituitaries (Fig. 2d–g). Rarely, in the *Tg(Lhb-cre)1Sac*;*Rosa26LacZ* pituitaries, overlapping expression is detectable with a GH- or TSHβ-producing cell, which may represent normal dual-hormone producing cells (Burrows *et al*., 1999). The penetrance and specificity of *cre* reporter activity within LHβ positive cells suggests this line will be an effective tool in the deletion of pituitary transcription factors in gonadotropes.

Tg(Lhb-cre)1Sac;*Rosa26LacZ* mice were used to determine when *cre* excision begins (Fig. 2h–j). At e16.5, very little to no *cre* activity is detected by X-gal staining (n = 3/3). At e17.5, when endogenous LHβ is detectable by immunohistochemistry, *cre* activity is detectable (n = 3/3). X-gal staining is also present at e18.5 (n = 5/5) and in the adult pituitary (Fig. 2a).

Adult tissues were examined from *Tg(Lhb-cre)1Sac*; *Rosa26LacZ* mice (n = 4–8) to ascertain the degree of transgene leakiness in nonpituitary tissues (see Fig. 3). There was no evidence of transgene activity in the heart, lung, liver, pancreas, spleen, or skeletal muscle (data not shown). Trophoblast-derived placental tissues are negative, and the visceral yolk sac and chorioallantoic plate are positive at e16.5 (n = 3/4, data not shown). No transgene activity is detected in the hypothalamus at e17.5 (a). Transgene activity is detected at very low levels in a few cells in the cortex of the brain (b) and the kidney (c), but not in the adrenal gland (d). Faint X-gal staining is detectable in some corpora lutea and follicles of the ovary in *Tg(Lhb-cre)1Sac*;*Rosa26LacZ* mice (e) as well as in nontransgenic mice (f) suggesting background β-galactosidase activity. Activity is evident in some seminiferous tubules of the testes in *Tg(Lhb-cre)1Sac*;*Rosa26-LacZ* mice (g) but not in nontransgenics. Because active *Lhb* transcription has been found in both the rat and human testis, it is possible that the X-gal staining in the testis results from appropriate activity of the *Lhb* promoter (Berger *et al*., 1994; Zhang *et al*., 1995a,b).

To determine the importance of *Pitx2* in gonadotrope maintenance, we crossed the *Tg(Lhb-cre)1Sac* mice to *Pitx2*^{tm2Sac} (*Pitx2*$^{+/-}$). The resulting *Pitx2*$^{+/-}$;*Tg(Lhb-cre)1Sac* progeny were mated to *Pitx2*$^{flox/flox}$ mice to delete *Pitx2* post germ cell development, generating gonadotrope-specific *Pitx2*-deficient offspring (*Pitx2*$^{flox/-}$; *Tg(Lhb-cre)1Sac*). To verify that *Pitx2* deletion was effective in the gonadotropes of the knock-out mice, double-immunohistochemistry was used to detect the colocalization of PITX2 and LHβ in adult pituitaries. In the wild type mouse pituitary, nearly all LHβ cells clearly express PITX2 (Fig. 4a). As expected, not all PITX2-positive cells express LHβ since PITX2 is also expressed in thyrotropes (Charles *et al*., 2005). In the knock-out pituitaries, no PITX2 immunoreactivity was detected in the majority of LHβ cells. The remaining PITX2 expression is likely coming from thyrotropes (Fig. 4b). Hence, the *Tg(Lhb-cre)1Sac* line effectively knocked out *Pitx2* in gonadotropes.

To assess the effect of gonadotrope-specific *Pitx2* deletion on fertility, four *Pitx2*$^{flox/-}$;*Tg(Lhb-cre)1Sac* mice were each mated with four 6-week-old C57BL/6J female mice. All the females mated to the *Pitx2*$^{flox/-}$;*Tg(Lhb-cre)1Sac* males presented a copulation plug and carried full-term pregnancies. Four *Pitx2*$^{flox/-}$;*Tg(Lhb-cre)1Sac* female mice were mated with adult C57BL/6J male mice, and all females were able to carry a full-term pregnancy, give live births, and care for pups.

