Mechanisms of activin and inhibin action on FSH synthesis *in vivo*

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Abstract

Follicle-stimulating hormone (FSH), a product of pituitary gonadotrope cells, is an essential regulator of ovarian follicle development and fertility in females and of quantitatively normal spermatogenesis in males. According to the current dogma, FSH synthesis is stimulated by intra-pituitary activins and is suppressed by gonadal inhibins. Activins, members of the transforming growth factor family, regulate FSH production by inducing FSHβ subunit (*Fshb*) transcription. Based on *in vitro* evidence, activins signal through type I and II serine/threonine kinase receptor complexes. Once bound by activins, the type II receptors phosphorylate cytoplasmic effector protein SMAD3, which then complex with SMAD4, and accumulate in the nucleus. In the nucleus, the SMADs for complexes with transcription factor forkhead box L2 (FOXL2) and bind directly to the promoter region of *Fshb*. *In vivo* studies are both consistent with and contradictory to *in vitro* observations. First, gonadotrope-specific *Smad4* knockout mice are sub-fertile and produce FSH at reduced levels relative to controls. I hypothesized that SMAD3 may compensate for the loss of SMAD4, thus maintaining fertility in these mice. To test this hypothesis, I generated mice that lack SMAD3 DNA binding activity as well as SMAD4 in their gonadotropes. In the absence of both SMAD3 and SMAD4, males and females are profoundly FSH-deficient and hypogonadal, and the females are sterile. This phenotype is consistent to that of *Fshb* knockout mice. A similar phenotype was also observed in gonadotrope-specific *Smad4/Foxl2* knockout mice. However, the Cre recombinase driver used in these mice and my SMAD3/SMAD4 model is first active during pituitary development (E12.75). Therefore, it was unclear whether the diminished FSH synthesis in these mice reflected impaired gonadotrope development or the necessity for intact activin signaling molecules in adult gonadotropes. To discriminate between these possibilities, I generated a new mouse line that enables the tamoxifen-

inducible deletion of *Foxl2* and *Smad4* in gonadotropes of adult mice. All animals exhibited normal fertility prior to treatment, suggesting that there was no precocious Cre activity under physiologic conditions. One month after tamoxifen injections, however, both males and females produced significantly reduced levels of FSH. In females, reduced FSH results in an adult-onset arrest in folliculogenesis and infertility. In males, spermatogenesis appears to be intact. These data suggest that FOXL2 and SMAD4 are required for FSH synthesis in gonadotrope cells of adult mice. Finally, I investigated the mechanism of inhibin antagonism on FSH synthesis *in vivo*. According to the current model, inhibins suppress FSH by acting as competitive activin receptor antagonists, particularly in the presence of the co-receptor, TGFBR3 (betaglycan). However, the role of TGFBR3 in inhibin action and FSH synthesis in gonadotropes *in vivo* has not been determined, in part due to embryonic lethality of *Tgfbr3* global knockout mice. Therefore, I established a novel 'floxed' *Tgfbr3* murine model, allowing us to ablate the gene specifically in gonadotropes *in vivo* (hereafter, T3cKO). My data indicate that, inhibin A antagonism is largely impaired in the absence of TGFBR3 both *in vivo* and *ex vivo*. Furthermore, T3cKO females are supra-fertile, ovulating more eggs per cycle and producing approximately two more pups per litter than controls. Surprisingly, T3cKO mice synthesize and secrete normal levels of FSH. The mechanism underlying the enhanced fertility in T3cKO mice is currently unresolved. Collectively, this research significantly advanced our understanding of the mechanisms of activin and inhibin action on FSH synthesis and secretion in murine gonadotropes *in vivo*.

Résumé

L'hormone folliculo-stimulante (FSH), un produit des cellules gonadotropes de l'hypophyse, est un régulateur essentiel du développement du follicule ovarien et de la fertilité chez les femelles et, quantitativement, de la spermatogenèse chez les mâles. Selon le dogme actuel, la synthèse de FSH est stimulée par les activines intra-pituitaires et est inhibée par les inhibines gonadiques. Les activines, membre de la famille des facteurs de croissance transformant, régulent la production de FSH en induisant la transcription du gène *Fshb*, codant pour la sous-unité FSHβ de la protéine FSH. Selon les évidences *in vitro*, les activines signalent à travers le complexe des récepteurs sérine/thréonine kinase de type I et II. Une fois liés par les activines, le récepteur de type I phosphoryle la protéine cytoplasmique SMAD3, qui, à son tour, s'interagit avec la protéine SMAD4 pour ensuite s'accumuler dans le noyau. Dans le noyau, les SMADs forment un nouveau complexe avec le facteur de transcription forkhead box L2 (FOXL2) et se lient directement au promoteur du gène *Fshb*. Cependant, Les études *in vivo* sont à la fois cohérentes et contradictoires avec ces observations *in vitro*. Par exemple, les souris knock-out du gène Smad4 spécifiquement dans les gonadotropes sont sous-fertiles et leur production de la FSH mais est réduits comparé aux témoins. Suite à ces constatations, J'ai formulé l'hypothèse que SMAD3 pourrait compenser la perte de SMAD4, réservant ainsi la fertilité chez ces souris.

Pour tester mon hypothèse, j'avais invalidé d'une manière conditionnelle le domaine de liaison à l'ADN du gène *Smad3* ainsi que le gène Smad4. En absence de SMAD3 et SMAD4, les souris mâles et femelles étaient profondément déficient en FSH et hypogonadiques, et les femelles étaient infertiles. Ce phénotype était consistant avec le phénotype observé chez les souris knock-out du gène *Fshb*. De plus, un phénotype similaire était observé chez les souris ayant les deux gènes, *Foxl2* et *Smad4*, invalides spécifiquement dans les gonadotropes. Toutefois, la Cre

recombinase dans cette lignée de souris ou dans la lignée de SMAD3/SMAD4 est active durant le développement de l'hypophyse (E12.75). Par conséquent, c'est difficile de distinguer si la diminution en production de la FSH est due à cause d'une altération des cellules gonadotropes durant le développement, ou est une conséquence du manque de la signalisation par les activines. Pour distinguer entre ces deux possibilités, j'ai généré une nouvelle lignée de souris qui permet la délétion inductible par la tamoxifène de *Smad4* et *Foxl2* dans les gonadotropes de souris adultes. Les souris présentaient une fertilité normale avant le traitement par la tamoxifène, suggérant qu'il n'y avait pas d'activité Cre précoce dans des conditions physiologiques normales. Par contre, un mois après les injections de tamoxifène, les mâles et les femelles ont produit des niveaux significativement réduits de l'hormone FSH. Chez les femelles, la réduction de la FSH entraîne un arrêt de la folliculogenèse et l'infertilité. Chez les mâles, la spermatogenèse reste intacte. Ces données suggèrent que FOXL2 et SMAD4 sont nécessaires pour la synthèse de FSH dans les cellules gonadotropes de souris adultes. Enfin, j'ai étudié le mécanisme par lequel l'inhibine exerce son effet antagoniste sur la synthèse de la FSH *in vivo*. Selon le modèle actuel, les inhibines répriment la FSH en agissant comme antagonistes compétiteurs des récepteurs de l'activine, en particulier en présence du co-récepteur, TGFBR3 (bétaglycan). Toutefois, le rôle du TGFBR3 dans le mécanisme d'action de l'inhibine et la synthèse de la FSH dans les gonadotropes n'a pas été déterminé *in vivo,* en partie due à la mortalité des embryons des souris ayant une délétion globale du gène *Tgfbr3*. Pour contourner ce problème, j'ai établi un nouveau modèle murin oú le gène Tgfbr3 était «floxé», ce qui nous permet de recombiner et invalider le gène spécifiquement dans les gonadotropes (ci-après, T3cKO). Mes données indiquent que l'effet inhibiteur de l'inhibine A est largement altérée en absence de TGFBR3, à la fois *in vivo* et *ex vivo*. De plus, les femelles T3cKO sont supra-fertiles, ovulant plus d'oocytes par cycle et produisant environ deux souris

chiots de plus comparé aux souris témoins. Par contre et d'une façon surprenante, les souris T3cKO synthétisent et sécrètent des niveaux normaux de FSH. Le mécanisme qui pourrait expliquer l'augmentation de la fertilité, tout en maintenant un niveau normal de la FSH, chez les souris T3cKO est actuellement non résolu.

Collectivement, mes recherches durant mon doctorat ont significativement avancé nos compréhensions des mécanismes de l'action de l'activine et de l'inhibine sur la synthèse et la sécrétion de FSH dans les gonadotropes murines *in vivo*.

Contribution of authors

This thesis is presented as a collection of scholarly papers, and reformatted and submitted following the "Thesis Guidelines" of the Faculty of Graduate and Postdoctoral Studies, McGill University. Please note that the formatting of the experimental **Chapters** (**Ch. 2-4**) is not uniform and reflects the standards of the journals to which the manuscripts were or will be submitted.

Chapter 2

SMAD3 regulates follicle-stimulating hormone synthesis by pituitary gonadotrope cells *in vivo*.

Yining Li, Gauthier Schang, Ulrich Boehm, Chu-Xia Deng, Jonathan Graff and Daniel J. Bernard *J Biol Chem. 2017 Feb 10;292(6):2301-2314*

I was responsible for the experimental design and data analyses. I conducted the majority of the experiments, except for the promoter-reporter assay (Fig. 2.2), which was performed by Gauthier Schang. The original mouse strains were provided by our collaborators, Drs. Boehm, Deng, and Graff. All the work was performed under the supervision of Dr. Bernard. I wrote the first draft and collaborated with Dr. Bernard to prepare the final version of the manuscript.

Chapter 3

SMAD4 and FOXL2 are required for FSH synthesis in adult mice

Yining Li, Gauthier Schang, Ying Wang, Xiang Zhou, Alexandre Boyer, Adrien Levasseur, Ulrich Boehm, Chu-Xia Deng, Mathias Treier, Derek Boerboom, and Daniel J. Bernard. The manuscript was submitted to *Endocrinology*.

I was responsible for the experimental design and data analyses. I conducted most of the experiments in this study. Gauthier Schang helped with evaluating recombination efficiency (Fig. 3.1B); Ying Wang made the targeting vector (Fig. S3.1); Xiang Zhou produced the iGRIC mice from the chimera stage; Drs. Boyer and Boerboom performed the gene targeting in ES cels (Fig. S3.1); Adrien Levasseur performed the immunohistochemistry staining in the testes (Fig. 3.3E); Drs. Deng and Treier provided floxed mouse strains; Dr. Boehm provided the GRIC targeting vector from which iGRIC construct was generated. I wrote the first draft and collaborated with Dr. Bernard to prepare the final version of the manuscript.

Chapter 4

Enhanced fertility, but not FSH secretion, in female mice with a gonadotrope-specific deletion of the inhibin co-receptor TGFBR3 (betaglycan)

Yining Li, Jérôme Fortin, Luisina Ongaro, Ulrich Boehm, Daniel J Bernard[&] and Herbert Y. Lin[&]

Data in Figs. 4.2, 4.3A-B, 4.4A, S4.1A, and S4.2 were generated by Jérôme Fortin; data presented in Fig. 4.4C-E were mostly generated by Yining Li with assistance from Luisina Ongaro; blood sample collection was assisted by Xiang Zhou; Drs. Ulrich Boehm (iGRIC) and Herbert Y. Lin (floxed *Tgfbr3*) generated mouse models used in this project. All other experiments were conducted by me under the supervision of Dr. Daniel J Bernard. I wrote the first draft and collaborated with Dr. Bernard to prepare the final version of the manuscript.

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κ-EOPR: dynorphin receptor
4-OHT: 4-hydroxytamoxifen
ABP: androgen binding protein
AC: adenylyl cyclase
ACTH: adrenocorticotropic hormone
ACVR: activin receptor
Ad: adenovirus
AF: antral follicle
ALK: activin receptor-like kinase
AMH: Anti-Müllerian hormone
AP-1: activator protein-1
AR: androgen receptor
ARC: arcuate nucleus
Asn: asparagine
AVPV: anteroventral periventricular nucleus
BDNF: brain-derived neurotrophic factor
BMP: bone morphogenetic protein
BMPR1/*Bmpr1*: bone morphogenetic protein receptor type 1
bp: base-pair
C-tail: cytoplasmic tail
Ca^{2+}: calcium
cAMP: cyclic AMP
CASA: computer-assisted sperm analysis
CC: Clomiphene citrate
CG: chorionic gonadotropin
CGA/Cga: chorionic gonadotropin alpha
cKO: conditional knockout
CL: corpus luteum
CMV: cytomegalovirus
co-: common
COCs: cumulus-oocyte complexes
CRE: cAMP response element
Cre: Cre-recombinase
CREB: cAMP response element-binding protein
CYP11A1: cytochrome P450 family 11A1
CYP19A1: cytochrome P450 family 19A1
DAG: diacylglycerol
DHT: didydrotestosterone
dNTP: deoxynucleotide triphosphate
dpc: day post coitum
E2: 17β-estradiol
EDN2: endothelin-2
EGF: epidermal growth factor
Egr-1: early growth response protein 1

ER: endoplasmic reticulum
ERE: estrogen response element
ERα: estrogen receptor alpha
ERβ: estrogen receptor beta
ESC: embryonic stem
Ex: exon
eYFP: enhanced yellow fluorescent protein
FBE: forkhead box protein L2 binding element
FBS: fetal bovine serum
FEZF1: FEZ zinc finger 1
FGF8: fibroblast growth factor 8
FOXL2: forkhead box protein L2
FSH: follicle-stimulating hormone
FSHR: follicle-stimulating hormone receptor
FSHβ/Fshb: FSH beta subunit
fx: floxed
GalNAc: N-Acetylgalactosamine
GDF: growth differentiation factor
GFP: green fluorescent protein
GH: growth hormone
Gly: glycine
GnRH: gonadotropin-releasing hormone
GnRHR: gonadotropin-releasing hormone receptor
GRIC: GnRH receptor-IRES-Cre
GS: glycine and serine
hCG: human chorionic gonadotropin
HH: hypogonadotropic hypogonadism
hpg: hypogonadal
HPG: hypothalamic-pituitary-gonadal
HRP: horseradish peroxidase
HRT: hormone replacement therapy
HSD17β: 17β-hydroxysteroid dehydrogenase
HSV: Herpes simplex virus
i.p.: intraperitoneal
iGRIC: inducible GnRH receptor-IRES-Cre
Inducible F2S4 cKO/F2S4 icKO: inducible *Smad4* and *Foxl2* conditional knockout
Inha: inhibin α subunit
IP_3: inositol 1,4,5-trisphosphate
IRES: internal ribosomal entry site
JRE: junctional regulatory element
KISS1R: kisspeptin receptor
KNDy: kisspeptin-neurokinin B-dynorphin
KS: Kallmann syndrome
LBD: ligand-binding domain
Leu: leucine
LH: luteinizing hormone

LHR: luteinizing hormone receptor
LHX3: LIM-homeodomain transcription factor 3
LHβ/*Lhb*: LH beta subunit
LoxP: locus of x-over P1
MAPK: mitogen-activate protein kinase
ME: median eminence
MH1: MAD homology N terminus
MH2: MAD homology C terminus
Neo: neo cassette
NFκB: nuclear factor κB
NK3R: neurokinin B receptor
NKB: neurokinin B
OCT: optimal cutting temperature
OVX: ovariectomy
PBS: phosphate-buffered saline
PCOS: polycystic ovary syndrome
PEI: polyethylenimine
PFA: paraformaldehyde
PGR/*Pgr*: progesterone receptor
Phe: phenylalanine
PIP_2: phosphatidylinositol 4,5-bisphosphate
PIT1: pituitary-specific transcription factor 1
PITX: *paired*-like homeodomain transcription factor
PKA: protein kinase A
PKC: protein kinase C
PLC: phospholipase C
PMSG: pregnant mare's serum gonadotropin
POMC: pro-opiomelanocortin
PRL: prolactin
PROK2: prokineticin 2
PROKR2: prokineticin 2 receptor
PROP1: *paired*-like homeodomain factor 1
rtTA: reverse tetracycline transactivator
s.c.: subcutaneous
S3/4 cKO/cKO in chapter 2: *Smad3* and *Smad4* conditional knockout
F2S4 cKO: *Smad4* and *Foxl2* conditional knockout
SARA: SMAD anchor for receptor activation
SBE: SMAD-binding element
SEMA-3a: class 3 semaphorin family
Ser: serine
SERM: selective estrogen receptor modulator
SF1: steroidogenic factor 1
SgII: secretogranin II
SHH: sonic hedgehog
SMAD: homolog of Drosophila mothers against decapentaplegic
SOX9: SRY box 9

SP1: specificity protein 1
SRC1: steroid receptor co-activator 1
SRD5A1: 3-oxo-5α-steroid 4-dehydrogenase 1
StAR: steroidogenic acute regulatory protein
T: testosterone
T3cKO: *Tgfbr3* conditional knockout
TAM: tamoxifen
TBX/Tpit: T box factor
Tet: tetracycline
TetR: Tet repressor
TGFBR3: transforming growth factor beta receptor type 3
TGFβ: transforming growth factor beta
TRE: Tet responsive element

Chapter 1 General introduction

Follicle-stimulating hormone (FSH), one of the pituitary gonadotropins, plays a fundamental role in regulating mammalian gamete maturation and reproduction. FSH and the other gonadotropin, luteinizing hormone [1], are both glycoproteins produced by gonadotropes located in the anterior pituitary gland. Synthesis and secretion of FSH are regulated by multiple factors in the hypothalamic-pituitary-gonadal (HPG) axis, including gonadotropin-releasing hormone (GnRH) from the brain, activins from the pituitary gland, and steroid hormones (e.g. androgens, estrogens, and progestagens) and inhibins from the gonads. Among these factors, activins and inhibins, which are transforming growth factor beta (TGFβ) superfamily members, selectively stimulate or suppress FSH production, respectively, whereas GnRH and steroid hormones regulate production of both gonadotropins. Although the molecular basis for the regulation of gonadotropin synthesis has been thoroughly investigated, it is not yet completely understood. The goals of my PhD studies were to further investigate the actions of activins and inhibins on FSH synthesis *in vivo*. For the purposes of this research, it is important to recognize that the regulation of gonadotropin production occurs in the context of a complex hormonal network within the HPG axis. Thus, in **Chapter 1** of my thesis, I will present our current knowledge of the HPG axis, with emphasis on how GnRH and TGFβ superfamily members regulate the synthesis and release of gonadotropins. At the end of this chapter, I will introduce the gaps between what is known and what remains to be understood about this subject, leading to the rationale for the experiments that I performed in the research chapters (**Chapters 2 to 4**).

