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

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Abstract

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 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.

Gonadotropes are one of 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 towards 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.*, 2006; Charles *et al.*, 2005; Kumar *et al.*, 2003; Suh *et al.*, 2002; Vesper *et al.*, 2005; 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

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Author contributions: MAC prepared the transgene construct and identified the best cre line by breeding with the GFP reporter strain as part of his PhD thesis research. AHM used the *Tg(Lhb-cre)* line to analyze the role of *Pitx2* in gonadotropes. MAP analyzed the developmental, tissue-specific, and cell-specific expression of *Tg(Lhb-cre)* and played the leading role in preparation of the manuscript.

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).

In adult mouse pituitaries, the majority of *Pitx2*-positive cells co-express 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 (Figure 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)26Sor*^{tm2Sho}/J), generating *Tg(Lhb-cre)1Sac*;RosaGFP progeny. Cre activity was estimated from GFP fluorescence in whole pituitaries (Figure 1b). In the highest expressing line, only the anterior lobe had detectable fluorescent signals. Furthermore, the proportion of discrete RosaGFP positive cells in the anterior lobe is consistent with the gonadotrope population size (Figure 1e, h). We assessed cell specificity by examining colocalization of GFP with LHβ using immunohistochemistry (Figure 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 (Figure 1g, j).

We utilized a separate cre-reporter strain to analyze expression of the transgene in other pituitary cell types because colocalization with other pituitary hormones requires 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 (Figure 2a, c). For each pituitary, 3-6 slides were examined for each hormone. Little to no colocalization is detectable between the lacZ-positive cells and GH, ACTH, PRL or TSHβ in most pituitaries (Figure 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 (Figure 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 (Figure 2a).

Adult tissues were examined from *Tg(Lhb-cre)1Sac*;Rosa26LacZ mice (n=4-8) to ascertain the degree of transgene leakiness in non-pituitary tissues (see Figure 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 non-transgenic mice (f), suggesting background β-galactosidase activity. Activity is evident in some seminiferous tubules of the testes in *Tg(Lhb-cre)1Sac*;Rosa26LacZ mice (g) but not in non-transgenics. Because active Lhβ transcription has been found in both the rat and human testis, it is possible that the lacZ staining in the testis results from appropriate activity of the *Lhβ* promoter (Berger *et al.*, 1994;Zhang *et al.*, 1995a;Zhang *et al.*, 1995b).

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 (Figure 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 (Figure 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 reductions 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, 1999b; 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 pituitary 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 gene (Keri *et al.*, 1994; Virgin *et al.*, 1985). We generated a construct for microinjection that uses the bovine LHβ 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 poly adenylation signal and intron from the rabbit β-globin gene for efficient nuclear processing of the transcript (Figure 1a). The complete construct was linearized and microinjected into F_2 zygotes from (C57BL/6J × SJL) F_1 (Hogan, 1994). Live progeny were genotyped via PCR for the presence of cre recombinase in from 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′-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. For transgene analysis, experimental animals carried one allele of the *cre* transgene and one allele of the reporter gene, while controls were negative for the *cre* transgene but positive for a reporter gene. B6;129-*Gt(ROSA)26Sor*^{tm2Sho}/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*^{tm2Sho}/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 minutes, followed by 35 cycles of 94°C for 30 seconds, 64°C for 60 seconds and 72°C for 60 seconds, and a final 10 minute extension at 72°C. The B6;129-*Gt(ROSA)26Sor*^{tm2Sho}/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 six weeks of age or later and fixed for 1 hour 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 5μm 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 Co., Torrance, CA) and cryosectioned at 16μm thickness. After X-gal staining, sections were counterstained for 2 minutes 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* × B6;129-*Gt(ROSA)26Sor*^{tm2Sho}/J progeny was achieved using a Leica MZFL III stereo/dissecting fluorescent microscope. Pituitaries were fixed for 1 hour in 4% formaldehyde and rinsed in 1× PBS. Immunohistochemistry for GFP and fluorescent LHβ expression was performed on 8-10μm 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 3 times for 5 minutes 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 hour at room temperature. Washing with 0.5% Triton-X100 in PBS was followed by a one hr incubation with biotinylated anti-guinea pig secondary antibody (Jackson Immunoresearch), 0.5% Triton-X100/PBS washes. Streptavidin-TRITC was added for 1 hour 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 above and 100 μl was placed on each slide over night at 4°C. Secondary detection was performed as described above 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.