A thorough comparison of the physical phenotype of gonadotrope-specific *Pitx2* knock-out mice and wild type mice was performed. Gonadotropin deficiency can cause males to exhibit a diminished growth spurt resulting in adult males and females of equivalent weight (Davey *et al*., 1999). Average weights of gonadotrope-specific *Pitx2* knock-out mice and their normal, *cre*-negative litter mates (*Pitx2*$^{flox/+}$) were recorded for males and females aged 2–12 weeks. There was no evidence of growth differences in the mutant and normal mice, and males grew larger than females (data not shown). Gonadotropin deficiency can cause striking reduction in the weight of the testes and seminal vesicles (Cunha, 1972). The size and weight of the gonads from both sexes of knock-out and wild type mice were similar. Misregulation of gonadotropins can cause precocious puberty or delayed sexual maturation (Cushman *et al*., 2001; Kendall *et al*., 1991; Vesper *et al*., 2006). The vaginal openings in female knock-out and wild type mice occurred at the same time, as did the detection of major urinary proteins (MUPs) in the urine of male mice (data not shown). Taken together, these data suggest that *Pitx2* expression in gonadotropes is dispensable for pituitary-gonadal axis function after birth.

It was surprising that deletion of *Pitx2* in pituitary gonadotropes had no apparent effect on gonadal development, puberty, or fertility. Many studies have shown that *Pitx2* transactivates the genes encoding gonadotropin subunits *Cga*, *Lhb*, and *Fshb* (Suszko *et al*., 2003; Tremblay *et al*., 2000). It is possible that *Pitx2* is dispensable in differentiated gonadotropes because *Pitx1* is capable of compensation. *Pitx1* and *Pitx2* have overlapping functions in the activation of *Lhx3* in Rathke's pouch development at e10.5 and in establishing a normally sized pituitary anlage (Charles *et al*., 2005; Gage *et al*., 1999a; Suh *et al*., 2002). *Pitx1* and *Pitx2* also have overlapping functions in other organs (Gage *et al*., 1999a,b; Lanctot *et al*., 1999; Marcil *et al*., 2003; Suh *et al*., 2002). Because *Pitx1* mutant pituitaries appear minimally affected, and *Pitx2* null pituitaries are extremely underdeveloped, *Pitx2* is more important than *Pitx1* in pituitary development (Suh *et al*., 2002). *Pitx1* might be more critical in adult pitui-