1.1 Central control of mammalian reproduction within the hypothalamic-pituitary-gonadal (HPG) axis

1.1.1 Hypothalamus

The hypothalamus is a small brain region found in all vertebrates and is located on the ventral surface of the brain between thalamus and pituitary gland [2, 3]. The main function of the hypothalamus is to maintain biological homeostasis, such as relating to hunger, body temperature, circadian rhythms, stress and levels of circulating hormones [2-4]. It connects the nervous system in the brain to the endocrine system via the pituitary (also known as the master gland), or it communicates with other neurons in the brain [3]. There are two kinds of neurosecretory cells in the hypothalamus [5]. One set of cells produces neurohypophyseal hormones (e.g., oxytocin and vasopressin), which are released directly into the bloodstream from the posterior pituitary lobe [5]. The other set of cells produces "releasing" hormones that reach the anterior pituitary lobe (see section 1.1.2.2), including corticotrophin-releasing hormone, dopamine, growth hormone-releasing hormone, somatostatin, thyrotropin-releasing hormone, and gonadotropin-releasing hormone (GnRH) [6, 7]. These releasing hormones subsequently control the synthesis and/or release of pituitary hormones that control adrenal cortex, mammary gland, liver, thyroid, and gonadal functions [6, 7].

1.1.1.1 GnRH neurons

To stimulate synthesis of gonadotropin production by gonadotropes cells, the hypothalamus releases GnRH from GnRH neurons [8, 9]. GnRH neurons are born outside the brain at the level of the olfactory placode [10, 11]. During embryonic development, they migrate caudally into the hypothalamus [10, 12]. Once they arrive in the hypothalamus, GnRH neurons

project their axons towards the median eminence (ME), with their terminals reaching the hypophyseal portal system, where they release the neuropeptide (GnRH) to communicate with the anterior pituitary gland [13, 14] (Fig. 1.1). In mice, the migration of GnRH neurons to the hypothalamic region starts around embryonic day 12.5 (E12.5) and lasts until right before birth. [10, 12, 15]. Similarly, human fetal GnRH neurons depart from the nasal area during the mid-gestation period [16]. The proper migration of GnRH neurons from the olfactory placode to ME ensures a functional HPG axis [14]. Failure of GnRH neuron migration causes hypogonadotropic hypogonadism (HH) and infertility, such as Kallmann syndrome (KS). Indeed, almost all patients of KS are invariably sterile and approximately 50% are identified as having anosmia [13]. The cause of Kallmann syndrome is associated with various loss-of-function mutations in several genes that are responsible for proper GnRH neuron migration. These genes include fibroblast growth factor 8 (*FGF8*) and its receptor *FGFR1* [17-19]; prokineticin 2 (*PROK2*) and its receptor *PROKR2* [20, 21]; and FEZ zinc finger 1 (*FEZF1*) [22], a brain-derived neurotrophic factor (BDNF) [23] that is a secreted guidance cue of the class 3 semaphorin family (SEMA-3a) [24]. Mutations of these genes primarily alter olfactory placode development, thus disrupting GnRH neuron migration from the olfactory placode into the hypothalamus [25, 26].

GnRH neurons are unique in the mammalian nervous system, as they do not have discernible axons and dendrites. Rather, they are bipolar cells, with both processes projecting to the ME and possessing both axonal and dendritic propertiess [14, 27]. These unique neuronal processes are termed ‘dendrons’ and are presumed to underlie important aspects of GnRH neuronal function [28].

1.1.1.2 GnRH pulsatile and surge release

GnRH, a highly conserved decapeptide found in all vertebrates, is released at the ME by the axon terminals of GnRH neurons [29, 30]. GnRH is secreted in a pulsatile manner with varied amplitudes and frequencies [31, 32]. The intermittent pattern of GnRH release is essential for its ability to stimulate gonadotropin synthesis and secretion by the pituitary gland [33-37]. The importance of GnRH pulsatile release has been demonstrated in many species, including humans and non-human primates [33, 38-41]. In rhesus monkeys with hypothalamic lesions, which do not secrete endogenous GnRH, administration of exogenous GnRH in pulsatile (once per hour), but not constant pattern, rescued gonadotropin secretion [33]. In contrast, sustained GnRH stimulation desensitizes or downregulates gonadotrope function [33]. Moreover, GnRH pulse frequencies determine the preferable stimulation of FSH or LH synthesis and release during reproductive ages [42]. Specifically, a higher frequency (e.g. once per hour) favours LH release; whereas, a slower frequency (e.g. once every 3-4 hours) favours FSH release [34, 42-46]. Moreover, the amount of LH release corresponds to the amplitude of GnRH stimuli, whereas FSH is not as sensitive to GnRH amplitude [45]. The pulse frequency exhibits dynamic changes during each reproductive cycle in females, with a relatively high frequency and amplitude during the follicular phase (every 1.5 hrs) and a slow frequency during the luteal phase (every 6 hrs) in women (see section 1.1.3.3.2) [30].

A normal adult murine brain contains around 1,000 GnRH neurons, which are distributed from diagonal band, medial septum, preoptic area (POA) and anterior hypothalamic area to establish appropriate neurosecretory contacts with the ME [47, 48]. The pulsatile release of GnRH suggests that the activity of dispersed GnRH neurons must somehow be synchronized for coordinated secretion of the hormone [49, 50]. Previously, mounting evidence has shown that

GnRH pulses are remotely controlled by a 'pulse generator' located in the arcuate nucleus (ARC) of the hypothalamus, as complete destruction of the ARC abolishes gonadotropin release [51-54]. In the ARC, a subset of neurons that co-express three neuropeptides, including kisspeptin [55-58], neurokinin B (NKB), [1, 59], and dynorphin (DYN) [60, 61], the so-called KNDy neurons, have been strongly implicated in the control of pulsatile release by GnRH neurons [62]. KNDy fibers contact with both GnRH cell bodies and terminals in the ME [52, 63-65]. The direct interaction of KNDy cells with GnRH neurons appears highly conserved in many species, including rats [66, 67], mice [68], rhesus monkeys [69], and humans [63]. However, it has been clearly demonstrated that KNDy cells mainly project to the ME to establish close contact with GnRH terminals [67, 69, 70]. Additionally, the KNDy cells reciprocally interconnect at the ARC, and the establishment of this network may play a role in the generation of pulsatile GnRH release [71-73]. However, only the neurokinin B receptor (NK3R) and the dynorphin receptor (κ-EOPR) have been detected post-synaptically on KDNy cells, whereas the kisspeptin receptor (KISS1R or GPR54) is predominantly found in GnRH cells [70, 74-81]. Based on these and other observations, the proposed model for KNDy synchronous activity is modulated by reciprocal regulation of NKB and DYN and their output to GnRH neurons is primarily mediated by kisspeptin. Specifically, NKB has been demonstrated playing stimulatory role in GnRH release in sheep [82] and human [59]; whereas DYN appears inhibiting GnRH pulse frequency in sheep and human [61, 83]. However, these regulatory roles of NKB and DYN in rodent is less clear [52].

It has been demonstrated that pulsatile release of GnRH mediated by KNDy neurons is regulated by circulating steroid levels [84, 85]. The sex steroid hormones are produced by the gonads in responds to FSH and LH stimulations, which in turn, send either positive or negative feedback effect to the hypothalamus and pituitary levels to regulate gonadotropin production [86-

88]. Indeed, KNDy neurons express high levels of gonadal steroid hormone receptors, including estrogen receptor α (ERα), progesterone receptor (PGR) and androgen receptor (AR) [71-73, 89-91]. In mice, sheep, and primates, estrogen replacement following ovariectomy decreases the level of kisspeptin coding gene (*Kiss1*) expression in the ARC [92-96]. This evidence suggests that estrogen negative feedback effect in the hypothalamus is through the regulation of KNDy neuron activity rather than at the level of GnRH neurons. Indeed, GnRH neurons do not express steroid receptors [97-99]. In addition, estrogens inhibit *Kiss1* mRNA level in the ARC via nonclassical mechanism that does not involve estrogen response element signaling (see section 1.2.4.2) [100]. Whereas, the progesterone feedback effect is mediated via inhibiting DYN from KNDy neruons in ewes and primates [60, 61, 72, 101]. However, it is less clear whether DYN mediates progesterone negative feedback in rodents.

Whether the same population of KNDy neurons are also responsible for preovulatory GnRH surge, which trigger ovulation event in females, is not clear. However, compelling evidence suggests that a distinct population of kisspeptin neurons located in the POA regulates the GnRH surge in several species, including pig, sheep, and non-human primate but not in rodent (Fig. 1.1) [102-112]. Whereas, estrogen acts on kisspeptin neurons located in the anteroventral periventricular nucleus (AVPV) and adjacent areas to induce the preovulatory GnRH surge in rodents [113]. Like the KNDy neurons in ARC, kisspeptin neurons in the AVPV or POA also express ERα [113]. The levels of *Kiss1* mRNA in the AVPV or POA are also dependent on estrogen levels throughout the estrous cycle (see section 1.1.3.3.2), and is activated at the time of preovulatory surge [113-115]. Additionally, GnRH surge release can be influenced by various external and internal factors, such as day lights, stress and metabolic status [113, 116-120].

Taking together, GnRH secretion from the hypothalamus is regulated by neuronal networks. The pulse and surge modes of GnRH release are mediated by different subgroups of kisspeptin neurons. Specifically, KNDy neurons located in the ARC synchronize GnRH neurons and send stimulatory signals that generate GnRH pulses and mediate gonadal steroid negative-feedback on GnRH release (see section 1.2.4) [71-73, 75, 89-91, 95, 121-123]. On the other hand, a distinct subpopulation of kisspeptin neurons located in the AVPV or POA control pre-ovulatory GnRH/LH surge evoked by positive-feedback actions of estrogen (Fig. 1.1). However, more questions remain to be answered, such as the precise molecular basis of KNDy cells responses to steroid hormones and whether additional components are involved in the regulation of GnRH release. Intriguingly, the discovery of kisspeptin's control of GnRH secretion has promoted the application of kisspeptin receptor agonists and antagonists in clinical trials for the treatment of multiple reproductive disorders, such as polycystic ovary syndrome PCOS, and in assisted reproduction [124-126]. PCOS is common, affecting 2-20% women of reproductive age, and most patients exhibit abnormally elevated GnRH-pulse frequency, leading to an imbalance in the ratio of LH to FSH [127-129]. The elevated pulse frequency may be a result from reduced sensitivity to the gonadal steroid negative feedback or the malfunction of GnRH pulse generator (e.g. KNDy neurons). Therefore, gaining knowledge of the molecular mechanisms of the GnRH pulse generator will provide therapeutic opportunities to patients with PCOS and related disorders of gonadotropin secretion.

1.1.2 Pituitary

1.1.2.1 Pituitary cell lineages

The pituitary, or "master gland", homeostatically controls metabolism, growth, reproduction, and lactation by secreting hormones that target various peripheral tissues. The pituitary gland is composed by three independent parts: the anterior pituitary lobe, the posterior pituitary lobe, and the intermediate lobe. The anterior pituitary lobe produces endocrine hormones, which are controlled by the hypothalamus [130]. In turn, it receives feedback from the peripheral endocrine organs including the gonads, the thyroid gland, and the adrenal glands to maintain hormonal homeostasis [130]. The anterior pituitary contains five hormone-producing cell types that are defined by the hormones they synthesize and secrete, as well as one non-endocrine cell type, the folliculo-stellate cells [131-133]. Somatotropes secrete growth hormone (GH), which acts on liver, muscle and bone tissues; thyrotropes secrete thyroid-stimulating hormone, which acts on the thyroid gland; corticotropes make adrenocorticotropic hormone (ACTH), which regulates adrenal function; lactotropes produce prolactin (PRL), which promotes milk production; and gonadotropes synthesize the two gonadotropins, FSH and LH [1, 134], which controls gonadal development and function [130]. Generally, the entire pituitary population is composed of approximately 50% of somatotropes, 10-25% of lactotropes, 10-20% corticotropes, 10% of thyrotropes and 10% of gonadotropes [133, 135]. The function of posterior lobe is distinct from that of the anterior lobe. Rather than synthesizing hormones, it is composed of axons and terminal of oxytocin and anti-diuretic (vasopressin) neurons [136]. The intermediate lobe secretes several bioactive peptides, such as alpha-melanocyte-stimulating hormone (α-MSH). In some species, α-MSH is involved in the process of skin colour adaptation [137].

1.1.2.2 Anterior pituitary development

The anterior pituitary (hereafter, pituitary) is differentiated from oral ectoderm during embryonic/fetal development [6, 138]. Pituitary organogenesis begins at E8.5 in mice, marked as the formation of Rathke's pouch, which is the primordium of the anterior pituitary [139-141]. In the following day of development until E17.5, the five pituitary endocrine cell types emerge in a temporally- and spatially-defined fashion [139]. The initial differentiation involves a combination of many signaling molecules and transcription factors, whereas subsequent terminal differentiation is controlled by cell-specific genes (Fig. 1.2) [139].

At E9, the initiation of pituitary cell differentiation requires signals from the ventral diencephalon, which directly contacts with Rathke's pouch, including WNT family 5A (WNT5A), bone morphogenetic protein 4 (BMP4), fibroblast growth factor 8 (FGF8) and FGF10. The onset of these gene expression coincides with the initial development of Rathke's pouch [139]. And the oral ectoderm provides a ventral Sonic hedgehog (SHH) [142-144]. By E11.5, the ventral → dorsal BMP2 and SHH and the opposing BMP4, FGF8/10 form signaling gradients result in positional commitment of pituitary cell lineages [139, 142, 144]. Between E11.5 to E13.5, activation of LIM-homeodomain transcription factor 3 (LHX3) and *paired*-like homeodomain factor 1 (PROP1) is required for all cell lineages except corticotropes, which instead are determined by the expression of T box factor 19 (TBX19 or Tpit) [145, 146]. During the terminal differentiation, Tpit induces pro-opiomelanocortin (*POMC*) expression, which enables the differentiation of pituitary cells into POMC-expressing corticotropes [147]. Due to the early expression of POMC (E12.5 in mouse), the corticotropes are the first hormone-producing cells of the pituitary to reach terminal differentiation [138]. Between E12.5 and E14.5, the pouch robustly expresses a PROP1, which regulates the POU domain, class 1, transcription factor 1 (PIT1, also known as POU1F1) [148].

Timely expression of PIT1 determines the terminal differentiation, growth, and survival of somatotropes, lactotropes and thyrotropes [138, 149, 150]. Additionally, PROP1 may also contribute to gonadotrope differentitation, as *Prop*-deficient mice failed to initiate both proliferation of the three PIT1-dependent cell types and proliferation of gonadotropes [140, 148]. The last cell type that reaches terminal differentiation is gonadotropes [151, 152]. The initial high level of PIT1 expression occurs on E13.5 under the control of PROP1, and then the expression is reduced between E16.5-17.5 [153]. Coincidently, BMP2-mediates the highest level of GATA2 expression during E16.5-E17.5, suggesting that GATA2 may contribute to the final differentiation of gonadotropes by inhibiting PIT1 expression at the same time [154]. Additionally, steroidogenic factor 1 (SF1) was shown to be involved in the terminal differentiation of gonadotropes [151, 155-157]. However, exogenous administration of GnRH in *Sf1* knockout mice stimulates gonadotropin production, excluding essentiality of SF1 for gonadotrope-specification [158]. Overall, expression of critical transcription factors determines the final differentiation of pituitary cell lineages. The five cell types emerge between E12.5 (corticotropes) and E17.5 (gonadotropes) (Fig. 1.2) [5, 139, 142, 143, 159, 160].

1.1.3 Gonadotropins and their roles in reproduction

1.1.3.1 FSH and LH

Pituitary gonadotropes produce two gonadotropin hormones, FSH and LH, which play fundamental roles in mammalian reproduction [161]. FSH and LH are dimeric glycoproteins composed of a shared α subunit (chorionic gonadotropin alpha, CGA) noncovalently linked to unique β subunits (FSHβ and LHβ), the latter of which confers its biological specificity [162, 163]. In humans, horses and non-human primates, an additional gonadotropin, chorionic gonadotropin

(CG), is synthesized by the placenta, and its β subunit is encoded by the *CGB* gene [164, 165]. CG contains LH-like activity, and is essential in maintaining the corpus luteum, which produces progesterone during pregnancy [166]. In both males and females, the main functions of gonadotropins are to stimulate the maturation of gametes, to produce steroid production and to maintain reproductive functions [162]. The hormones elicit their biological functions through their cell-surface receptors, which are mainly expressed in the gonads [167, 168].

1.1.3.2 Gonadotropin assembly and glycosylation

Gonadotropin α and β subunit genes are independently translated as pre-prohormones that contain signal peptides of 18-24 amino acids, which are cleaved during translation [162, 169]. Additionally, each gonadotropin subunit has carbohydrate chains attached to asparagine [170] residues [165, 170]. In the rough endoplasmic reticulum (ER), α and β subunits are noncovalently lined, and oligosaccharides are attached to the Asn [165, 171]. After glycosylation, the heterodimers are transported to the Golgi apparatus via vesicles, where hormone-specific modifications in carbohydrate structure occur [165]. Free α subunits are also released from ER because they are produced in excess to the β subunits, suggesting that β subunit synthesis is rate-limiting in the production each specific hormone [172-174]. The α chain contains two and the β subunits contain either one or two (FSH) Asn residues that can be glycosylated, respectively [175]. Deglycosylation of the α subunit impacts the specific receptor signaling on gonadotropin-responsive cells; however, hormone-receptor binding is intact, suggesting that the level of α subunit glycosylation is not required for receptor binding [176].

In the Gogi apparatus, oligosaccharides on the terminal β chain of FSH are differently modified from those on LH [169, 177-181]. Specifically, LHβ is predominantly modified with

sulfate and GalNAc (a common sugar in O-linked carbohydrates, but not commonly found on Asn-linked oligosaccharides) [180], whereas FSHβ is mostly modified with sialic acid and galactose [169].

This distinct modification (sulfonation vs sialylation) determines the half-lives of FSH and LH proteins in circulation, as well as their bioactivities in the gonads [169, 182-184]. Upon release from gonadotropes, LH is rapidly (~20-30 min in humans) broken down in hepatic endothelial cells, which express an enzyme that recognizes and binds to the attached sulfated carbohydrates [185]; by contrast, the presence of sialic acid attached to FSH extends the time for liver enzymatic recognition and prolongs FSH half-life (3-4 hours in humans) in the circulation [186]. In addition, the levels of glycosylation on the β chain differentiate each glycoprotein into an array of isoforms, which differ in bioactivity [169, 187-189]. Human studies have categorized multiple isoforms of FSH by their levels of glycosylation [190-193]. The abundance of glycosylation influences FSH binding to FSHR, thereby determines its potency in the gonads [194]. Specifically, hypoglycosylated FSH (FSH^{21} or FSH^{18}) is more efficient than fully glycosylated FSH (FSH^{24}) in stimulating FSHR downstream signaling and in promoting steroid production [195]. The numbers reflect the molecular sizes of the proteins. Different isoforms of FSH co-exist in all women, however, the ratio of these isoforms exhibits dynamic changes throughout normal menstrual cycle and at different ages [159, 196]. In particular, $FSH^{21/18}$ is predominantly expressed in young girls and shows peak abundance during the mid-cycle stage [159, 196], whereas, FSH^{24} is predominantly expressed in post-menopausal women and exhibits a high plateau without a mid-cycle peak during normal reproductive cycles [159, 190, 196, 197]. The mechanism responsible for determining the ratio of these isoforms has yet to be defined. One study has suggested that it is associated with the available oligosaccharyltransferase in the ER, which transfers oligosaccharide

to a nascently translated polypeptide (e.g. FSHβ) [196]. In contrast to FSH, LH is secreted with a more uniform post-translational modification at all times [193]. However, there is a dramatic transition from less glycosylated isoform to more glycosylated LH at male puberty [193].