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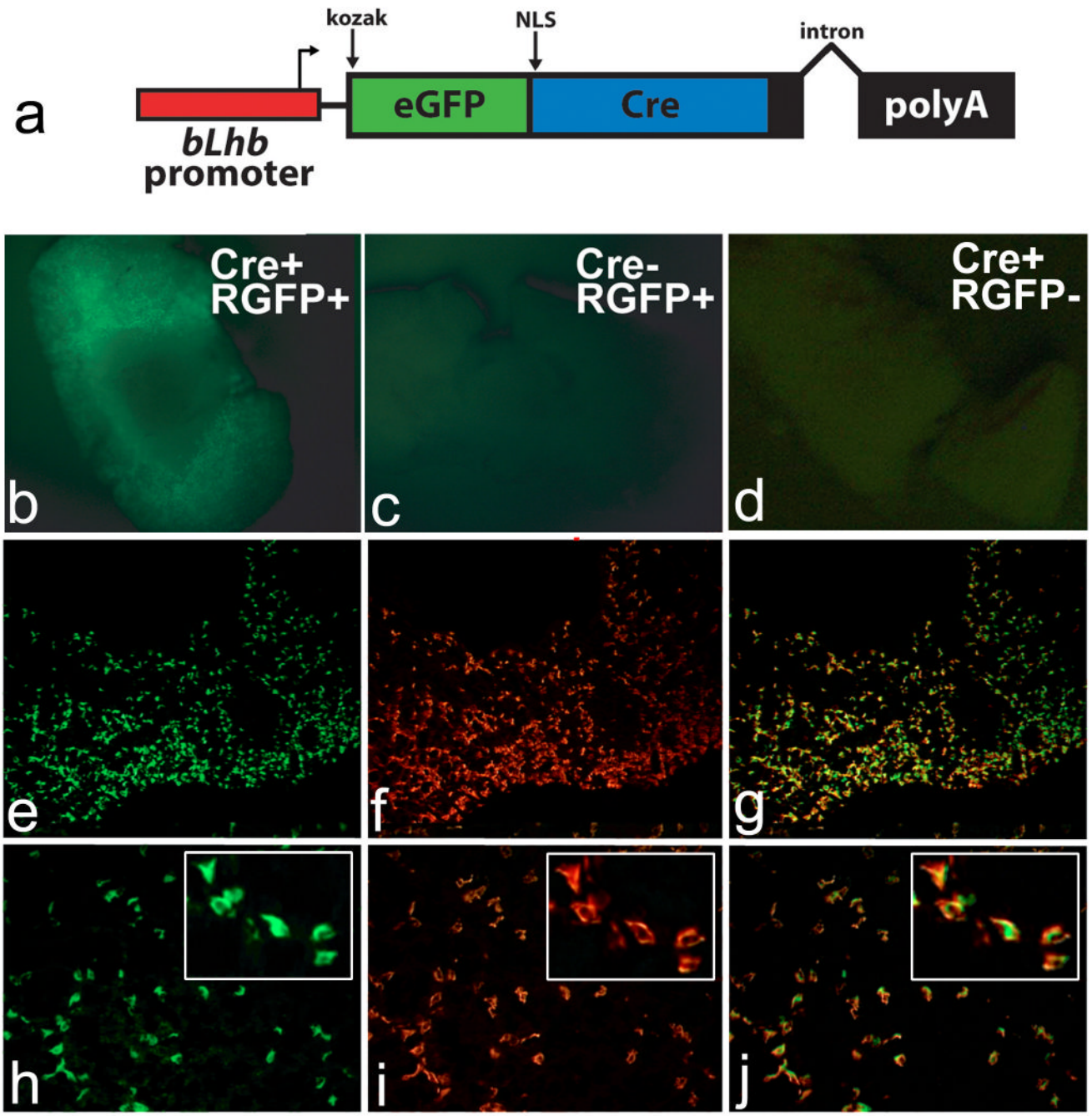

Figure 1. Gonadotrope-specific cre transgenic construct and expression

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.