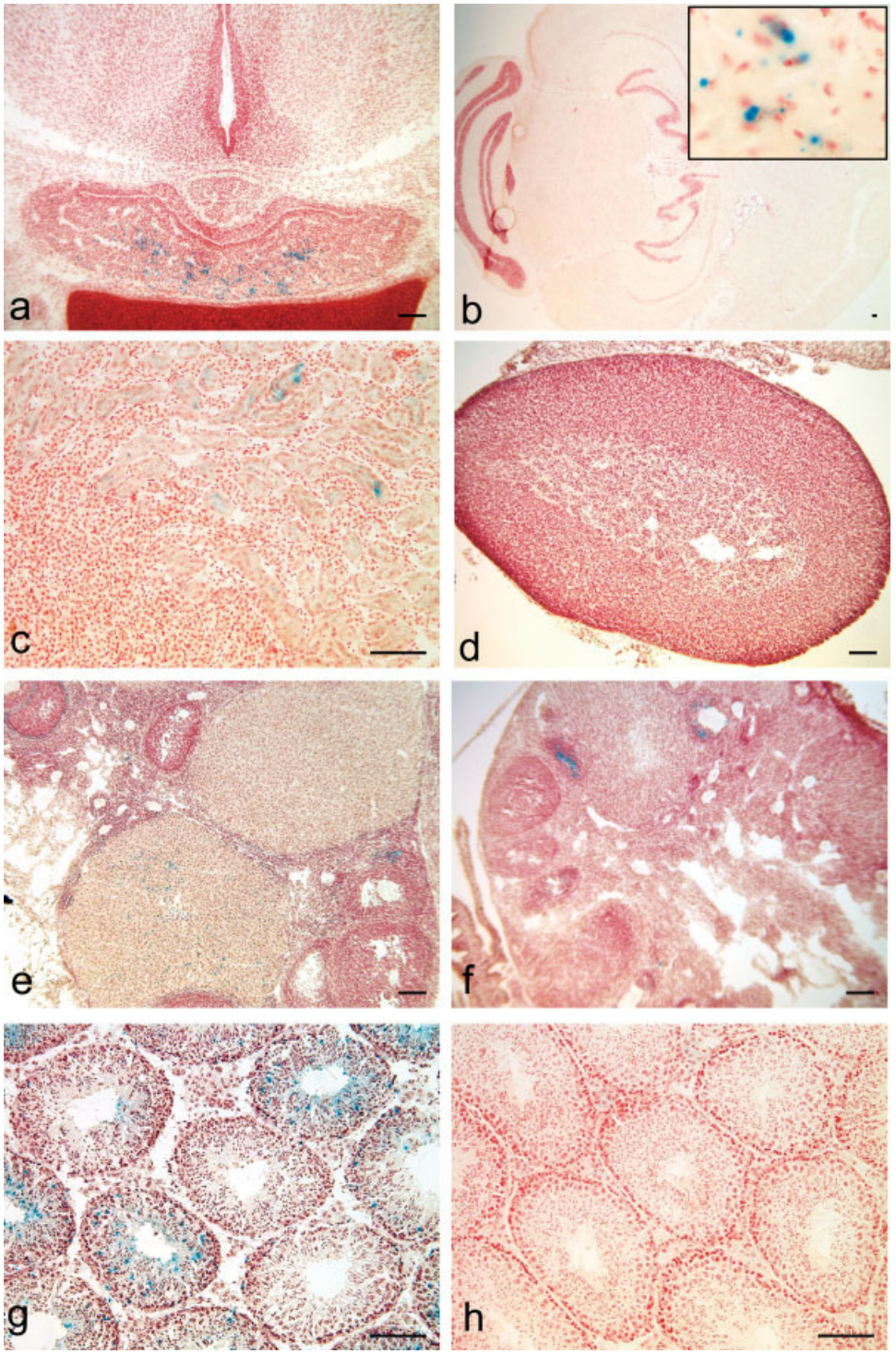

FIG. 3. Limited ectopic activity of *Tg(Lhb-cre)1Sac* transgene. Tissues from *Tg(Lhb-cre)1Sac*; *Rosa26-LacZ* reporter animals stained with X-gal reveal that *cre* activity is high in the e17.5 anterior pituitary, but is absent from the hypothalamus (**a**). Limited evidence of cre activity is detected by X-gal staining in rare cells in the adult brain (**b**) and kidney (**c**), but not in the adrenal gland (**d**). Low levels of X-gal staining are detected in a few ovarian follicles and corpora lutea of from *Tg(Lhb-cre)1Sac*; *Rosa26LacZ* double transgenic mice (**e**) above background X-gal staining in *Rosa26-LacZ* mice lacking the *cre* transgene (**f**). X-gal staining is present in some seminiferous tubules of the double-transgenic mice (**g**), but not in wild-type testis (**h**). Scale bars represent 100 μm for all panels. Inset (b) width represents 100 μm.

tary function than *Pitx2*. This prediction could be tested with a floxed allele of *Pitx1* and the *Tg(Lhb-cre)1Sac* line that we report here.

In this study, we characterize a cre transgenic line that is a valuable tool for deleting genes in gonadotropes and demonstrate its effectiveness by using it to assess *Pitx2* function in mature gonadotropes.

METHODS

Generation of Transgene Construct

Tg(Lhb-cre)1Sac mice were generated using a 776-bp sequence from the bovine *Lhb* promoter. The gonadotrope specificity of the promoter was demonstrated using a herpes simplex virus thymidine kinase reporter