Upon leaving Golgi apparatus, both gonadotropins are stored in secretory granules prior to release from gonadotropes [198, 199]. However, LH is dominantly packaged together with secretogranin II (SgII) in small, dense core granules and is sorted into the regulated secretory pathway [200-204], whereas FSH is mainly packed in larger, more diffuse granules that can be constitutively secreted [165, 200, 205-208]. The precise molecular basis for the distinct packaging following budding from trans-Golgi network is not fully understood, however, because the two gonadotropins share an identical α subunit, the differentially regulated intracellular trafficking must be determined by sequences within their specific β subunits. Indeed, some studies has reported that the LHβ, but not FSHβ, contains a hydrophobic C-terminal heptapeptide (Leu-Ser-Gly-Leu-Leu-Phe-Leu), which functions as a sorting signal for LH entry into the regulated pathway [209, 210]. An LHβ mutant lacking the heptapeptide is secreted constitutively from gonadotrope cells [209, 210]. Likewise, adding the heptapeptide sequence to FSHβ results in the rerouting of FSH to the regulated secretory pathway [211]. Remarkably, a recent study demonstrated that redirecting FSH to the regulated secretory pathway in mice dramatically increases ovarian responsiveness to FSH stimulation [212].

1.1.3.3 Gonadotropin actions on the gonads

It has been known for nearly a century that the anterior pituitary secretes hormones that control gonadal steroidogenesis and promote germ cell maturation in both males and females [213-

217]. In the gonads (testis and ovary), gonadotropins target the somatic cells that surround the germ cells (Fig. 1.3).

1.1.3.3.1 Gonadotropin functions in the testis

Both LH and FSH contribute to spermatogenesis by stimulating Leydig cells and Sertoli cells, respectively (Fig. 1.3A) [218]. LH stimulates testosterone (T) production by Leydig cells. T actions in Sertoli cells are essential for spermatogenesis [219-221]. The essentiality of LH is best demonstrated in mice lacking the LHβ subunit or the LH receptor (LHR). Knockout males exhibit significantly reduced testicular mass, prominent Leydig cell hypoplasia, defects in the expression of steroidogenic enzymes, reduced testosterone levels, and arrested spermatogenesis at the spermatid stage [220, 222]. LH acts on its receptor LHR, a G-protein coupled receptor, expressed on the Leydig cells [223, 224]. Binding of LH to LHR activates the adenylate cyclase-cyclic AMP (cAMP)-protein kinase A (PKA) pathway [225-227]. Activated PKA translocates to the nucleus and induces gene expression that is essential for steroidogenesis (e.g. steroidogenic acute regulatory protein (StAR)) [228-230]. Following de-esterification, StAR promotes translocation of cholesterol from the outer to inner mitochondrial membrane, where cholesterol is metabolized to pregnenolone by the cytochrome P450 side chain cleavage enzyme (CYP11A1) [225, 231]. Pregnenolone is then transported from mitochondria to the smooth ER, where it is metabolized into testosterone by a series of enzymes, including CYP17, 3β-hydroxysteroid dehydrogenase (HSD3β), and 17β-hydroxysteroid dehydrogenase (HSD17β) [232, 233]. Upon synthesis, testosterone is secreted from Leydig cells into circulation or into the neighboring Sertoli cells. In the circulation, testosterone exists either free (~4%) or bound to extracellular proteins (the remaining population), such as androgen binding protein (ABP), the testis-produced sex hormone-

binding protein (SHBG) [234, 235]. ABP is produced by Sertoli cells in the seminiferous tubules. The function of ABP has been ambiguous, mostly due to the lack of null mutation models to date. However, some researchers have proposed that ABP carries testosterone to the epididymis, and concentrates testosterone in the seminiferous tubules, a critical step for spermatogenesis [236-239]. Additionally, ABP has been proposed to regulate 3-oxo-5α-steroid 4-dehydrogenase 1 (SRD5A1), an enzyme that converts testosterone to a more potent androgen, dihydrotestosterone (DHT) [240-242]. Importantly, the expression of ABP is stimulated by FSH in Sertoli cells [243]. Unlike LH, FSH targets Sertoli cells in the seminiferous tubules. In addition to ABP, FSH also stimulates inhibin B production in Sertoli cells, which negatively feeds back to the pituitary and specifically decreases FSH synthesis (see section 1.2.2.1 and 1.1.2.6) [244]. Furthermore, FSH stimulates prenatal and pre-pubertal proliferation of Sertoli cells and determines their final cell numbers in the entire life, thereby determining testicular size and spermatogenic potential [245, 246]. The requirement of FSH to stimulate sperm production and to support male fertility in the adult appears to be species-dependent, as discussed in detail in **Chapter 3**.

1.1.3.3.2 Gonadotropin functions in the ovary

Unlike the life-long spermatogenesis in males, the final number of oocytes (the female germ cell) is determined during embryonic development [247]. At birth, the ovary contains a pool of primordial follicles. Each unit is formed by one oocyte surrounded by a single layer of flattened somatic cells, namely granulosa cells (Fig. 1.3B) [248]. Shortly after birth, primordial follicles gradually but continually grow. Although the nature of the signals initiating follicular development is unknown, we know that this initial growth is gonadotropins-independent [249, 250]. After female puberty, one or a few follicles will be selected during each reproductive cycle. Once

selected, the follicles undergo development and eventually meet one of two fates: ovulation or atresia [251]. Once the follicle develops an antrum, a cavity filled with fluid, granulosa cells respond to FSH stimulation leading to proliferation. The dominant follicles also promote the oocytes growing advance of antral stage [252]. Meanwhile, a layer of cells become evident outside the follicls, called theca layer [253]. The precise origin of theca cells is still unclear, but they are thought to be recruited from surrounding stromal tissue by factors secreted from the follicles [253]. Theca cells act like Leydig cells in the testis, which respond to LH stimulation and metabolize cholesterol into androgens (testosterone and androstenedione), most of which diffuses through the basement membrane to the neighbouring granulosa cells [253, 254]. Granulosa cells express FSH receptor (FSHR). The binding of FSH to FSHR directly stimulates aromatase (encoded by *CYP19A1*) expression, which subsequently converts androgens into estrogens [253, 255, 256]. Like in male Sertoli cells, FSH also stimulates inhibin B production from the growing granulosa cells [257]. Ovarian-derived steroids and inhibin B, in turn suppress gonadotropin secretion from the pituitary (see section 1.2.2.1, 1.2.2.6 and 1.2.4).

During each cycle, multiple follicles reach early antral stages; however, only a few become dominant follicles and continuously grow to the next stage, while the others undergo atresia [258-260]. Although the selection of the dominant follicle is not completely understood, it is thought to be dependent on the duration of FSH stimulus as well as FSHR expression in each follicle [261, 262]. As the antrum develops, the granulosa cells differentiate into two types. The type immediately associated with the oocyte are called cumulus cells; the other type, located underneath the theca layer, are called “mural” granulosa cells. Mural cells act distinctly from cumulus cells. Instead of talking with the oocyte, mural granulosa cells up-regulate LHR expression in response to FSH stimulation [263]. As the antrum grows and granulosa cells proliferate, a dramatically large

amount of estrogen is produced, which subsequently sends a positive feedback signal to the pituitary and the hypothalamus, thereby triggers LH surge and subsequently initiates ovulation [29]. Just prior to ovulation, LH stimulates mural granulosa cells to synthesize and secrete epidermal growth factor (EGF) family members, amphiregulin, epiregulin and beta-cellulin into the antrum [264, 265]. These factors reach cumulus cells to promote the synthesis and secretion of a hyaluronic acid-rich extracellular matrix, which mediates cumulus expansion and results in dissociation of cumulus cells from tight contact with the oocyte [266-269]. Meanwhile, mural granulosa cells express other factors, such as endothelin-2 (EDN2), a potent vasoconstrictor, which diffuses into the theca externa, where it initiates the final follicular rupture and meiosis resumption. [270, 271]. During fetal development, oocytes enter prophase stage of meiosis I and are arrested at the diplotene stage, until LH surge-mediated ovulation [272, 273]. Once leave the ovary, oocyte(s) spontaneously resumes meiosis without hormonal stimulation, however, the process will only complete when fertilize, a phenotype conserved in all mammalian species [274-277]. The precise mechanism for the immediate meiosis resumption following ovulation is not clear, however, it may associate with the removing of meiosis inhibitory once leave the ovary [274, 278].

Furthermore, following ovulation, mural granulosa, theca, capillaries and fibroblasts rapidly form a new transient structure in the ovary called corpus luteum (CL). The main function of the CL is to produce progesterone, a steroid hormone required for pregnancy maintenance [279, 280]. Additionally, in primates, the CL secrets inhibin A, which acts like inhibin B produced by growing granulosa cells, to negatively regulate FSH production in the pituitary gland. If fertilization does not occur, the CL regresses (luteolysis), allowing a new cycle to begin [279].

Follicular development program is highly conserved in mammals, though the duration of each reproductive cycle varies depending on the species. For example, each reproductive cycle

lasts for ~28 days (a menstrual cycle) in women but only lasts for 4-5 days (an estrous cycle) in rodents. The human menstrual cycle can be divided into two phases separated by ovulation and menstruation: the follicular phase and the luteal phase (Fig. 1.4A). In contrast, the rodent estrous cycle can be divided into four stages: proestrus, estrus, metestrus, and diestrus [281]. The high levels of estrogen on the afternoon of proesturs stimulates reproductive behavior, and LH surge in rodents occurs precisely in the proestrus afternoon, followed by ovulation in the early morning (estrus). In addition to the GnRH-mediated FSH/LH surge that induces ovulation, a secondary FSH (but not LH) surge occurs in the early estrus morning in rodents (Fig. 1.4B). The secondary FSH surge is critical in recruiting multiple ovarian follicles and in preparing for the next ovulation [282, 283]. Importantly, the secondary FSH surge is GnRH-independent, and is instead mediated by activins produced by the pituitary gland. Compelling evidence has demonstrated that enhanced activin stimulation during the early estrus morning is coincident with the dramatic decline of inhibin produced in the ovary, suggesting an opposing relationship between pituitary activins and gonadal inhibins (Fig. 1.4B) (see section 1.2.2.1, 1.2.2.3 and 1.2.2.6) [257, 283-287].

1.2 Regulation of FSH synthesis

Synthesis and secretion of gonadotropins by pituitary gonadotropes are regulated by hormones produced in the HPG axis, including hypothalamic GnRH, pituitary activins, as well as the gonadal steroid hormones and inhibins. GnRH and steroids regulate both FSH and LH synthesis and release, whereas, activins and inhibins specifically stimulate or inhibit FSH production in the gonadotropes, respectively. Additionally, some BMPs are implicated in FSH regulation; however, most of their effects were only observed *in vitro* but not *in vivo*.

1.2.1 GnRH signaling

GnRH is essential for gonadotropin synthesis and release. Loss-of-function mutations in *GNRH1*/*Gnrh1* (the GnRH encoding gene) [288, 289] or in its receptor genes (*GnRHR*/*Gnrhr*) [290-293] cause delayed or absent of puberty and infertility. Pituitary sensitivity to GnRH stimulation is dependent on the level of its receptor's (GnRHR) presence on gonadotropes (Fig. 1.5) [294-297].

The GnRH receptor (GnRHR) is a member of G protein-coupled receptor family and is predominantly expressed on gonadotropes in the anterior pituitary [298]. The GnRHR extracellular ligand binding domain, as well as the seven transmembrane domains are highly conserved between many species [299]. However, mammalian GnRHR lacks the cytoplasmic tail (C-tail), which is present in the GnRHR of non-mammalian species [300]. The absence of the C-tail from the mammalian GnRHR decreases the rate of receptor internalization after GnRH binding, thereby it was proposed to be responsible for the longer lasting LH surge in mammals [300-304]. The binding of GnRH to GnRHR dissociates and activiates the Gα-proteins coupled to the receptor, including Gαq/11 and/or Gαs [305-310]. Activation of Gαq/11 activates phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into two second messengers: inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 then binds to its receptor on the endoplasmic reticulum (ER) to trigger release of intracellular-stored calcium (Ca^{2+}) from the ER into the cytosol [311-314]. The intracellular Ca^{2+} mobilization in response to GnRH stimuli is biphasic and oscillatory, with a rapid initial peak followed by a lower, but sustained, cytosolic Ca^{2+} increase during continued exposure to GnRH [311, 315]. This rapid initial Ca^{2+} peak leads to a large release of gonadotropins from vesicles (mostly LH, but also some FSH) [316-321]. Evidence suggests that the initial Ca^{2+} response (phase I) is partially caused by an extracellular Ca^{2+} influx,

whereas the sustained LH synthesis and secretion (phase II) is mainly mediated by an extracellular Ca^{2+} influx through voltage-gated L-type Ca^{2+} channels during membrane depolarization [311, 313, 320]. Elevated Ca^{2+} together with DAG activate protein kinase C (PKC), which then activates mitogen-activated protein kinase pathways (MAPKs) [322-324]. The activated MAPKs and subsequently phosphorylated ERK1/2 promotes expression of the LHβ subunit (*Lhb*) and the common α subunit (*Cga*) (Fig 1.5) [325, 326].

Although early observations demonstrated that GnRH increases pituitary cyclic adenosine monophosphate (cAMP) accumulation, there was no proof to support the idea that GnRH receptor couples to Gαs, therefore the receptor was thought to bind uniquely to Gαq/11 [327, 328]. Following significant advancement in biological tools during the past decades, more studies have demonstrated the activation of cAMP/protein kinase A (PKA) pathway in GnRH-mediated gonadotropin synthesis (Fig. 1.5) [228, 329-333]. Interestingly, the activation of cAMP occurs in response to GnRH stimulation appears exclusively at proestrus stage during the rodent estrus cycle [334]. Consistent with this observation, blocking GnRH action at diestrus blocks cAMP increase in the following proestrus, suggesting that the cAMP pathway is only recruited during preovulatory surge [333]. Importantly, activation of cAMP by GnRH increases LH release [335-337]. However, the molecular mechanism for this increase has not been characterized [338]. Moreover, some studies suggested that cAMP accumulation by GnRH appears Ca^{2+}-dependent, and requires phosphorylation of ERK1/2 for stimulating *Lhb* expression, suggesting that GnRH may signal through both Gαq/11 and Gαs [339]. Currently, the relative roles of Gαs and Gαq/11 in GnRH signaling in physiological conditions remain unclear and the relevance of the two pathways *in vivo* remains to be reported.

Clearly, GnRH stimulates FSH synthesis *in vivo*; the mechanism of this synthesis, however, is poorly characterized [340, 341]. Studies using heterologous cells (e.g. HeLa and COS7), identified two activator protein-1 (AP-1) like enhancers in the ovine *Fshb* promoter, which are required for GnRH induction *in vitro* [342, 343]. Additionally, these AP-1 like sequence are likely conserved in many other species, such as human, cow and pig [343]. However, *Fshb* promoter activity in transgenic mice lacking both AP-1 sites appears no different to the wild-type control group, excluding their function in GnRH-mediated *Fshb* transcription *in vivo* [344]. Recently, some other studies suggested that GnRH pulses at low frequency stimulate *Fshb* transcription through activation of cAMP/PKA and thereby activating cAMP response element-binding protein (CREB) in gonadotrope like cell lines, reflecting Gαs activation but not conventional Gαq/11 [345, 346]. However, this mechanism has not yet been confirmed *in vivo*.

1.2.2 Inhibin and activin receptor signaling

Activins and inhibins, members of TGFβ superfamily, play pivotal roles in the regulation of mammalian reproduction. Unlike GnRH, which regulates both FSH and LH production, activins and inhibins specifically regulate FSH, but not LH synthesis by gonadotropes. Pituitary-derived activins stimulate *FSHβ*/*Fshb* transcription, whereas gonad-derived inhibins suppress FSH synthesis by antagonizing the actions of activins.

1.2.2.1 Discovery and purification of inhibins, activins, and follistatins

The inter-relationship of testis and pituitary was first described by Mottram and Cramer in 1923 [347]. In their reports, male rats exhibited pituitary hypertrophy and obesity after exposure to radium. They proposed that the changes in the pituitary anterior lobe of after irradiarion of the

testes can be attributed to the absence of an internal secretion of testicular origin [347]. In 1932, McCullagh dubbed this testicular-derived, water-soluble hormone as 'inhibin' [348]. The evidence of inhibin-like activity in follicular fluid in females was first reported by De Jong et al. in 1976 [349]. In their study, intraperitoneal injection (i.p.) injection of bovine follicular fluid (from the antra of large follicles) specifically suppressed FSH but not LH in male rats [349]. These observations suggested the gonads secret factors that is required for maintaining normal reproductive biology and endocrine systems, thereby encouraging a large global collaboration/competition for characterizing the inhibin protein.

Fifty-three years after inhibin-activity was first reported, a 32 kDa protein with inhibin-like activity was first successfully isolated from porcine follicular fluid [350]. The isolated molecule contained two disulfide-linked chains, one of 18-kDa (inhibin α) and the other of ~14 kDa (inhibin β) molecular weight [350]. A following study further discovered two isoforms of the β chains, one of 14.7 kDa (inhibin βA) and the other one of 13.8 kDA (inhibin βB) [351, 352]. By chance, the researchers observed that homo- and hetero-dimers of the β chains positively stimulated FSH release in cultured pituitary cells [353, 354]. Based on their opposite biological effects to inhibins, the proteins were named activins [353]. The structural organization of the β subunit were found to be homologous to that of the TGFβs, therefore, both activins and inhibins were categorized into the large TGFβ superfamily [351, 355]. Like other TGFβ superfamily ligands, inhibin α and β subunits are produced as preproproteins [356]. The α subunit precursor contains two polyarginine cleavage sites and β subunit precursors each contain one cleavage sequence [357]. The prodomains are critical for dimeric assembly of the two subunits and for appropriate protein folding [358]. According to the activin A structure, the two βA subunits dimerization was mediated by both disulfide (covalent) bonding but also by hydrophobic

(noncovalent) interactions [359]. The structure of Activin B, activin AB, inhibin A and B, however, have yet to be solved, therefore, the dimeric assembly of these molecules are currently unclear. Notably, free α subunit isoforms are also secreted in excess of inhibin dimers, but are biologically inactive [360]. Before secretion, all precursors are cleaved by furin-like proprotein convertases from the mature domains to form the biologically active protein [358]. Due to the multi-step biosynthetic pathway, purification of inhibins results in a poor yields for several reasons [356]. The main reason for this is that inhibin preparations always contain 'contaminating' activins due to the dimerization of β subunits [356]. Encouragingly, a recent study reported that modification of protease cleavage sequences in both α and βA subunit yields a better bio-active form of inhibin with a dramatic bias assembly toward a higher ratio (~50 fold) of inhibin A over activin A production [361]. The modification of the βB subunit has yet to be reported; however, it is theoretically promising [361].