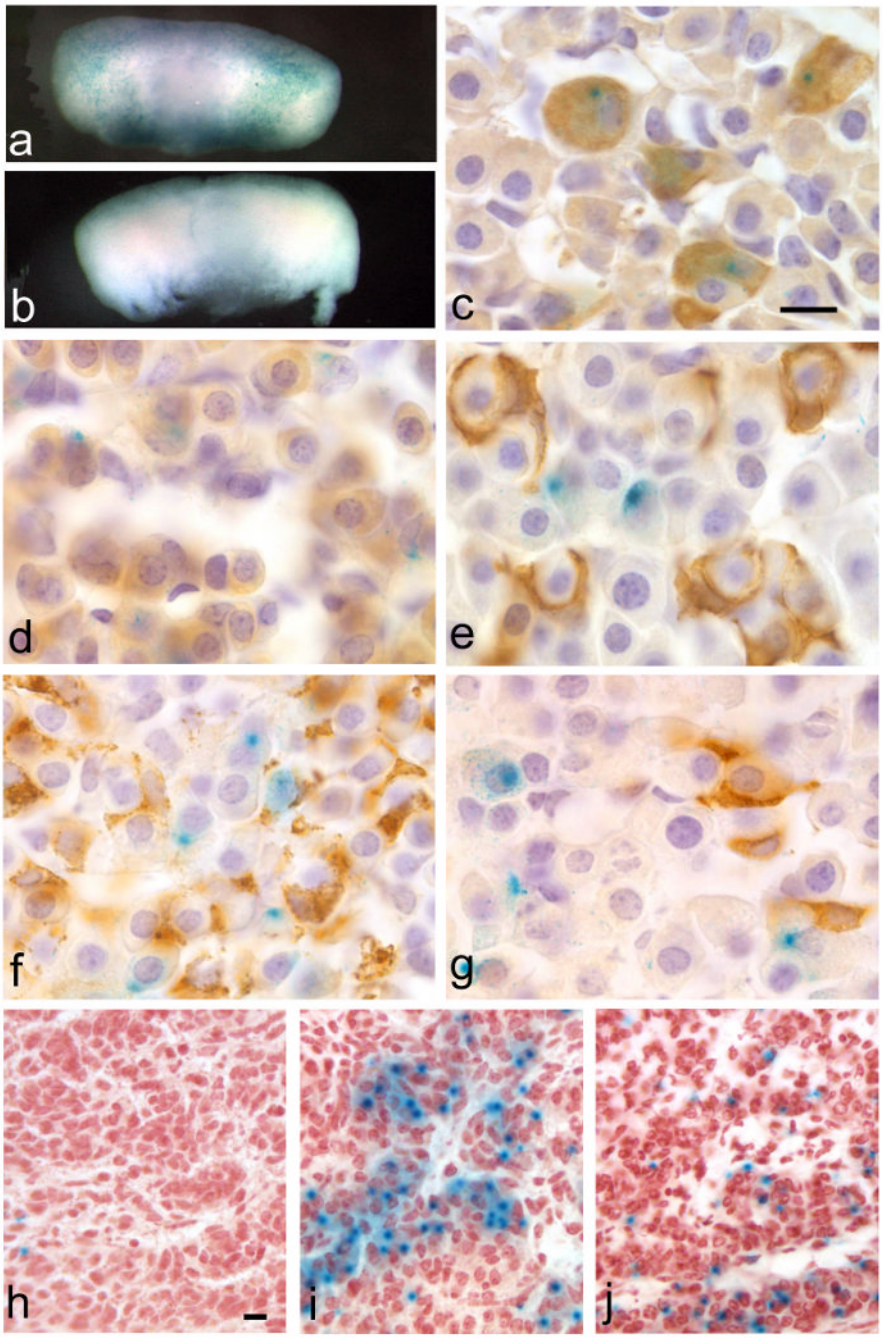

Figure 2. *Tg(Lhb-cre)1Sac* transgene is active in late gestation and is specific to pituitary gonadotropes

Adult pituitaries of *Tg(Lhb-cre)1Sac*; Rosa26LacZ reporter progeny (a) and non-transgenic (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 non-transgenic (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 10μm for panels c-g (100× magnification) and panels h-j (40× magnification).