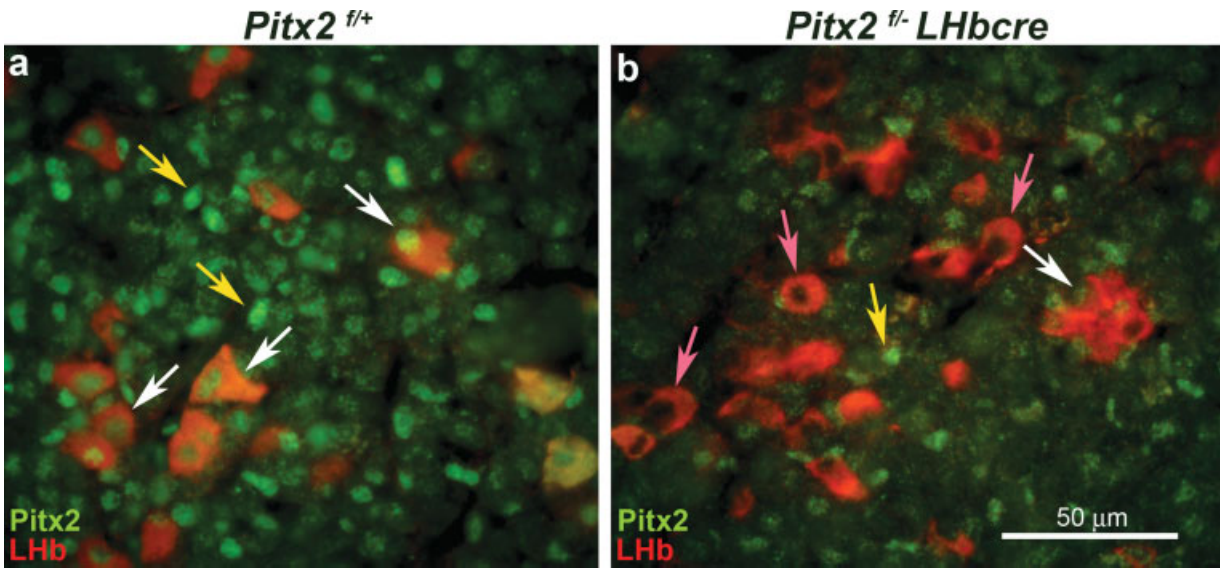

FIG. 4. Normal LH expression in gonadotrope-specific *Pitx2* knockout mice. (**a**) Most LHβ-immunostained cells (red) costain with PITX2-specific antibodies (green) in *Pitx2*$^{flox/+}$ adult mouse pituitary (white arrows), but about half of the PITX2 immuno-positive cells do not costain with LHβ-specific antibodies (yellow arrows). (**b**) *Pitx2*$^{flox/-}$; *Tg(Lhb-cre)1Sac* adult mouse pituitary lacks cells that costain with antibodies for PITX2 and LHβ. Cells immunostained for PITX2 only (yellow arrow) or LHβ only (pink arrows) predominate, whereas PITX2 and LHβ costained cells are quite rare (white arrow).

gene (Keri *et al*., 1994; Virgin *et al*., 1985). We generated a construct for microinjection that uses the bovine *Lhb* subunit gonadotrope-specific promoter to drive the expression of an enhanced *GFP-cre* recombinase fusion gene (Kaspar *et al*., 2002). We engineered a consensus KOZAK sequence (GCCGCCACC*ATG*G) to encourage efficient translation of the transgene mRNA transcript into protein and inserted a polyadenylation signal and intron from the rabbit β-globin gene for efficient nuclear processing of the transcript (Fig. 1a). The complete construct was linearized and microinjected into F_2 zygotes from (C57BL/6J X SJL) F_1 (Hogan, 1994). Live progeny were genotyped via PCR for the presence of cre recombinase in genomic DNA prepared from a tail biopsy.

Mice

Cre mice were maintained by crossing with C57BL/6J mice from The Jackson Laboratory. All mice were maintained at the University of Michigan under the guidelines of the Unit for Laboratory Animal Medicine and the University Committee for Care and Use of Animals. *Cre* mice were identified by PCR amplification of genomic DNA with primers 5′-gcataaccagtgaaacagcattgctg-3′ and 5′-gga catgttcagggatcgccaggcg-3′ under the following conditions: 94°C for 3 min, followed by 32 cycles of 94°C for 30 s, 60°C for 60 s, and 72°C for 90 s, and a final 10-min extension at 72°C. For transgene analysis, experimental animals carried one allele of the *cre* transgene and one allele of the reporter gene, whereas controls were negative for the *cre* transgene but positive for a reporter gene. B6;129-*Gt(ROSA)26Sor*^{tm2Sbo}*/J* and B6;129S4-*Gt(ROSA)26Sor*^{tm1Sor}*/J* reporter mice were obtained from The Jackson Laboratory and maintained as homozygotes. Genotyping of B6;129-*Gt(ROSA)26Sor*^{tm2Sbo}*/J* mice was performed by PCR using primers 5′-ggcttaaaggctaacctgatgtg-3′, 5′gcgaagagtttgtcctcaacc-3′, and 5′-ggagcgggagaaatggatatg-3′ under the following conditions: 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 64°C for 60 s, and 72°C for 60 s, and a final 10-min extension at 72°C. The B6;129-*Gt(ROSA)26Sor*^{tm2Sbo}*/J* band is 1,146 bp and wild-type band is 374 bp. *Pitx2*$^{flox/-}$;*Tg(Lhb-cre)1Sac* mice were generated by mating B6-*Pitx2*$^{+/-}$ mice with *Tg(Lhb-cre)1Sac* positive mice. The *Pitx2*$^{+/-}$;*Tg(Lhb-cre)1Sac* offspring were mated to B6-*Pitx2*$^{flox/flox}$ mice, and genotyping was performed as previously described (Gage *et al*., 1999a).