In addition to activins, the purification procedure from porcine ovarian follicular fluid also unexpectedly led to the isolation of another FSH-inhibitory protein, follistatin [362-364]. Follistatin is a single chain glycoprotein derived from one of the two polypeptide core sequences of 288 or 315 amino acids (FS315 or FS288) and encoded by an alternatively spliced mRNA [365, 366]. Between the two isoforms, FS315 is the dominant form of circulating follistatin [367], though FS288 is 6-10 times more potent at inhibiting FSH secretion [365].

1.2.2.2 TGFβ signaling

Both activins and inhibins belong to TGFβ superfamily. This family is composed of a group of structurally related growth factors, including TGFβs (TGFβ1-3), bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs) as well as activins and inhibins. The whole family,

except inhibins, functions through transmembrane heterotetrameric serine/threonine kinase receptors [368, 369]. The receptors are classified into two types: type I and II. Both types of receptors are composed of a short extracellular domain that binds to the ligands, a single transmembrane domain, and a long cytoplasmic tail that contains kinase activities [370]. Thirty-three TGFβ ligands have been identified; however, only five type II receptors and seven type I receptors have been characterized for the entire family, suggesting that the ligands share receptors. TGFβ ligands first bind to either the type I or the type II receptor dimer, and then they recruit the other type of receptor dimers into the ligand-receptor complex [371-373]. Regardless of the order of binding, upon formation of the ligand-receptor complex, type II receptors phosphorylate the associated type I receptors at the serine residues at the glycine and serine-rich sequence (GS region) in the juxtamembrane domain [374, 375]. The activated kinase activity of type I receptors then phosphorylate two serine residues at the C-terminal end of receptor-regulated homologs of the *Caenorhabditis elegans* protein SMA and the *Drosophila* mothers against decapentaplegic homolog (MAD), or R-SMAD [370, 376]. There are two subclasses of R-SMADs that transduce signals for the TGFβ superfamily: SMAD2/3 for TGFβs/activin/nodal branch and SMAD1/5/8 for applicable BMP/GDF ligands [377]. In basal conditions, the R-SMADs are retained in the cytoplasm due to their interactions with the SMAD anchor for the receptor activation protein (SARA) [378, 379]. Receptor-mediated phosphorylation at carboxyl-terminal serine residues releases R-SMADs from SARA and increases their interaction with SMAD4 (also known as DPC4), a shared SMAD (co-SMAD) of the whole TGFβ superfamily [380]. The R-SMADs/co-SMAD complex then translocates and accumulates in the cell nucleus, where it works either directly or in partnership with other DNA-binding cofactors to bind to the DNA and initiate target gene transcription (Fig. 1.6) [380-382].

1.2.2.3 Activin type I and type II receptors

Activins can bind to three type II receptors: activin receptor type IIA (ACVR2A), IIB (ACVR2B), and the type II BMP receptor (BMPR2) [359, 383-386]. The necessity of ACVR2A for FSH synthesis has been clearly demonstrated in *Acvr2a*-knockout mice, which are FSH-deficient, hypogonadal, and the females are subinfertile [385]. This evidence suggests that ACVR2A is required for activin regulation of FSH synthesis *in vivo*. And the other potential type II receptors, including ACVR2B and BMPR2, cannot fully compensate for the loss of ACVR2A for activin induced FSH synthesis [387]. In fact, ACVR2B appears dispensable for activin-mediated *Fshb* transcription *in vitro* [386, 388], the role of ACVR2B *in vivo* has not been reported. Although activin stimulates FSH production using BMPR2 in immortalized gonadotrope cell line, LβT2 cells, the role of BMPR2 *in vivo* has yet to be confirmed [386]. At present, two of the type I receptors, activin receptor-like kinase 4 (ALK4 or ACVR1B) and 7 (ALK7 or ACVR1C), have been shown to be involved in activin-mediated *Fshb* transcription [389, 390]. The relative importance of these receptors is unclear, and may depend on the isoform of the activins (activin A, B or AB) to which they bind.

1.2.2.4 SMAD signaling molecules

Once activins bind to the receptor complexes, the type I receptor phosphorylates and activates SMAD2 and 3 and recruit SMAD4 signaling molecules [382]. All R-SMADs and SMAD4 are composed of two functionally independent domains, termed Mad-homology domains MH1 and MH2, respectively [391]. The two domains are linked by a proline-rich linker sequence [391]. The MH1 domain is responsible for SMAD binding to DNA, whereas the MH2 domain interacts with other SMADs and with other transcription factors [391]. Both SMAD3 and SMAD4

can directly bind to DNA [392, 393]. SMAD2, however, contains 30 additional amino acids located at the N-terminal MH1 domain, which sterically disrupts its DNA binding capacity [394]. Interestingly, a naturally occuring splice variant of SMAD2 (SMAD2Δexon3) lacks these residues and therefore enables SMAD2 to bind to DNA [394, 395]. In fact, SMAD2Δexon3 is functionally similar to SMAD3 *in vitro* [396]. SMAD2 and SMAD2Δexon3 are expressed at a ratio ranging from 3:1 to 10:1 in cells [397]. The C-terminus of the MH2 domain of all R-SMADs, but not SMAD4, contain a Ser-Ser-X-Ser (SSXS) motif that can be phosphorylated by the type I receptors [398].

In the nucleus, the SMAD complex recognizes and binds to SMAD-binding elements (SBEs, 5'-GTCT-3', its complement 5'-AGAC-3' or a palindromic 8-bp SBE 5'-GTCTAGAC-3') at the promoter regions of target genes [399-402]. In the mouse and rat *Fshb* promoter, an 8-bp SBE is functional for activin-induced transcription [400, 401]. To reach a maximum transcriptional activity, it requires simultaneous binding of both SMAD3 and SMAD4 to each half of the 8-bp SBE in LβT2 cells [403]. However, a mutation in this SBE attenuates, but does not abolish all activin-mediated *Fshb* promoter activity [403, 404]. Moreover, this sequence has been only observed in rodent *Fshb* promoters [402-404], but is missing in the other species including humans, pigs, and sheep [405]. These observations suggest that the 8-bp SBE may not be the dominant activin-responsive element.

It has been demonstrated that SMAD3 is critical for *Fshb* promoter reporter activity of many species, including rats [406], pigs [392], and sheep [407] in LβT2 cells. SMAD2, however, appears dispensable for FSH production [406, 408]. Phosphorylated SMAD3 associates with SMAD4, translocated and accumulate in the nucleus, where the SMAD complex initiates *Fshb* gene transcription [382]. Consistent with this view, SMAD4's essential role in activin-mediated

FSH production has been confirmed both *in vivo* and *in vitro* [409, 410]. Surprisingly, gonadotrope-specific *Smad2* and *Smad3* double-knockout mice exhibited normal FSH production and fertility [408]. The authors further showed that these mice produce a novel (residual) *Smad3* transcript, which encodes a truncated but functional SMAD3 protein [408]. Therefore, it is currently still unclear whether SMAD3 is essential for FSH production *in vivo* (see **Chapter 2**).

1.2.2.5 FOXL2 transcription factor

Although specific, the binding of SMAD3 and SMAD4 to DNA appears to be of low affinity, suggesting that other cofactors are involved in stabilizing the SMAD complex binding to DNA [391, 411, 412]. In addition, the potential DNA-binding cofactor(s) should confer the cell type and gene specificity [413, 414]. Previously, our laboratory found that porcine and human proximal *Fshb*/*FSHB* promoter sequences possess ~90% identity [405]. However, the porcine *Fshb* promoter is much more sensitive to activin A stimulation than its human equivalent [378, 415]. Notably, neither the human nor porcine *FSHB/Fshb* promoter contains the 8-bp palindromic SBE [378, 415]. Using a comparative approach to map the activin-responsive element at the proximal promoter region, a single bp difference between human and porcine *FSHB*/*Fshb* promoters was discovered [392]. Importantly, the single-bp difference appeared responsible for the strong activin responsiveness of the porcine promoter [392]. We further identified that this sequence confers high affinity binding of the transcription factor forkhead box protein L2, FOXL2 [405]. Although activins strongly stimulate *Fshb* expression in murine gonadotropes, the murine *Fshb* promoter contains a 2-bp mismatch in the porcine FOXL2 binding site, suggesting that if FOXL2 also regulates murine *Fshb*, it does so elsewhere in the promoter [405]. Indeed, an *in silico* analysis identified an alternative FBE (5'-TAAACA-3') located at a more proximal region of

murine *Fshb* promoter, which is also conserved in pigs and humans [405]. Intriguingly, both FBEs are found adjacent to a 4-bp SBE, suggesting a cooperative action between SMADs and FOXL2 [392, 405]. These discoveries shed light on the role of SMAD/FOXL2 complexes in the regulation of murine *Fshb* transcription. Indeed, immunoprecipitation experiments have demonstrated that FOXL2 physically interacts with SMAD3, but not with SMAD4, whereas, SMAD4 preferably binds to the SBE adjacent to the proximal FBE in the murine *Fshb* promoter (Fig. 1.6) [392, 416]. In the absence of SMAD4, however, SMAD3 can bind to the half SBE and synergistically stimulate *Fshb* transcription with FOXL2 *in vitro* [393].

Recently, our laboratory has further confirmed the essentiality of FOXL2 *in vivo* using a conditional knockout strategy [417]. Briefly, in the absence of FOXL2 in the gonadotropes, mice exhibited significantly reduced FSH production and impaired fertility, establishing FOXL2 as the first transcription factor required for *Fshb* expression *in vivo* [417]. A similar but not identical phenotype was observed in *Smad4* conditional knockouts [409]. The combined loss of *Foxl2* and *Smad4* in the gonadotropes causes a more dramatic reduction in FSH synthesis *in vivo*, and the females in this case exhibited similar reproductive defects to *Fshb* knockouts [408]. These observations confirm the essential role of SMAD4 and FOXL2 for FSH synthesis *in vivo*. More recently, another FBE was reported in a distal region of murine *Fshb* promoter; however, FOXL2's action appears independent of SMAD action at this binding site [418]. Taken together, these data suggest that FOXL2 is a critical transcription factor required for *Fshb* transcription across multiple species [393, 403, 405]. At the murine proximal promoter, the synergistic action of FOXL2 with SMAD3 and SMAD4 proteins is critical for *Fshb* expression *in vitro* and *in vivo*. However, FOXL2 may also function through another mechanism independently of the SMADs.

Although the human *FSHB* is ~90% similar to porcine *Fshb* promoter and ~70% similar to the murine *Fshb* promoter, it has exhibited the weakest activin-responsiveness in promoter-reporter analyses [405]. This may be due to the absence of the consensus 8-bp SBE at the human *FSHB* promoter. Consistent with this idea, adding the murine 8-bp SBE to human promoter increases both SMAD-sensitivity and acute regulation by activin A, but not to the same levels as observed with the murine promoter-reporter [403]. However, the human *FSHB* promoter contains at least three FBEs, and one of these is conserved with the murine SBE/FBE core elements [405, 418]. One report has demonstrated that mutations in two of the human FBEs significantly impair activin-induced human *FSHB* promoter activity in LβT2 cells [405]. However, *FSHB* basal activity is largely intact in human promoters with FBE mutations [392, 418]. Therefore, it is currently unclear whether and to what extent FOXL2 contributes to the stimulation of human FSH synthesis.

In addition to FBE, a highly conserved binding site for the *paired*-like homeodomain transcription factors PITX1 and PIX2, which are located in the proximal promoter region, has been identified in many species, including mice, rats, and humans [419, 420]. The binding of PITX1 or 2 to DNA appears important for endogenous and exogenous activin-stimulated *Fshb* transcription *in vitro* [419-421]. Additionally, depletion of endogenous PITX proteins by RNA interference impairs activin-induction of *Fshb* expression in LβT2 cells [419, 422]. Moreover, PITXs and SMAD proteins physically interact, further implicating PITXs in activin-induced FSH production [420, 422]. Interestingly, there are no SBEs in proximity to the binding elements of PITXs, thus it is currently unclear how the PITX/SMAD complex binds to DNA in response to activin stimulation [387].

Although SMAD-dependent signaling has been more documented, TGFβ ligands, including activins, have also been shown to function through SMAD-independent pathways, such

as MAPKs [423-425]. However, the current results appear controversial therefore inconclusive [426]. That said, there is currently no solid evidence suggesting that activins stimulate *Fshb* transcription through pathways other than SMADs.

1.2.2.6 Inhibin action

Although the fact that inhibins act in an endocrine manner to specifically suppress pituitary FSH synthesis in the pituitary gland was first observed during the 1970s [349], the mechanism underlying this regulation has not been fully resolved. Many reasons contribute to the obstacles in elucidating the inhibin cellular mechanism, foremost among them was the failure to identify inhibin receptors [427]. Unlike the other TGFβ ligands, inhibins do not appear to transduce intracellular signals; rather, they appear to antagonize activin actions through competitively bind to activin type II receptors [428, 429]. Because they share the common β subunits, inhibins are able to bind to activin type II receptors (Fig. 1.6) [427]. Binding of activins to their type II receptors propagates intracellular signaling cascades as described above [430]. In contrast, when inhibins bind to the activin type II receptors, the type I receptor is not recruited, and thereby inhibins block activin signaling (Fig. 1.6) [431, 432]. However, the binding affinity of inhibins to the type II receptors is much weaker than that of activins (~10 fold less) [433-435]. Therefore, effective antagonism would require greater quantities of inhibins relative to activins. Surprisingly, however, inhibins can robustly antagonize activin action at equivalent or even at lower concentration in many contexts; whereas on many other occasions, inhibins fail to antagonize activins' action even when present at excess molar concentrations [436-438]. This suggests that additional components are involved in the inhibin-activin type II receptor complex. After 10+ years of searching, this idea

finally lead to the identification of transforming growth factor β (TGFβ) type III receptor (TGFBR3, also known as betaglycan) as a *bona fide* inhibin co-receptor [434, 439].

1.2.2.7 TGFβ type III receptor (TGFBR3)

TGFBR3 has a domain structure distinct from that of type I and II receptors [440, 441]. It is composed of two functionally independent, structurally-linked extracellular ligand binding domains, a single hydrophobic transmembrane region, and a short intracellular tail lacking known functional motifs. It is therefore not considered a signaling receptor [440, 442]. TGFBR3 can bind to many TGFβ superfamily ligands, including all TGFβ isoforms [443, 444], some BMPs [445], and inhibins [434, 446-448]. The three TGFβs and BMPs bind to both extracellular binding sites, whereas inhibins only bind to the juxtamembrane portion of the extracellular domain [440, 443, 446, 449]. Additionally, inhibins bind to TGFBR3 through the α subunit, explaining why activins do not share this co-receptor [434, 450]. No evidence indicates a signaling cascade for TGFBR3 [440, 441]. Therefore, TGFBR3 was originally considered an accessory receptor to the TGFβ family, which concentrates TGFβ ligands on the cell surface and presents them to their specific receptors [440, 441, 443, 444]. However, later studies have indicated that TGFBR3 functions as more than a simple co-receptor for the TGFβ ligands; moreover, it plays indispensable roles in reproduction [447, 451, 452], in fetal development [453-455], and in tumor development [448, 456-462].

TGFBR3 is expressed in gonadotropes, somatotropes, and lactotropes in the anterior pituitary as well as in the intermediate lobe [463, 464]. According to *in vitro* studies, in the presence of the co-receptor TGFBR3, inhibins form high affinity ternary complexes with activin type II receptors, blocking the binding of activins [429, 434, 447, 465]. Therefore, it is reasonable

to predict that inhibins antagonize the action of activins in FSH synthesis through binding to TGFBR3-type II receptor complexes. However, this idea has never been assessed in adult animals *in vivo*, due to embryonic lethality in *Tgfbr3* null mice [454]. To investigate TGFBR3's function in inhibin antagonism, a conditional knockout model is required (see **Chapter 4**).

1.2.2.8 Follistatin action

Gonad-derived inhibins act as endocrine hormones that negatively regulate FSH in the pituitary gland. In contrast, the bioactivity of gonad-derived activins are neutralized by activin-specific binding proteins called follistatins (see section 1.2.2.1) [466]. Although both follistatins and inhibins block activin action, their mechanisms of action are distinct. Follistatins selectively and irreversibly binds to activin with high affinity at a 2:1 molar ratio, thus interfering with activin access to their receptors [466-469]. Moreover, follistatins also accelerate the endocytosis and degradation of activins [470]. Follistatins are expressed in a variety of tissues, including the gonads and the pituitary gland [362, 466, 471, 472]. In the pituitary, follistatins are mainly secreted by folliculostellate and gonadotrope cells and act in a paracrine or autocrine manner to bind to activins [205, 473-475]. Furthermore, the expression of follistatins in gonadotropes is regulated by activin [205, 473, 476-480]. Activins stimulate follistatin expression in gonadotropes, which in turn inhibit activin action. Moreover, activin-regulated follistatin expression requires the activation of SMAD3, but not SMAD4, and recruits FOXL2 as the transcription co-factor in gonadotrope cultures [416].

In summary, the current thinking for activin/inhibin-mediated FSH production is that pituitary local activins act on their type I and II serine/threonine kinase receptors and recruit SMAD3 and SMAD4 signaling molecules as well as FOXL2 to stimulate *Fshb* transcription.

Inhibins, in contrast, do not signal. Instead, they competitively bind to activin type II receptors and thereby blocking activin access, particularly in the presence of TGFBR3. It is important to note, however, that inhibin antagonism through TGFBR3 *in vivo* has not been reported (see **Chapter 4**).

1.2.3 BMP regulation of FSH

Another subgroup of the TGFβ superfamily, the BMPs, have also been shown to influence FSH synthesis and secretion in many contexts *in vitro*. For example, BMP6 and BMP7 can stimulate ovine *Fshb* promoter activity in transiently transfected LβT2 cells [481]. The induction is enhanced ~10-fold when treated with BMP15, BMP4, or BMP2 in the same cell line [378, 482, 483]. In contrast, BMP4 and BMP6 suppress FSH secretion in primary cultured pituitary cells prepared from ewes. In addition, BMP4 and BMP6 attenuate activin's action on *Fshb* expression and FSH release in these cells [484, 485]. In LβT2 cells, however, BMP2 stimulates murine *Fshb* transcription through the type I receptor BMPR1A (also known as activin receptor-like kinase 3 or ALK3) and SMAD1/5 signaling [486]. Surprisingly, gonadotrope-specific *Bmpr1a* knockout mice exhibit normal FSH production and fertility [487]. Overall, BMP regulation of FSH synthesis appears to be ligand-, context-, and species-specific. Additionally, the stimulatory effect of BMPs on FSH synthesis *in vivo*, if any, may be through receptors other than BMPR1A (or the related ALK2).