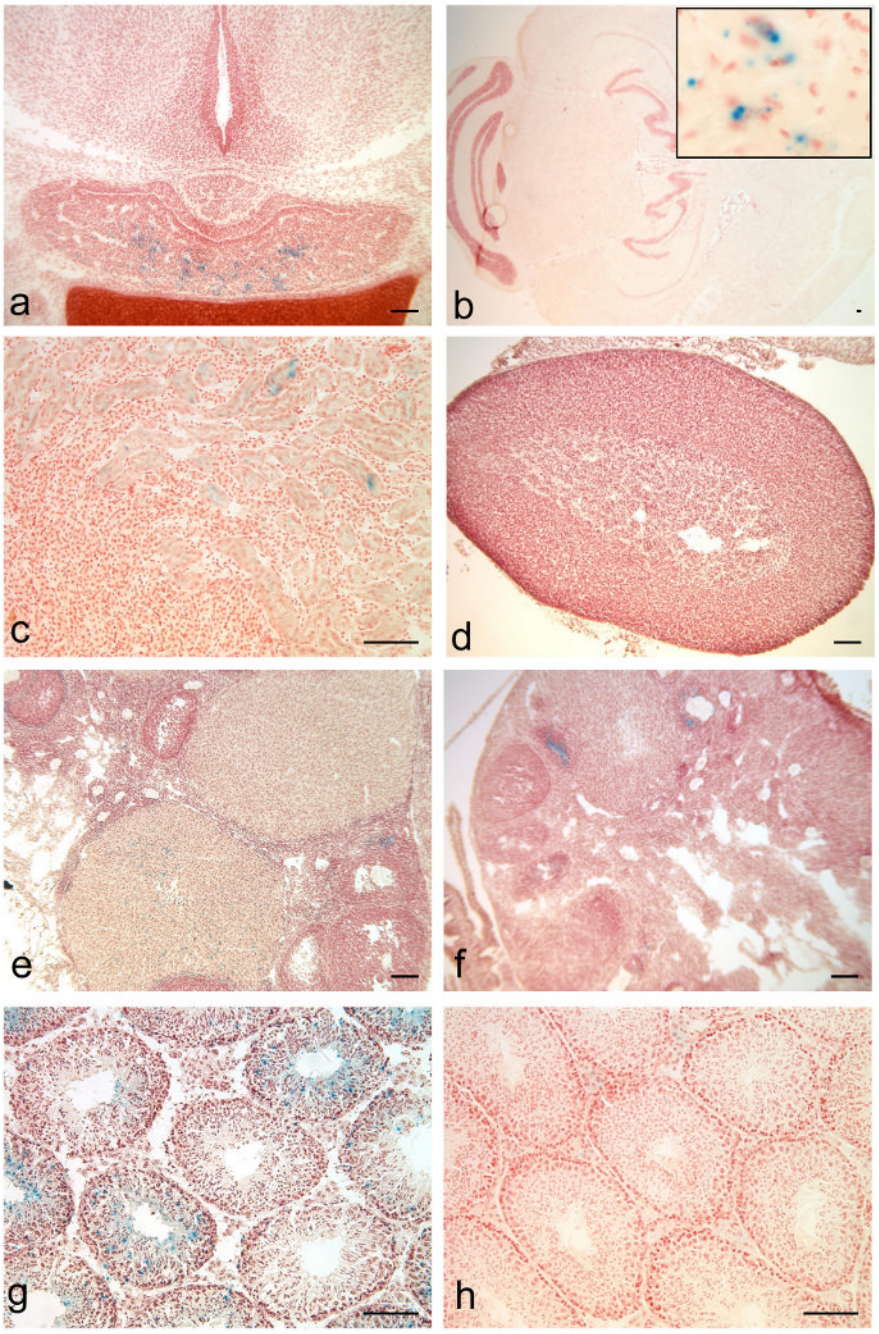

Figure 3. Limited ectopic activity of *Tg(Lhb-cre)1Sac* transgene

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 Rosa26LacZ 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 100μm for all panels. Inset (b) width represents 100μm.

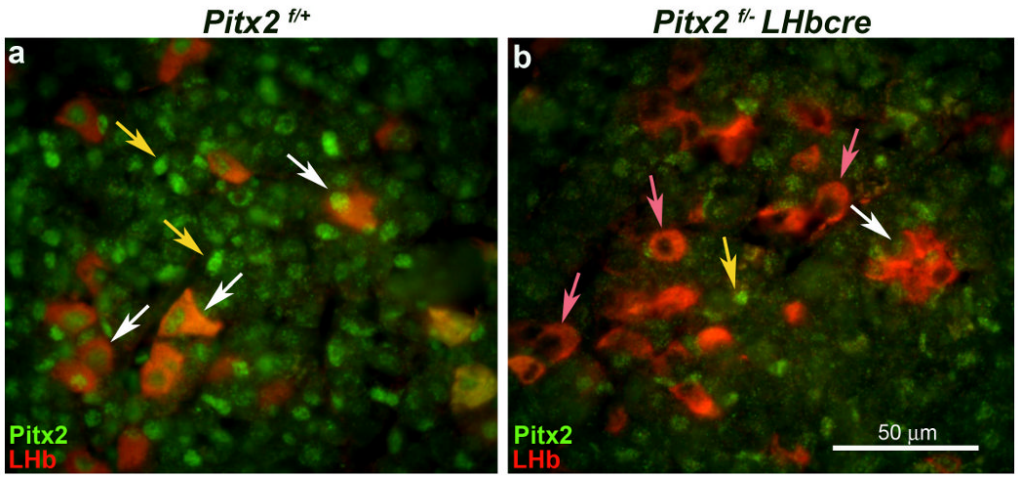

Figure 4. Normal LH expression in gonadotrope-specific *Pitx2* knockout mice

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