Tissue Preparation and Histology

Adult pituitaries were collected at 6 weeks of age or later and fixed for 1 h in 4% formaldehyde in PBS. X-gal staining was performed as previously described (Brinkmeier *et al*., 1998). After staining, pituitaries were fixed in 4% formaldehyde overnight, rinsed in PBS, dehydrated and embedded in a Citadel 1000 (Thermo Electric, Chesire, England) paraffin-embedding machine, and sectioned coronally at 5-μm thickness. Immunohistochemistry for the pituitary hormones was performed as previously described (Kendall *et al*., 1991). For embryos, noon of the day of the vaginal plug is designated as embryonic day 0.5. Embryos and adult organs were dissected, frozen on dry ice and stored at −80°C. Embryos were embedded in OCT (Sakura Finetek, Torrance, CA) and cryosectioned at 16-μm thickness. After X-gal staining, sections were counterstained for 2 min with 1% neutral red stain plus 4% sodium acetate:glacial acetic acid.

Sections were dehydrated and mounted with xylene:permount 1:2 (Fisher) mounting media. Whole mount capture of GFP fluorescence of freshly dissected *Tg(Lhb-cre)1Sac* x B6;129-*Gt(ROSA)26Sor*^{tm2Sho}/J progeny was achieved using a Leica MZFL III stereo/dissecting fluorescent microscope. Pituitaries were fixed for 1 h in 4% formaldehyde and rinsed in 1× PBS. Immunohistochemistry for GFP and fluorescent LHβ was performed on 8- to 10-μm pituitary cryosections using a rabbit anti-GFP Alexa Fluor 488 (Molecular Probes, Eugene, OR) antibody overnight at 4°C, diluted in a blocking solution comprised of 3% normal donkey serum, 1% BSA, and 0.5% Triton-X100 in 1× PBS. Slides were washed three times for 5 min using 0.5% Triton-X100 in 1× PBS. Guinea pig anti-LHβ antibody (NHPP) was diluted 1:100 in the same block and incubated for 1 h at room temperature. Washing with 0.5% Triton-X100 in PBS was followed by a 1-h incubation with biotinylated anti-guinea pig secondary antibody (Jackson Immunoresearch) and 0.5% Triton-X100/PBS washes. Streptavidin-TRITC was added for 1 h at 1:200 dilution. Rabbit-anti-PITX2 antibody was generated by Dr. Tord Hjalt (Lund University, Sweden) and provided by Dr. Philip Gage (University of Michigan, Ann Arbor). PITX2 antibody was diluted 1:100 in the same block described earlier, and then 100 μl was placed on each slide over night at 4°C. Secondary detection was performed as described earlier using biotinylated anti-rabbit antibody (Vector Laboratories) then washed in PBS/Triton, mounted with fluorescent mounting media, and images were captured using a Leica DMRB fluorescent microscope.

ACKNOWLEDGMENTS

The authors thank the University of Michigan DNA Sequencing Core, Dr. John Nilson for providing the *Lhb* promoter, Dr. Parlow at the National Hormone and Pituitary Program for providing the pituitary hormone antibodies, Drs. Philip Gage and Donna Martin for the *Pitx2*$^{flox/flox}$ mice, Drs. Fred Gage and Hoonkyo Suh for supplying the *eGFPcre* fusion construct, Kailey Owens for help with genotyping the mice, Galina Gavrilina for preparation of transgenic mice, and the University of Michigan Transgenic Animal Model Core.

Author contributions: MAC designed and cloned the transgene and identified the best *Lhb-cre* line. AHM carried out the analysis of the *Pitx2*$^{flox/-}$, *Lhb-cre* mice. MAP carried out the cell-specific and developmental analysis of cre activity and wrote the paper.

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