1.2.4 Steroid regulation of gonadotropin synthesis

In the gonads, FSH and LH stimulate sex steroid production, including androgens, estrogens, and progesterone [221, 222]. These steroid hormones in turn travel back to the brain and pituitary levels to either positively or negatively regulate gonadotropin production, thereby

forming a homeostatic loop [488-491]. In the hypothalamus, these hormones target their receptors located in several brain regions including the ARC, AVPV, and POA, where they influence kisppeptin expression and GnRH release and subsequently control gonadotropin secretion (see section 1.1.1.2). Although less well-established, evidence has indicated that steroid hormones also directly mediate gonadotropin synthesis at the pituitary level [95, 492-496]

1.2.4.1 Androgens

The androgens, including testosterone and 5α-dihydrotestosterone (DHT), directly stimulate *Fshb* expression at the pituitary level and have been shown to do so both in cultured pituitary cells and *in vivo* [497-499]. Testosterone and DHT are produced by Leydig cells and Sertoli cells, respectively (see section 1.1.3.3.1), and impart their effects on target cells by binding to the androgen receptor (AR) [500]. Androgens dose- and time-dependently stimulate murine *Fshb* promoter-reporter activity in LβT2 cells transfected with wild-type AR [498]. However, the induction is severely diminished when using receptors that lack DNA binding ability, suggesting that androgen-mediated *Fshb* expression requires that AR binds to DNA [498]. Many androgen-responsive elements have been discovered in *Fshb*/*FSHB* promoters, though they appear in a species-dependent manner [498, 501]. Interestingly, androgens synergistically stimulate *Fshb* promoter activity with activins in a SMAD3-dependent manner [501-503]. This synergism requires both AR and SMAD3 to bind to the promoter at their specific responsive elements and likely involves physical interactions between AR and SMAD3 proteins [502, 504]. In male rats, testosterone acts in combination with GnRH receptor antagonist to selectively increase *Fshb* mRNA and serum FSH levels, but not LH, to a comparable level as in castrated controls [499, 505, 506]. Similarly, testosterone administration to the hypogonadotrophic hypogonadal (*hpg*) mice, in

whom hypothalamic GnRH is deficient or absent [507], significantly increases pituitary FSH levels, though to a lesser extent than GnRH injections [340]. Conversely, androgens have either no effect or inhibitory effects on FSH expression in humans or non-human primates [508-512]. In primary cultured pituitary cells prepared from male monkeys or human fetuses, testosterone has no effect on FSH or LH secretion [508, 509]. Furthermore, testosterone administration suppresses both FSH and LH secretion in adult men [512, 513]. Taken together, while it is known that androgens stimulate rodent FSH expression and secretion both *in vivo* and *in vitro,* the effect of androgens on FSH in the other species is less clear. The species-specific regulation might derive from different AR responsive elements in the promoters of different species. Further studies are required to resolve these inter-species discrepancies.

In contrast to its stimulatory effect on *Fshb* expression, testosterone negatively regulates both *Cga* and *Lhb* mRNA levels in the gonadotropes devoid of hypothalamic input [499, 514]. The inhibitory effect on the *Cga* promoter is due to the direct binding of ligand-activated AR to the junctional regulatory element (JRE) between a cAMP response element (CRE) and the α subunit specific CCAAT box [515-518]. Ligand-bound AR suppresses *Lhb* promoter activity through its interaction with several transcription repressors, including specificity protein 1 (SP1), EGR1, SF1, and PITX1 [519-521]. Consistent with this finding, testicular Feminisation (Tfm) mice, which contain null AR alleles and little testosterone production, produce elevated levels of LH [522]. Furthermore, LH levels are significantly increased in humans with androgen insensitivity syndrome (AIS), an X-linked AR mutation disease [523]. In short, testosterone differentially regulates the two gonadotropins, with stimulatory effect on FSH (at least in some species) but inhibitory effect on LH production in gonadotropes.

1.2.3.2 Estrogens

Estrogens, including estrone, estradiol, and estriol, are primary female sex hormones that are required for developing female secondary sexual characteristics during puberty, maintaining appropriate female reproductive organs (the ovary, uterus, and vagina), and regulating feedback effects on gonadotropin synthesis throughout each cycle [524, 525]. Additionally, synthetic estrogen preparations (often in combination with progesterone) have been applied in the clinic (hormone replacement therapy, HRT) for the treatment of symptoms that are caused by low levels of estrogen, such as hot flashes and night sweats in postmenopausal women [526]. Although estrogen levels are significantly lower in males compare to females, estrogens play pivotal role in many tissues and organs in males [527, 528].

Like all steroid hormones, estrogen can diffuse across the cell membrane, and act through the estrogen receptors, which are characterized as a ligand-inducible transcription factor [529]. Mammals express two kinds of estrogen receptor: ERα and ERβ [530-536]. The ER subtypes are differentially distributed throughout the brain, and in many tissues their distribution differs between males and females [537-542]. However, both estrogen-positive and estrogen-negative feedback effects on gonadotropin production are mediated via ERα, but not ERβ [543-546]. The necessity of ERα for estrogen-negative feedback has been demonstrated in global ERα knockout (αERKO) animals. In this study, female αERKO, but not ERβKO mice exhibited increased pituitary *Cga* and *Lhb* expression and a chronic increase in circulating LH levels [543]. Furthermore, three-weeks after ovariectomy, LH concentration was significantly increased in wild-type but not in αERKO females [544]. Whereas, brain specific αER-deficient females failed to launch LH surge and therefore anovulation, indicating that the ERα also mediates estrogen positive feedback effect in the brain [547]. Interestingly, unlike in the global αERKO mice, brain

specific αER-deficient mice showed normal responsiveness to estrogen-negative feedback, suggesting that the negative regulation of estrogen is at least in part mediated at the pituitary level [547]. In fact, gonadotrope-specific ERα knockout females exhibited phenotypes similar to those of the global αERKO mice, though to a lesser extent, confirming that the estrogen feedback effect occurs in both the hypothalamus and the pituitary gland [489, 548].

In addition to the clearly demonstrated phenotypes in knockout models, ER signaling in the regulation of gonadotropin production at the molecular level has also been thoroughly investigated, though yet to be fully understood. In the classical ERα signaling pathway, the ligand-bound receptor dimerizes and translocates into the nucleus, where it crosstalks with co-activator molecules, such as steroid receptor coactivator 1 (SRC1) and nuclear receptor coactivator 1 (NCoA1) [549-551]; or a co-repressor, for example nuclear corepressor (N-CoR) and silencing mediator of retinoic and thyroid hormone receptors (SMRT) [552-555], and then binds to an estrogen response element (ERE) to regulate transcription of target genes [556]. *In vitro* studies suggest that ligand-bound ER binds to a 284 bp region located between -1388 to -1105 upstream of the rat *Lhb* transcription start site, suggesting that estrogen directly regulates the transcription of *Lhb* in primary cultured rodent pituitary cells [514, 557-559]. In addition to the direct regulation of gene expression, ligand-bound ER rapidly activates many other signaling cascades (nonclassical pathway), including phospholipase C (PLC)/protein kinase C (PKC)/IP_3/Ca^{2+} [560-564], MAPK [565, 566], and cAMP/PKA [567-570] pathways. Genetic knock in of a mutant form of ERα, which lacks ERE but contains ERE-independent activity, onto ERα null background mice (ΔERE) exhibited ~70% lower serum LH compared to ERα knockouts. Notably, these mice only respond to non-classic ER regulation (i.e., DNA-binding-independent) [571]. Furthermore, post-ovariectomy rise in serum LH was significantly suppressed in wild-type and ΔERE mice when

they received estrogen replacement. However, estrogen failed to decrease LH in ERα null mice [571]. These data indicate that ERE-independent regulation is sufficient to mediate functional estrogen-negative feedback in mice [571, 572]. Additionally, LH surge only occurs in wild-type but not ERα or ΔERE mice, suggesting that the classical ER signaling is required for mediating estrogen positive feedback [571]. Recall that (see section 1.1.1.2) estrogen-positive and estrogen-negative feedback in the brain might occur at anatomically distinct regions (AVPV/POA vs ARC) [573, 574]. Taken together, the two sets of evidence suggest that estrogen action may be separable both spatially and at the molecular level. Further study is needed to clarify whether estrogens regulate LH productions through different mechanism in AVPV/POA and ARC, respectively.

In contrast to LH, estrogen's effects on *Fshb* and FSH levels are controversial, and may occur in a species-specific fashion. Specifically, estrogen suppresses *Fshb* expression in isolated ovine pituitary cell cultures. By contrast, *Fshb* expression is increased in rat pituitary cultures treated with estrogen [165, 575]. Furthermore, research conducted on αERKO mice reported either intact [543] or increased [576] *Fshb* mRNA levels using qPCR and northern blot analyses, respectively. Moreover, an *in vitro* study has also demonstrated no impact on *Fshb* promoter-reporter activity in the absence of ER (either one or both ERs) [498]. Consistently, another study also reported that gonadotrope-specific ERKO mice exhibited normal FSH levels [489]. Therefore, it is currently inconclusive whether, and if so, how, estrogen directly regulates FSH synthesis.

Although estrogen levels in males are much lower than in females, αERKO male mice exhibited impaired spermatogenesis and sperm motility when compared to wild-type controls, suggesting that estrogen plays a role in mediating male reproduction [577]. As with the females, pituitary *Lhb* and circulating LH levels were significantly higher in αERKO males compared to the wild-type controls, though *Fshb* and FSH levels remained intact [578]. However, estrogen

injection significantly suppressed both FSH and LH secretion in both normal and GnRH-deficient men, suggesting that estrogen negatively regulates the two gonadotropins' release at both hypothalamus and pituitary levels [510]. Therefore, estrogen may regulate human but not murine FSH synthesis [578].

While it is apparent that estrogen-positive and estrogen-negative effects occur in both hypothalamus and pituitary levels, the mechanism for these occurrences have not been fully resolved. In particular, whether estrogen directly stimulates or inhibits FSH is not clear.

1.2.3.3 Progestagens

Originally, the function of progesterone, the main product of corpus luteum, was considered the maintenance of pregnancy in mammals [166]. Now, however, more functions of progesterone have been discovered within the HPG axis. Like estrogen, progesterone both positively and negatively regulates gonadotropin production through its receptor (PGR) expressed in the brain and in the pituitary gland [579-582]. It is evident that progesterone participates in both GnRH-mediated preovulatory gonadotropin surge and in the second FSH surge on the following morning in female rodents (see section 1.1.3.3.2) [583-589]. Consistent with these findings, anti-progesterone (RU486) administration attenuates the primary gonadotropin surges on proestrus, and further suppresses the secondary FSH surge on the morning of estrus (see section 1.1.3.3.2) [586-589]

PGR expression is regulated by estrogen in many tissues, including kisspeptin neurons [590-592]. In this way, progesterone facilitates the LH surge in estrogen-primed ovariectomized wild-type mice; in contrast, the LH surge does not occur in *Pgr* null mice, suggesting that PGR is required for estrogen positive feedback [593, 594]. Accordingly, neither *Pgr* null nor kisspeptin

neuron-specific *Pgr* knockout mice show preovulatory FSH and LH surges or ovulation, suggesting that PGR in kisspeptin neurons contributes to progesterone positive feedback [592, 594, 595]. Interestingly, *Pgr* deletion in kisspeptin neurons does not impact kisspeptin mRNA expression, indicating that progesterone may affect kisspeptin secretion rather than synthesis [592]. In addition, the pituitary may also contribute to progesterone positive feedback effects. Prior to initiate preovulatory LH surge, GnRH goes through self-potentiation prior to trigger primary surge. It has been reported that PGR participates during this GnRH self-priming, thereby augmenting LH secretion in rat pituitary cells [596, 597].

In contrast, *Pgr* null mice exhibit elevated levels of LH, but not FSH, assessed at metestrus, suggesting that progesterone negatively regulates LH secretion [594]. Kisspeptin neuron-specific *Pgr* knockouts, on the other hand, produce normal gonadotropin levels, indicating that progesterone-negative feedback for LH secretion is mediated at the pituitary level [592]. Interestingly, progesterone-negative regulation of LH appears to be species-dependent. Specifically, progesterone suppresses LH amplitude but not frequency in ovariectomized female rats, whereas progesterone administration suppresses LH pulse frequency but not amplitude in sheep [598, 599]. However, the molecular mechanism underlining progesterone's effects is not clear. Further study of *Pgr* gonadotrope-specific knockouts will clarify whether, and how PGR contributes to gonadotropin secretion *in vivo*.

The stimulatory effect of progesterone on the secondary FSH surge also requires high estrogen presence during proestrus [589]. These data suggest that estrogen provides essential components for progesterone action during estrus morning, which is likely PGR [600]. Furthermore, progesterone, in combination with estrogen, produces an augmented effect on *Fshb* expression in response to GnRH stimulation, suggesting that both hormones act, at least in part,

directly on the gonadotrope to augment GnRH action in FSH synthesis [42]. Accordingly, progesterone synergistically stimulates FSH synthesis with activin in pituitary cultures [498, 601, 602]. Additionally, *in vitro* promoter-reporter studies identified multiple progesterone-responsive elements at *Fshb* promoter regions, indicating that the induction of *Fshb* requires PGR DNA-binding activity [498, 603, 604]. Indeed, the synergistic effect was largely impaired when DNA-binding ability was depleted from PGR or when mutations were introduced to the progesterone-responsive elements in the promoter [503]. Moreover, progesterone and activin synergism requires SMAD3 DNA binding activity and likely involves a physical interaction between PGR and SMAD3 proteins [503]. Whether the formation of the PGR-SMAD3 complex is essential for driving FSH synthesis *in vivo* remains unknown. Taken together, this evidence shows that progesterone stimulates FSH production in a synergistic fashion with activin in cultured pituitary cells and requires the intact DNA-binding activity of both PGR and SMAD3 proteins.

1.3 Inducible knockout system

1.3.1 Conditional knockout system

The conventional gene targeting technology was developed by Drs. Mario Capecchi, Martin Evans and Oliver Smithies, which has been demonstrated as a powerful method to precisely modify the mammalian genome in embryonic stem (ES) cells [605]. The principle of this technology is to replace native DNA with a modified sequence via homologous recombination [606-609]. However, many genes are indispensable during embryonic development and at the early post-natal stages. Thus, global loss of function mutations often cause embryonic/neo-natal lethality, thus preventing further investigation of gene function at later stages [610]. To circumvent this problem, site-specific recombination (e.g. Cre-loxP) strategies have been employed [611-615].

In this system, Cre recombinase of the P1 bacteriophage efficiently recognizes and excises the sequences between two consensus 34-bp locus of x-over P1 (loxP) sites [616], leaving a single loxP site. Although in this system Cre-recombinase is introduced into mammalian cells, its expression is under the control of a cell-specific promoter [617-619]. As a result, recombination only occurs in cells that express Cre recombinase, leaving gene function unperturbed outside of the Cre-expressing cell lineages [616].

1.3.2 Inducible knockout systems

Activation of Cre recombinase expression in the conventional conditional knockout system depends on the selected cell-specific promoter. Therefore, the timing of Cre-mediated recombination is determined by the timing of promoter activity, making it challenging to study gene function at a desired time points, especially when Cre is first expressed during embryonic development [615]. To circumvent this limitation, several inducible systems have been established in transgenic mice. The most innovative approaches, including the tamoxifen (TAM)-inducible recombination system and the tetracycline [620]-regulated transcriptional activation system [512, 621, 622] were developed during the 1990s.

The major active metabolite of TAM, 4-hydroxytamoxifen (4-OHT), is a selective estrogen-receptor modulator (SERM) that binds to the estrogen receptors in the cytoplasm and translocates the receptors into the nucleus (see section 1.3.3) [623]. Therefore, a system has been established by fusing estrogen receptor ligand binding domain (LBD) to Cre recombinase, therefore the Cre activity is dependent on the estrogen receptor activation [146]. To avoid the problem of endogenous estrogens binding to the fusion protein, a mutation of the LBD (G521→R, Cre-ERT) was introduced that prevents 17β-estradiol binding but leaves 4-OHT binding intact

[624]. In physiological conditions, the Cre-ERT fusion protein is sequestered by Hsp90 in the cytoplasm, thereby preventing Cre-mediated recombination events in the nucleus [625]. Binding of 4-OHT to ERT leads to the translocation of Cre recombinase into the nucleus, where it recombines the allele harbouring the two loxP sites [625, 626]. To improve the inducible efficiency and specificity, a triple mutation (G400V/M543A/L544A) in the LBD was subsequently constructed, and termed Cre-ERT2 [625, 627]. Indeed, the efficiency of 4-OHT induced Cre-ERT2 nuclear translocation is ~10 fold higher than with the original Cre-ERT system [625].

Another approach that enables temporal control of mammalian gene expression is the tetracycline-regulated gene expression system [620, 621, 628]. This strategy was established based on the mechanism of bacteria resistant to Tet antibiotic exposure [629, 630]. In the bacteria, a key component, the Tet repressor (TetR), recognizes and binds to a Tet-responsive element (TRE), thereby preventing gene transcription. In the presence of Tet or its analog (e.g. doxycycline), however, TetR preferably binds to Tet but not the TRE, permitting gene transcription [631]. The Tet-inducible gene expression system (Tet-on) was established based on the use of a fusion protein between a strong transcription factor (VP16 from *Herpes simplex virus* (HSV)) and the Tet repressor isolated from *E. coli*. The fusion of the reverse Tet transactivator (rtTA) can interact with the Tet operon sequence linked to a minimal cytomegalovirus (CMV) promoter in the targeting construct. Therefore, the transcription of the transgene is turned on only in the presence of exogenous Tet or its derivative, doxycycline [621, 628, 631, 632]. In contrast, there is also a Tet-off inducible suppressive system, which enables the turning off of transgene expression upon Tet administration. However, with this strategy, multiple disadvantages have been reported (including slow response), preventing its wider application in later studies [628].

Overall, several inducible systems have been established and have been well-characterized in the past 20 years. Depending on the experimental design, one of the inducible systems, either the Tet-regulated gene expression system, which is mainly employed for turning on gene transcription or the TAM-inducible system, which is mainly employed for turning off gene expression, can be employed in a study.

1.3.3 Tamoxifen

In addition to be used for inducing gene recombination, TAM (proprietary name Nolvadex) has been applied as a frontline drug for the treatment of breast cancer since 1970s [633]. Nearly 2 out of 3 breast cancers are hormone receptor-positive (ER-positive and/or PR-positive), and the presence of hormones (estrogen and/or progesterone) stimulate the growth and spread of mammary tumours [634, 635]. Drugs that antagonize estrogen action have therefore been attractive candidates for hormonal therapy, such as TAM. Long-term treatment (> 1 yr) of TAM had been proven to be an effective anti-estrogen drug that significantly extend the survival time of breast cancer patients [636]. During the decades of clinical application, several side-effects were also observed, and these side effects were mostly due to the fact that TAM functions as both an agonist and an antagonist to estrogen receptors (ER), which are now known as SERM [637-639]. For example, TAM is applied as an antagonist for preventing or inhibiting breast cancer growth, whereas, TAM is used as agonists for the treatment of infertility in women with anovulation disorders [640-643]. TAM is a prodrug, having nearly no affinity for estrogen receptors per se. *In vivo*, TAM is converted in the liver by cytochrome P450 enzymes, such as CYP2D6 and CYP3A4, into biologically active metabolites, including 4-OHT and N-desmethyl-4-hydroxytamoxifen [637, 644]. In the cytoplasm, when estrogen receptors (ERα or ERβ) bind to 4-OHT, TAM undergoes a

conformational change that permits its spontaneous dimerization and interaction with estrogen response elements (EREs) within target genes. In the nucleus, the ER-4-OHT complex also recruits coregulators at the EREs [640]. The nature of TAM as SERM in multiple tissues has raised investigators' awareness about off-target effects and precocious Cre activity when using TAM-inducible system in specific research [645-647]. Therefore, appropriate control conditions must be included when employ TAM-inducible system.

1.4 Rationale of this thesis

Although GnRH has been considered the main regulator of gonadotropin production, recently emerging data have demonstrated the indispensable role of activin signaling in the regulation of FSH production and fertility in mice. Though in the previous study *Smad3* (alone or in combination with *Smad2*) conditional knockout mice unexpectedly exhibited normal FSH and fertility [408]. However, further investigation showed that the *Smad3* mutation in this mouse model did not remove all the SMAD3 protein, leaving the role of SMAD3 *in vivo* unresolved [408]. Additionally, the residual FSH production in gonadotrope-specific *Smad4* knockout mice may derive from partial compensation by SMAD3's DNA-binding activity [409]. Therefore, we used a genetic approach (the Cre-loxP system) to revisit SMAD3's role in regulating FSH synthesis and fertility in mice in the absence of the SMAD4 protein (**Chapter 2**). Additionally, gonadotrope-specific deletion of SMAD4 and/or the FOXL2 cause(s) profound FSH-deficiencies and impaired fertility [408, 409, 417]. Phenotypes of these mice were similar, though not identical, to *Fshb* knockout mice [221]. Nevertheless, these two transcription factors were knocked out before gonadotrope terminal differentiation during development in this study [409, 648, 649]. Thus, it was unclear whether the diminished FSH synthesis in these mice reflected impaired gonadotrope

development or the necessity for SMAD4 and FOXL2 in adult gonadotropes. To differentiate between these possibilities and to assess whether activin signaling is necessary for ongoing FSH synthesis, we generated a new mouse line that enabled the inducible deletion of *Smad4* and *Foxl2* in gonadotropes of adult animals (**Chapter 3**). *In vitro* studies suggested that inhibin acts as an activin receptor antagonist, particularly in the presence of the co-receptor TGFBR3 (betaglycan) [434]. However, the role of TGFBR3 in inhibin's action and in FSH synthesis in gonadotropes *in vivo* has not been determined, in part due to embryonic lethality in *Tgfbr3* null mice [454]. Thus, we established the first 'floxed' *Tgfbr3* murine model, allowing us to ablate the gene specifically in gonadotropes *in vivo* (**Chapter 4**).

Figure legends

Figure 1.1 The hypothalamic-pituitary-gonadal (HPG) axis. The gonadotropin-releasing hormone (GnRH) neurons located in the hypothalamus send their axon terminals into the median eminence (ME), where they release GnRH peptide. GnRH is secreted in both pulsatile and surge manners, which are mediated by KNDy [kisspeptin (Kiss)-Neurokinin B (NKB)-dynorphin (DYN)] and Kiss neurons, respectively. In the anterior pituitary gland, GnRH stimulates the synthesis and secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) by gonadotrope cells. The released FSH and LH then target the gonads, the ovaries and the testes, where they stimulate gametogenesis and sex steroid hormone production. In turn, gonadal steroid hormones either positively or negatively feedback to the brain and pituitary gland to mediate GnRH release and LH/FSH production. In addition to GnRH, FSH synthesis is also stimulated by pituitary-derived activins (or related ligands), and this effect is antagonized by gonad-derived inhibins. The generation of this figure was inspired by Herbison AE (2016). *Nat Rev Endocrinol* 12(8):452-466.

Figure 1.2 Schematic representation of anterior pituitary cell lineage specification. Upon Rathke's pouch formation at E8.5 (in mouse), a ventral → dorsal gradient of bone morphogenetic protein 2 (BMP2), WNT familiy 5A (WNT5A), and Sonic hedgehog (SHH), as well as the opposing (dorsal→ ventral) BMP4 and fibroblast growth factors 8 and10 results in the positional commitment of cell lineages. In the following days until E17.5, the five pituitary endocrine cell types terminally differentiate under the control of specific transcription factors, as indicated in the figure. The five cell types include corticotropes (expressing corticotropin or ACTH), somatotropes (expressing growth hormone), lactotropes (expressing prolactin), thyrotropes (expressing thyroid –stimulating hormone) and gonadotropes (expressing the gonadotropins, FSH and LH).

Figure 1.3 Structure of testicular seminiferous tubules and ovarian follicles. A) Schematic representation of seminiferous tubules in a testicular cross section, where spermatogenesis occurs. B) Schematic representation of ovarian follicle development. Primordial and primary follicles contain an oocyte surrounded by a single layer of immature or mature granulosa cells (blue). As follicles begin to grow to the secondary stage, granulosa cells proliferate, the oocyte grows, and a layer of cells appears outside of each follicle, known as the theca layer (dark flat cells). The early follicle development is gonadotropin-independent. However, once follicles reach the early antral stage, the follicle development is driven by FSH. At the preovulatory stage, granulosa cells can be classified into two subgroups: cumulus cells, which immediately surround the oocyte; and mural granulosa cells, which express the LH receptor (LHR) in response to FSH stimulation. Following the GnRH-mediated LH surge, the oocyte(s) is ovulated from the dominant large antral follicle(s). The remnants of the follicle differentiate into a new structure called the corpus luteum (yellow).

Figure 1.4 Schematic representation of inhibin and FSH levels across the human menstrual (top) and rodent estrous cycles (bottom). The figure depicts the qualitative (not quantitative) relationship between FSH (red), inhibin A (blue), and inhibin B (green) in the circulation. The approximate ovulation time following GnRH-mediated LH surge (not shown in this figure) is represented with the grey boxes.

Figure 1.5 Model of the gonadotropin-releasing hormone (GnRH) signaling pathway. The GnRH receptor (GnRHR) couples to at least two Gα subunits, Gαq/11 and Gαs. Upon ligand binding and receptor activation, GTP-bound Gαq/11 activates membrane-bound phospholipase C

(PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates Ca^{2+} release from the endoplasmic reticulum (ER), which promotes exocytosis of vesicles containing gonadotropins (LH, red; FSH, greed). DAG activates protein kinase C (PKC), which eventually phosphorylates mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase 1/2 (ERK1/2). Whereas, activation of Gαs stimulates cyclic adenosine monophosphate (cAMP) accumulation, and subsequently activates protein kinase A (PKA) and cAMP responsive element-binding protein (CREB). Both pathways mediate LHβ (*Lhb*), the common α subunit (*Cga*), and possibly FSHβ subunit (*Fshb*) gene expression.

Figure 1.6 Schematic representation of activins and inhibins and their actions on pituitary gonadotrope cells. A) Top: Activins are produced via hetero- or homodimerization of two inhibin β subunits. Bottom: Inhibins are heterodimers of inhibin α and one of the two inhibin β subunits. B) Mechanisms of activin and inhibin regulation of FSHβ subunit (*Fshb*) transcription. Activins bind to complexes of type I and type II receptor serine-threonine kinases. Ligand-bound type II receptors phosphorylate (P in red circles) the type I receptors, the latter then phosphorylate receptor-regulated SMAD3. Phosphorylated SMAD3 then forms complexes with the common SMAD, SMAD4, which accumulate in the cell nucleus. SMAD3 and SMAD4 complexes partner with the transcription factor forkhead box L2 (FOXL2) and bind to the proximal promoter of *Fshb*. SBE, SMAD binding element. FBE, FOXL2 binding element. Inhibins also bind to activin type II, but not type I, receptors, and therefore do not generate intracellular signals. Instead, they acts as competitive receptor antagonists.

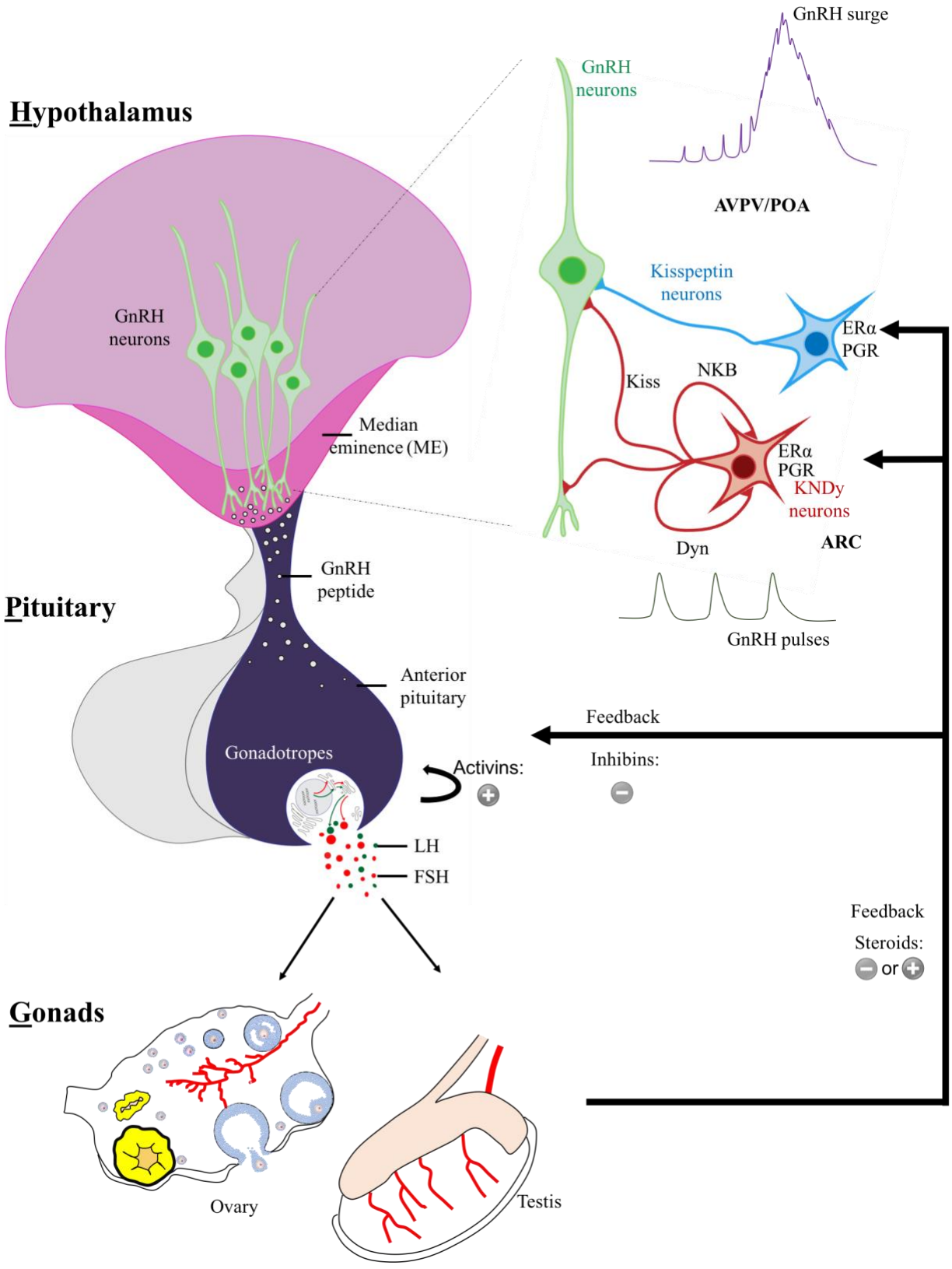

Hypothalamus
GnRH neurons
Median eminence (ME)
Pituitary
GnRH peptide
Anterior pituitary
Gonadotropes
LH
FSH
Gonads
Ovary
Testis
GnRH surge
GnRH neurons
AVPV/POA
Kisspeptin neurons
ERα
PGR
Kiss
NKB
ERα
PGR
KNDy neurons
Dyn
ARC
GnRH pulses
Feedback
Activins:
Inhibins:
Feedback
Steroids:
or

Figure 1.1

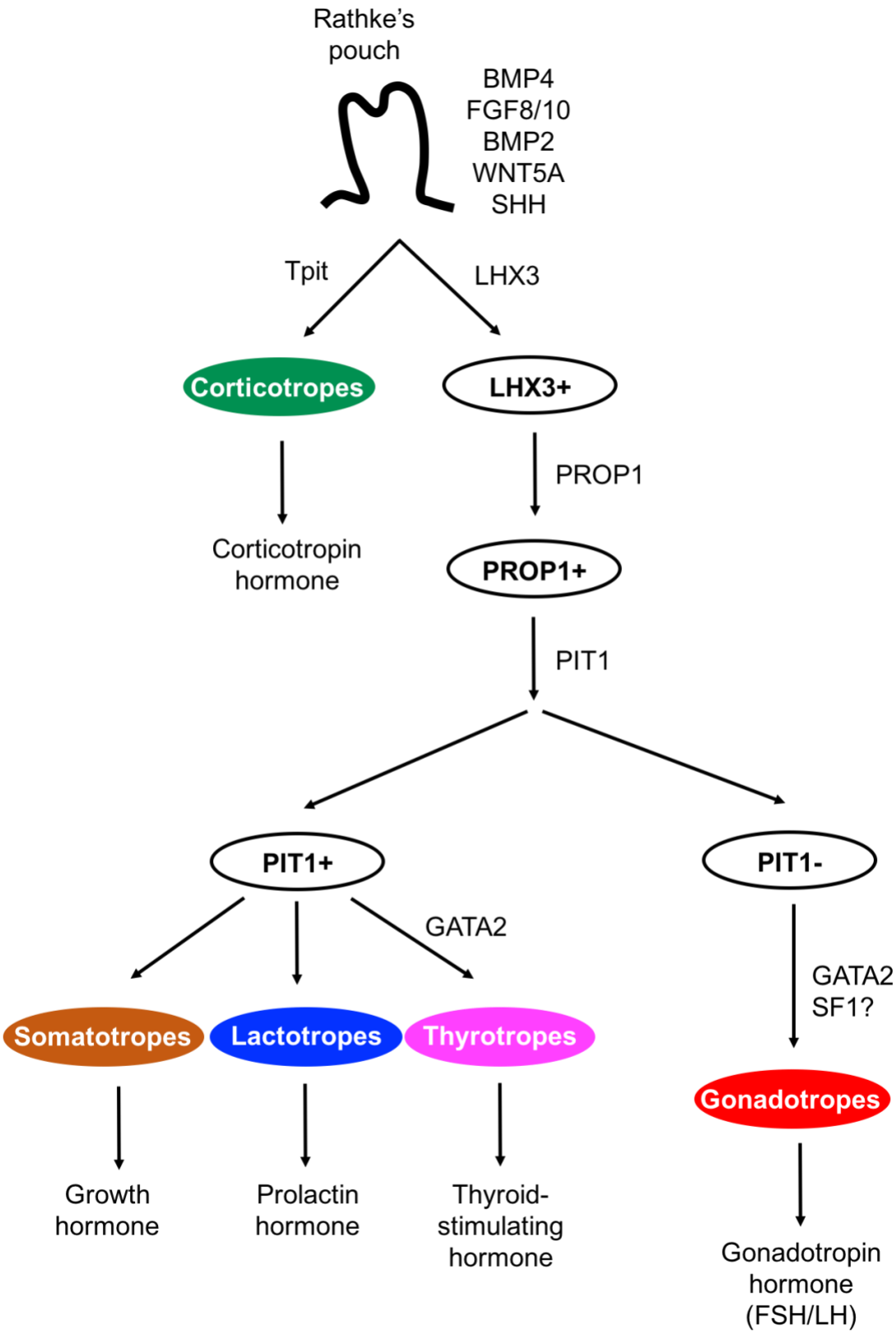

Rathke's pouch
BMP4
FGF8/10
BMP2
WNT5A
SHH
Tpit
LHX3
Corticotropes
LHX3+
PROP1
Corticotropin hormone
PROP1+
PIT1
PIT1+
PIT1-
GATA2
GATA2
SF1?
Somatotropes
Lactotropes
Thyrotropes
Gonadotropes
Growth hormone
Prolactin hormone
Thyroid-stimulating hormone
Gonadotropin hormone (FSH/LH)

Figure 1.2

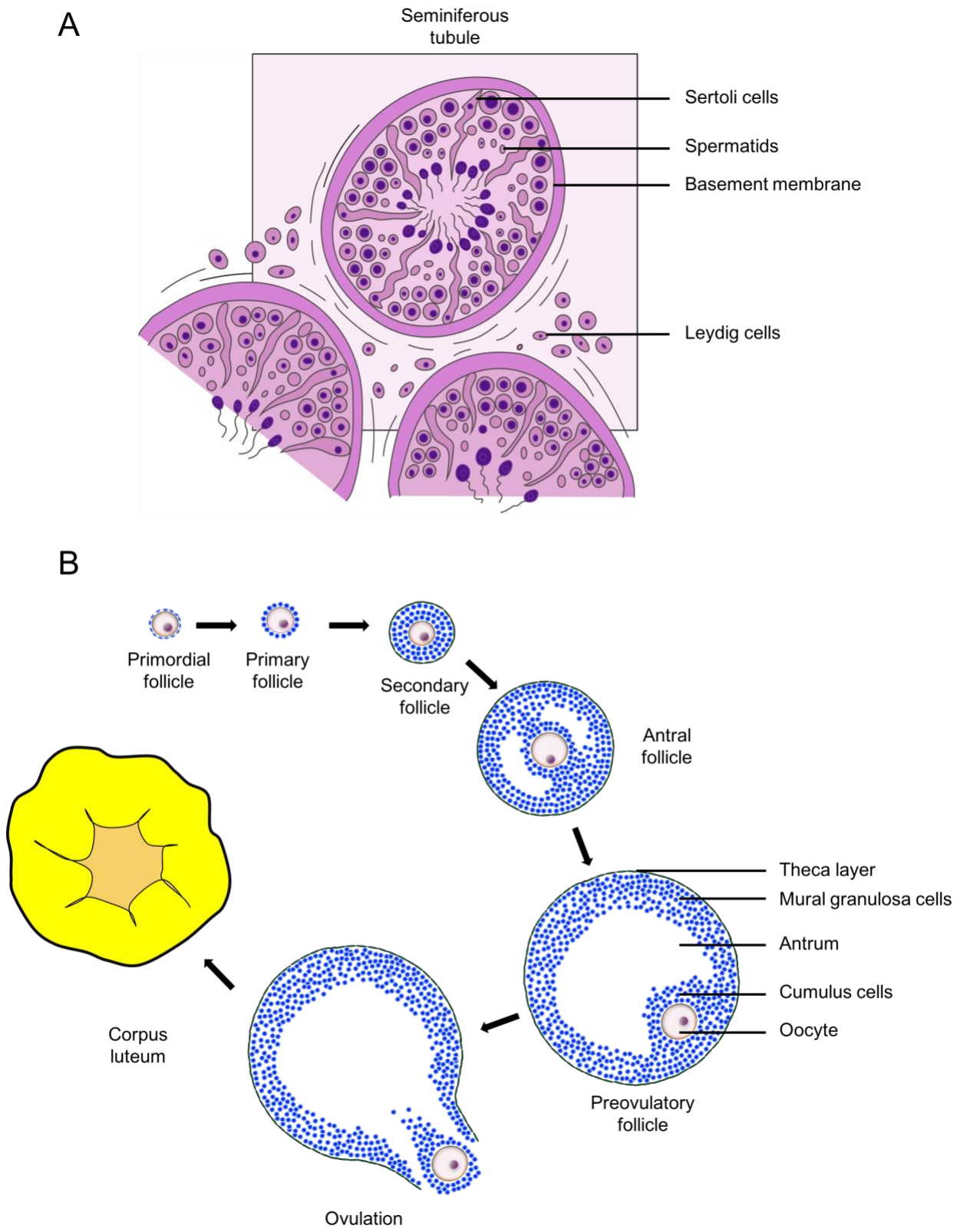
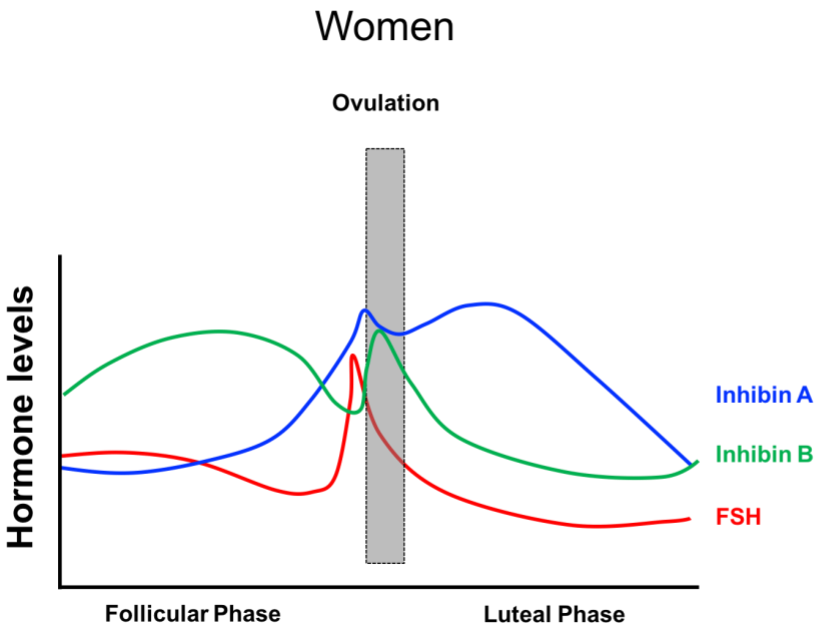

A
Seminiferous tubule
Sertoli cells
Spermatids
Basement membrane
Leydig cells
B
Primordial follicle
Primary follicle
Secondary follicle
Antral follicle
Theca layer
Mural granulosa cells
Antrum
Cumulus cells
Oocyte
Preovulatory follicle
Ovulation
Corpus luteum

Figure 1.3

A


Women
Ovulation
Hormone levels
Inhibin A
Inhibin B
FSH
Follicular Phase
Luteal Phase

B

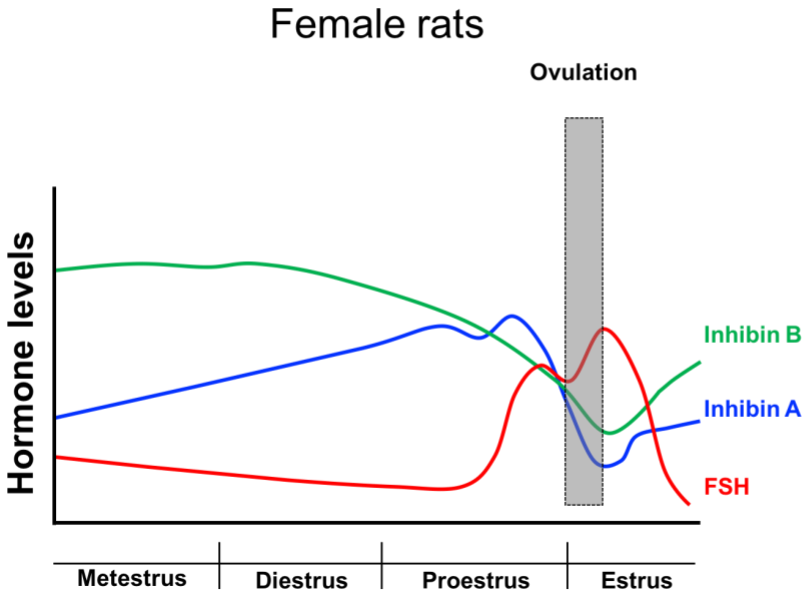

Female rats
Ovulation
Hormone levels
Inhibin B
Inhibin A
FSH
Metestrus
Diestrus
Proestrus
Estrus

Figure 1.4

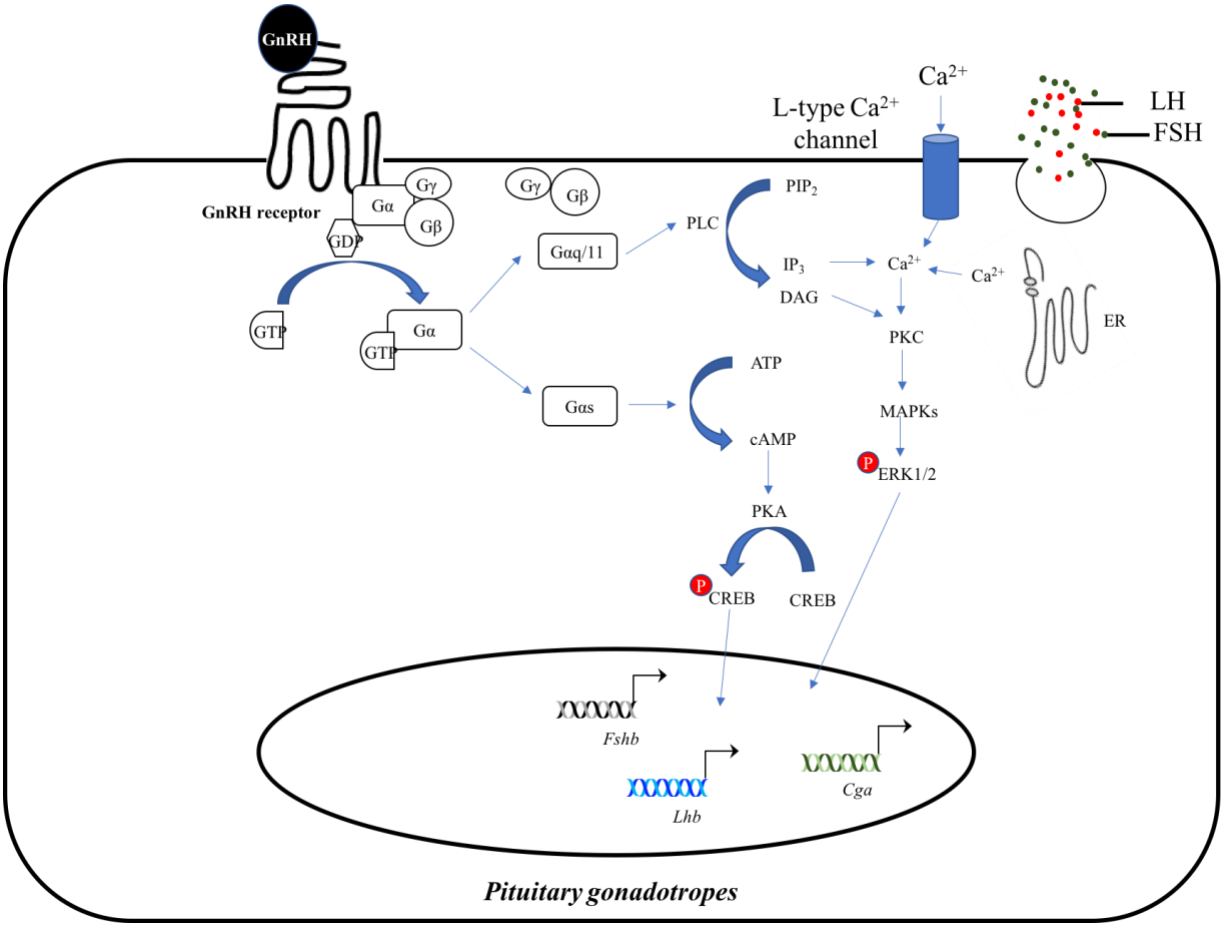
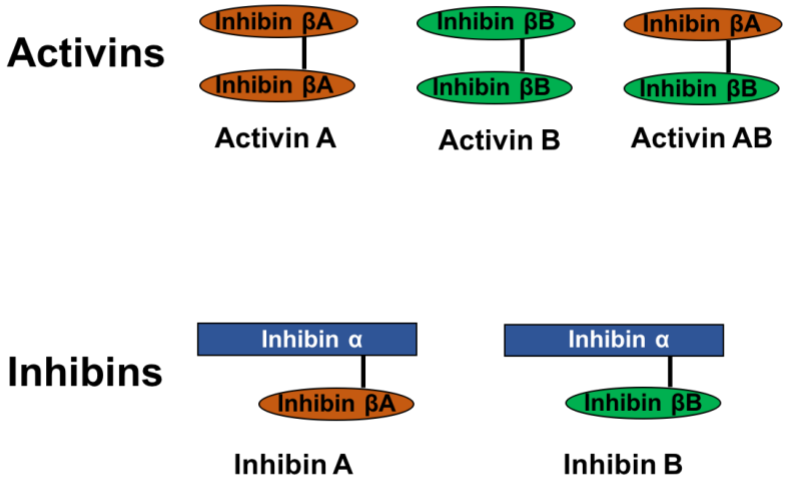

GnRH
GnRH receptor
Gα
Gγ
Gβ
GDP
GTP
Gαq/11
Gγ
Gβ
PLC
PIP2
IP3
DAG
Ca2+
L-type Ca2+ channel
Ca2+
LH
FSH
ER
PKC
MAPKs
ERK1/2
Gαs
ATP
cAMP
PKA
CREB
CREB
Fshb
Lhb
Cga
Pituitary gonadotropes

Figure 1.5

A


Activins
Inhibin βA
Inhibin βA
Activin A
Inhibin βB
Inhibin βB
Activin B
Inhibin βA
Inhibin βB
Activin AB
Inhibins
Inhibin α
Inhibin βA
Inhibin A
Inhibin α
Inhibin βB
Inhibin B

B

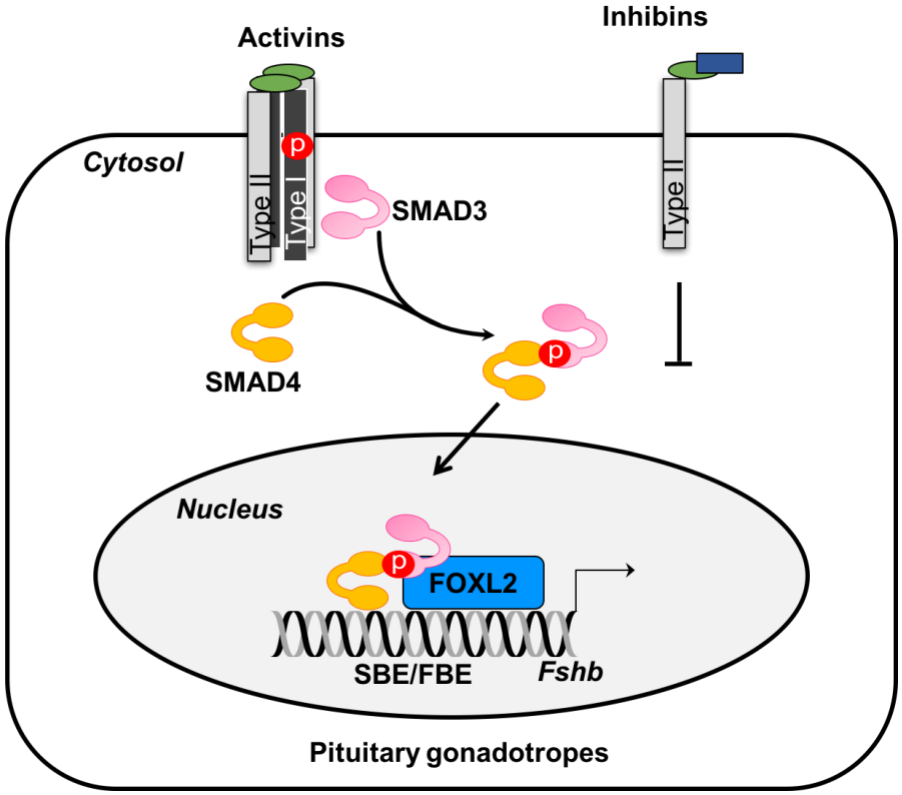

Activins
Inhibins
Cytosol
Type II
Type I
P
SMAD3
SMAD4
Type II
P
Nucleus
P
FOXL2
SBE/FBE
Fshb
Pituitary gonadotropes

Figure 1.6

Chapter 2

In **Chapter 2**, we investigated whether SMAD3 plays an essential role in FSH synthesis *in vivo*. Previous *in vitro* and *in vivo* data have been inconsistent in answering this question. The *in vitro* studies showed a clear role for SMAD3 in activin induction of *Fshb* transcription [393, 396, 403]. In contrast, the first attempt to examine SMAD3's role in gonadotropes *in vivo* suggested that SMAD3 was dispensable for FSH synthesis [408]. However, not all SMAD3 function was eliminated in this first *in vivo* model [408]. Here, I used a combination of different mouse strains that precluded compensatory actions of SMAD4, enabling us to more definitively interrogate SMAD3 function in FSH production *in vivo*.

SMAD3 regulates follicle-stimulating hormone synthesis by pituitary gonadotrope cells *in vivo*

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Abbreviated title: SMAD3 regulates FSH production in vivo

Keywords: FSH, SMAD transcription factor, activin, pituitary gland, gene knockout

Abstract

Pituitary follicle-stimulating hormone (FSH) is an essential regulator of fertility in females and of quantitatively normal spermatogenesis in males. Pituitary-derived activins are thought to act as major stimulators of FSH synthesis by gonadotrope cells. *In vitro*, activins signal via SMAD3, SMAD4, and forkhead box L2 (FOXL2) to regulate transcription of the FSHβ subunit gene (*Fshb*). Consistent with this model, gonadotrope-specific *Smad4* or *Foxl2* knockout mice have greatly reduced FSH and are subfertile. SMAD3's role *in vivo* is unresolved; however, residual FSH production in *Smad4* conditional knockout mice may derive from partial compensation by SMAD3 and its ability to bind DNA in the absence of SMAD4. To test this hypothesis and determine SMAD3's role in FSH biosynthesis, we generated mice lacking both the SMAD3 DNA binding domain and SMAD4 specifically in gonadotropes. Conditional knockout females were hypogonadal, acyclic, sterile, and had thread-like uteri; their ovaries lacked antral follicles and corpora lutea. Knockout males were fertile but had reduced testis weights and epididymal sperm counts. These phenotypes were consistent with those of *Fshb* knockout mice. Indeed, pituitary *Fshb* mRNA levels were nearly undetectable in both male and female knockouts. In contrast, gonadotropin-releasing hormone receptor mRNA levels were significantly elevated in knockouts in both sexes. Interestingly, luteinizing hormone production was altered in a sex-specific fashion. Overall, our analyses demonstrate that SMAD3 is required for FSH synthesis *in vivo*.

Introduction

Pituitary FSH is an essential regulator of gonadal function [221, 650]. FSH acts on ovarian granulosa cells to regulate follicle development and on testicular Sertoli cells to regulate spermatogenesis [221, 651, 652]. In both humans and rodents, mutations in genes regulating FSH synthesis or action cause primary amenorrhea due to the arrest of follicle development at the pre- or early antral stage [653-657]. In contrast, the effects of these mutations in males are species-specific. For example, FSH-deficient mice are oligospermic, but fertile [221], whereas FSH-deficient men are azoospermic and infertile [658-660].

FSH is a dimeric glycoprotein composed of the chorionic gonadotropin α subunit (CGA) non-covalently linked to the FSHβ subunit (FSHβ). The former is shared with other glycoprotein hormones; the latter is specific to FSH [161, 661-663]. Synthesis of the FSHβ subunit is rate-limiting in dimeric FSH production [664-666] and is regulated by several endocrine and autocrine/paracrine factors in the hypothalamic-pituitary-gonadal axis [437, 667]. Among these factors, the pituitary-derived activins have been the most thoroughly investigated, both *in vitro* and *in vivo*. In recent years, particular focus has been placed on the mechanisms through which activins regulate transcription of the FSHβ subunit gene (*Fshb*) [161, 396, 405, 406, 421, 668, 669].

As members of the transforming growth factor β (TGFβ) superfamily, activins signal through complexes of serine/threonine kinase receptors and SMAD signaling proteins [426, 670]. According to current *in vitro* models, activins stimulate the phosphorylation and nuclear accumulation of the receptor-regulated SMAD protein, SMAD3, in gonadotrope cells. In the nucleus, phospho-SMAD3 partners with the ubiquitous co-factor SMAD4 and the cell-specific forkhead box L2 (FOXL2) to promote transcription of the murine *Fshb* gene [393, 403]. Specifically, SMAD4 and FOXL2 bind adjacent *cis*-elements in the proximal *Fshb* promoter (Fig.

2.1, top, SBE2/FBE2). The two proteins are then linked through their mutual association with the C-terminal Mad homology 2 (MH2) domain of SMAD3 [392, 393]. The *in vitro* model further suggests that SMAD3 can regulate murine *Fshb* with SMAD4 at a canonical SMAD binding element (SBE1) located 144 bp upstream of SBE2/FBE2 [393] and with FOXL2 via a third response element (FBE1) approximately 85 bp upstream of the SBE [418] (see Fig. 2.1, top). Importantly, the actions of SMAD3 and SMAD4 at SBE1 appear to be independent of FOXL2, whereas the actions of SMAD3 and FOXL2 at FBE1 are independent of SMAD4 [393, 403]. Thus, only at SBE2/FBE2 do all three proteins work together, but SMAD3 represents the common mediator of activin signaling at all three *cis*-elements [393, 403].

Consistent with the *in vitro* model, conditional deletion of either *Smad4* or *Foxl2* from pituitary gonadotrope cells (hereafter, *Smad4* cKO and *Foxl2* cKO, respectively) produces profound FSH deficiency in mice *in vivo* [409, 417]. Female cKOs are subfertile, producing smaller litters (both models) at reduced frequency (*Foxl2* cKO) compared to wild-type littermates [409, 417]. This contrasts with the more extreme phenotype of *Fshb* knockout females, which are infertile [221]. However, it is important to note that in both *Smad4* and *Foxl2* cKOs, females still produce some FSH, which may be sufficient to drive some ovarian follicle development [409, 417]. This residual FSH production may reflect the fact that there are cis-elements in the *Fshb* promoter where FOXL2 and SMAD4 can act independently of one another (see above and Fig. 2.1). Consistent with this idea, ablation of *Smad4* and *Foxl2* together (hereafter, *Smad4*/*Foxl2* cKO), which would affect protein complex binding to all three *cis*-elements, results in a more extreme FSH deficiency as well as infertility in female mice [409].

In apparent contrast with the in vitro model, however, mice with a conditional deletion in the *Smad3* gene in gonadotropes (hereafter *Smad3* cKO) produce FSH at normal levels and are

fertile [408]. This was a surprising result as, a priori, these mice would be predicted to exhibit a phenotype comparable to that of *Smad4/Foxl2* cKOs. The absence of a phenotype is not explained by compensation via the related receptor-regulated SMAD2, as mice with conditional deletions in both *Smad2* and *Smad3* in gonadotropes exhibit normal fertility and quantitatively normal FSH levels [408]. Instead, the absence of a phenotype may be explained by the observation that the conditional deletion in the *Smad3* gene does not remove all *Smad3* expression (and hence function) in gonadotropes of these mice [408].

In the above described models, the Cre-lox system was used to remove functionally important exons in both the *Smad2* and *Smad3* genes in gonadotropes [408]. Recombination of the *Smad2* gene effectively abolished SMAD2 protein expression. In the case of *Smad3*, however, the floxed region (exons 2 and 3) encodes only one part of the SMAD3 protein: the N-terminal MH1 domain, which mediates SMAD3 binding to DNA [400]. In gonadotropes of *Smad3* cKO mice, the *Smad3* transcript lacked exons 2 and 3, as predicted, but contained the remaining 7 exons (exons 1 and 4-9). Moreover, there was an increase in the abundance of this novel mRNA. Though translation is predicted to start in exon 1 and for the protein to be translated out of frame in exon 4, the translation start in exon 1 does not conform to a consensus Kozak sequence [671]. In contrast, such a sequence exists in exon 4, allowing the C-terminal half of SMAD3 to be translated in frame. This includes the entirety of the MH2 domain, which is phosphorylated by activin type I receptors, and interacts with SMAD4 and FOXL2 [416, 672]. *In vitro*, this truncated protein functions identically to full-length SMAD3, in the presence of SMAD4 [408]. If this is also the case *in vivo*, this could explain the absence of an FSH or fertility phenotype in *Smad3* cKO mice. This possibility is further supported by the *in vitro* model (Fig. 2.1), which indicates that SMAD3 does

bind DNA at the FBE1 or SBE2/FBE2 cis-elements. Unfortunately, these data only indirectly suggest a role for SMAD3 in FSH synthesis *in vivo*.

To definitively rule in or out a role for SMAD3, we need to specifically and completely ablate SMAD3 protein function (including the MH2 domain) in gonadotropes of mice. To our knowledge, there are no other floxed *Smad3* strains currently available that would enable this approach. Still, existing models may allow an assessment of SMAD3 function in FSH synthesis. Specifically, residual FSH production in *Smad4* cKO mice may be explained by SMAD3's ability to partially compensate for SMAD4's absence (Fig. 2.1, bottom). *In vitro,* SMAD3 can bind to SBE2 in the *Fshb* promoter [393] (Fig. 2.1). Moreover, SMAD3-FOXL2 can stimulate transcription via SBE2/FBE2, although less potently than SMAD3-SMAD4-FOXL2 [393]. If SMAD3 does in fact partially compensate for the absence of SMAD4 at this element and must bind DNA to do so, mice lacking SMAD4 as well as SMAD3 DNA binding activity should exhibit more extreme FSH deficiency and fertility phenotypes than mice lacking SMAD4 alone. Here, we tested this hypothesis using the Cre-lox system to simultaneously remove SMAD4 and the SMAD3 MH1 domain in gonadotrope cells.

Results

Truncated SMAD3 stimulates *Fshb* transcription in the presence, but not absence of SMAD4 *in vitro*

Mice lacking *Smad3* exons 2 and 3 express a truncated form of SMAD3, which lacks the DNA binding MH1 domain (hereafter SMAD3-MH2) [408]. We proposed that this protein functions equivalently to full-length SMAD3, but only in the presence of SMAD4, enabling transactivation of the *Fshb* gene. To further test this idea, we compared the activities of full-length

SMAD3 and SMAD3-MH2 in a heterologous reporter assay. We co-transfected SW480.7 cells, a SMAD4-deficient human colon adenocarcinoma-derived cell line [673, 674] with a murine *Fshb* promoter-reporter and the indicated combinations of SMAD3 or SMAD3-MH2, SMAD4, FOXL2, and a constitutively active form of the canonical activin type I receptor (T206D or TD) [389]. The latter was used as a surrogate for ligand. Co-transfection of SMAD3 and FOXL2 stimulated reporter activity, which was further increased by ALK4TD (Fig. 2.2). In contrast, the combination of SMAD3-MH2 and FOXL2 failed to turn on the reporter, whether or not ALK4TD was co-transfected. SMAD3 and SMAD3-MH2 were expressed at comparable levels (data not shown). Remarkably, when SMAD4 was introduced, SMAD3 and SMAD3-MH2 were comparable in their abilities to potently stimulate the *Fshb* reporter in the presence of FOXL2. Thus, as predicted, SMAD3-MH2 can activate the *Fshb* promoter, but only in the presence of SMAD4 (and FOXL2). These results reinforce the idea that SMAD3-MH2 should be unable to promote *Fshb* expression in the absence of SMAD4 *in vivo*.

Smad3 and *Smad4* genes were efficiently and specifically recombined in gonadotropes

To ablate the SMAD3 MH1 domain and SMAD4 in gonadotropes, we crossed mice harboring floxed alleles for *Smad3* and *Smad4* (Fig. 2.3A) with GRIC mice, which express Cre from the GnRH receptor locus. As anticipated, we observed recombination of the floxed alleles in pituitaries of both male and female cKO mice, as well as in the testes and epididymides of cKO males (Fig. 2.3B). Note that, in addition to gonadotropes, the GRIC allele is active in the male germline [675]. An analysis of gonadotropes (YFP+ cells) isolated from these animals revealed highly efficient depletion of *Smad3* (Fig. 2.3C) and *Smad4* (Fig. 2.3E) mRNAs, when examined using primers against the targeted exons. As reported previously [408], there was an overall up-

regulation of the *Smad3* mRNA levels in gonadotropes of cKO mice, though this transcript lacked the targeted exons (Fig. 2.3D). Note that *Smad3* and *Smad4* mRNA expression in non-gonadotrope cells of the pituitary (YFP-) was equivalent between genotypes (white bars in Figs. 2.3C-E). The purity of YFP+ cells was 79.3% for the control and 78.3% for cKO mice, respectively. No YFP+ cells were observed in the YFP- population.

cKO mice are FSH-deficient

Serum FSH levels were significantly depleted in adult cKO mice relative to littermate controls (Figs. 2.4A-B). Similarly, intrapituitary FSH protein content was profoundly reduced in cKO animals as assessed by RIA (Figs. 2.4C-D) or immunofluorescence (Fig. 2.5). Reductions in FSH protein derived from a robust decrease in pituitary *Fshb* mRNA expression in cKOs (Figs. 2.6A-B).

Male cKO mice also showed diminished serum LH relative to controls (Fig. 2.4E). In contrast, serum LH was significantly elevated in cKO females (Fig. 2.4F). Consistent with the sex difference in LH secretion, pituitary *Lhb* and *Cga* mRNA levels were modestly decreased in cKO males but markedly increased in cKO females (Figs. 2.6A-B). Interestingly, these RNA differences were not reflected at the level of intrapituitary LH protein content (Figs. 2.4G-H and 5). In both sexes, pituitary *Gnrhr* mRNA expression was upregulated in cKOs relative to controls (Figs. 2.6A-B). Expression of *Smad3* or *Smad4* did not differ between genotypes of either sex, except for *Smad4*, which was modestly decreased in cKO males (Figs. 2.6A-B). Given the relatively small proportion of gonadotropes in the pituitary (~5-10%) and the ubiquitous expression of SMADs, we did not expect to detect changes in *Smad3* or *Smad4* in the analysis of the whole gland. Though, as described above, recombination in gonadotropes was highly efficient (Fig. 2.3).

Impaired fertility in cKO females and males

Eight-week-old male and female control and cKO mice were paired with age-matched wild-type C57BL/6 mice for a period of 6 (males) or 4 (females) months. Littermate controls and cKOs were mated on the same day. cKO and control males sired equivalent numbers of litters at similar intervals over 6 months (Figs. 2.7A); however, cKOs fathered smaller litters (Fig. 2.7B). Note that two of the six cKO males only sired one litter and were excluded from the analysis. Control females were fertile and produced an average of 4.5 litters and 6.25 pups per litter over the course of 4 months. In contrast, cKO females were uniformly sterile; none produced a single pup or showed any overt evidence of pregnancy during the mating trial (Fig. 2.7C).

Spermatogenesis is impaired in cKO males

Despite having equivalent body mass relative to littermate controls (Supplementary Fig. S2.1A), cKO males exhibited a 50% reduction in testicular mass (Figs. 2.8A-B). Cauda epididymidal sperm count was similarly reduced by 50% in cKO males relative to controls (Fig. 2.8C). In contrast, there were no differences between genotypes in progressive sperm motility (Fig. 2.8D) or seminal vesicle weights (Fig. 2.8E). Similar results were seen in control and cKO males at the conclusion of the fertility trials (data not shown). Consistent with the normal seminal vesicle weights, but in apparent contrast with the reduced serum LH, cKO males had statistically normal serum testosterone levels (Fig. S2.2). Despite impairments in spermatogenesis, cKO males showed overtly normal testicular histology (Fig. 2.8F).

cKO females are acyclic and hypogonadal

We next examined the underlying cause of infertility in cKO females. Adult cKO females exhibited normal body mass (Fig. S2.1B), but were profoundly hypogonadal with hypoplastic uteri (Figs. 2.9A,C). The latter condition was emblematic of estrogen-deficiency, though we did not measure levels of 17β-estradiol (E2) in these animals. In our experience, E2 ELISAs lack sufficient sensitivity to accurately measure the hormone on cycle stages other than proestrus in the mouse. Though cKO and control littermates exhibited vaginal opening at roughly the same postnatal age (Fig. 2.9D), we were unable to reliably measure estrous cyclicity in cKO females (data not shown). Indeed, as indicated in ovarian tissue sections, cKO females showed an arrest in follicle development at the pre-antral or early antral stage and the complete absence of corpora lutea (Fig. 2.9E). cKO mice also showed impaired responsiveness (ovulation) to exogenous gonadotropins when treated as juveniles (postnatal days 24-28; Fig. 2.9F).

Basal and activin A stimulated *Fshb* expression are reduced in cKO pituitaries

To more directly assess the effects of the targeted mutations on activin signaling in gonadotropes, we cultured pituitary cells from male and female cKO and control mice. Basal Fshb mRNA expression in murine pituitary cultures depends on activins or related ligands [388]. Here, basal *Fshb* expression was almost completely abolished in cKO cultures relative to controls (Figs. 2.10A-B). Activin A induced significant increases in *Fshb* expression in cultures from both control males and females (Figs. 2.10A-B). In contrast, activin A modestly stimulated *Fshb* in cultures from male but not female cKOs (Figs. 2.10A-B).

Discussion

SMAD3 regulates FSH synthesis *in vivo*

The data presented here provide the first conclusive evidence that SMAD3 regulates FSH synthesis *in vivo*. Previously, we reported that *Smad3* conditional knockout [672] mice are fertile with normal FSH synthesis [408]. However, these animals express a truncated and functional form of SMAD3 (i.e., the C-terminal MH2 domain) that lacks DNA binding activity (i.e., the N-terminal MH1 domain) but can still cooperatively regulate *Fshb* promoter activity with SMAD4 and FOXL2 (e.g., Fig. 2.2). Though we will eventually require a model that removes all SMAD3 function in gonadotropes, here we were able to combine two existing mouse strains to probe SMAD3's role in FSH synthesis: the *Smad3* mice described above (lacking the MH1 domain) and mice lacking *Smad4* in gonadotropes [409].

The latter have reduced FSH and are sub-fertile [409]. We argued that the residual FSH in these animals results from partial compensation by full-length SMAD3 and its ability to bind DNA via its MH1 domain [400] (Fig. 2.1, bottom). If this is true, then mice lacking the SMAD3 MH1 domain should be unable to compensate for the absence of SMAD4. Indeed, as we show here, *Fshb* mRNA and FSH protein are essentially undetectable in these mice. As a result, females are sterile; males are oligospermic and have small testes. These phenotypes are consistent with those of *Fshb* knockouts [221]. Our data therefore suggest that SMAD3 regulates FSH synthesis *in vivo*. They also suggest that SMAD3's MH2 domain is more critical for *Fshb* regulation than its MH1 domain and that SMAD complex binding to the *Fshb* promoter is mediated principally via SMAD4.

We contend that the SMAD3-MH1 domain plays a lesser role than its MH2 domain in *Fshb* regulation for at least two reasons. First, mice lacking the SMAD3-MH1 domain produce FSH at seemingly normal levels [408]. A role for the SMAD3-MH1 domain in FSH synthesis is only

observed when SMAD4 is absent (as demonstrated here). Second, the SMAD3-MH2 domain is phosphorylated by the type I receptor (in response to ligand binding and receptor complex activation) and mediates the protein's interactions with both SMAD4 and FOXL2 [392, 416, 672, 676, 677]. Based on these observations, one could argue that SMAD3 may not need to directly bind the *Fshb* promoter to regulate its transcription under normal conditions. Instead, SMAD complex binding activity is conferred principally by SMAD4 and FOXL2.

If this is true, the 8-bp SMAD binding element (SBE1 in Fig. 2.1) in the proximal promoter may not play a significant role in FSH synthesis *in vivo*, as SMAD3-SMAD4 complex binding to this site requires DNA binding activity of both proteins, at least *in vitro* [403]. The role, if any, of this *cis*-element *in vivo* should be assessed, perhaps with the aid of newer gene-editing technologies (e.g., CRISPR-Cas9). Interestingly, though this was the first activin responsive *cis*-element described in the *Fshb* promoter [406], it has only been observed in rodents thus far [161]. The *Fshb* promoters of other species, such as sheep and pigs, can be robustly stimulated by activins in the absence of this 8-bp SBE [405, 415, 678]. Moreover, adding this element to the human promoter only modestly increases its activin and SMAD sensitivity [403]. It is perhaps unsurprising then that it might not play an essential role in mice. Indeed, even point mutations in this element in *in vitro* promoter-reporter assays only partly attenuate activin responsiveness [393, 403, 664].

A potential role for SMAD2 in FSH synthesis?

Our contention that the SMAD3-MH2 domain is essential for FSH synthesis is challenged by earlier findings. To date, four mouse strains with targeted mutations in *Smad3* have been described. As we argued previously [408], we would predict that mice in two [679, 680], if not

three [681], of these strains likely produce *Smad3* transcripts that encode the MH2 domain, akin to what we describe in the present model. A fourth strain (hereafter *Smad3*$^{\Delta ex8}$), however, lacks exon 8 (of the 9 exon gene) and, as a result, the terminal 89 amino acids of the MH2 domain of the protein [682]. This includes the critical C-terminal serines that are phosphorylated by type I receptors. FSH has been measured in these mice, but not in great detail and with seemingly conflicting results. Female *Smad3*$^{\Delta ex8}$ mice appear to have increased circulating FSH, which may result from the loss of estrogen and/or inhibin feedback (i.e., these animals also have ovarian defects [683]). In contrast, pituitary *Fshb* mRNA levels are reduced by about 30% in 8-week-old *Smad3*$^{\Delta ex8}$ males [684]. Though the basis of the sex difference is not yet clear, the fact remains that these mice lack a functional SMAD3-MH2 domain and can still produce FSH protein or transcript at slightly reduced to elevated levels.

The prediction of our model is that complete loss of SMAD3 (and in particular the MH2 domain) should abrogate FSH production, as we see in *Smad4/Foxl2* cKO mice [409] and the *Smad3/4* cKO animals described here. The *Smad3*$^{\Delta ex8}$ mice challenge this idea and raise the possibility that SMAD2 might compensate for the loss of functional SMAD3 in these animals. This is plausible for a few reasons. First, SMAD2 is abundantly expressed in gonadotropes and is phosphorylated in response to activin stimulation, at least in immortalized gonadotropes [396, 406]. Second, SMAD2 interacts with both SMAD4 and FOXL2 [392, 672]. The latter interaction appears to be weaker than the association between SMAD3 and FOXL2, but is still measureable. Moreover, the interaction might be enhanced in the absence of SMAD3. Recall that *Smad3*$^{\Delta ex8}$ mice would not express a functional SMAD3-MH2 domain, which is required for the FOXL2 interaction. Third, SMAD2 was previously discounted because it lacks DNA binding activity [408]. However, as we discuss above, SMAD3 does not need to bind to DNA to regulate *Fshb*. Thus, if

SMAD2 is indeed partially compensating for the loss of SMAD3 function in *Smad3*$^{\Delta ex8}$ mice, then crossing these animals with those lacking SMAD2 in gonadotropes [408] should lead to severe FSH deficiency.

We would still argue that, under normal circumstances, SMAD2 plays little to no role in *Fshb* regulation. Instead, SMAD3 is likely the primary transducer of type I receptor activation to the *Fshb* promoter. This concept is supported by the absence of an FSH phenotype in gonadotrope-specific *Smad2* knockout mice [408]. Rather, SMAD2's ability to regulate *Fshb* may only be manifested when SMAD3 function is compromised, as in *Smad3*$^{\Delta ex8}$ mice. If this is true, it begs the question of why SMAD2 does not compensate in the *Smad3/4* cKO mice described here. The simple answer is likely because it lacks DNA binding activity. That is, in *Smad4* cKO mice, SMAD3 can partially compensate because it can bind DNA via its MH1 domain. In *Smad3/4* cKO mice, SMAD2's inability to bind DNA and its weaker FOXL2 interaction would likely preclude compensation. There is a naturally occurring splice variant of SMAD2 that can bind DNA, but it is expressed at far lower levels than the full-length isoform [394-396, 406].

Shared and unique phenotypes of *Smad3/4* vs. *Smad4/Foxl2* knockout mice

The phenotypes of *Smad3/4* cKO mice are highly similar, but not identical to those of *Smad4/Foxl2* cKO mice [409] (see Table 2.1). In both models, FSH production is severely impaired, leading to oligospermia in males and sterility in females. Females exhibit a block in folliculogenesis at the pre-antral or early antral stage akin to what was described in *Fshb* knockout mice [221]. In both models, LH secretion and *Lhb* mRNA expression are increased in females. *Cga* mRNA expression and LH secretion are reduced in males of both models. Interestingly, however, *Lhb* mRNA levels are decreased in *Smad3/4* cKO males, but elevated in *Smad4/Foxl2*

cKO males. *Lhb* mRNA is not altered in males lacking *Smad4* alone, suggesting that SMAD3 and FOXL2 play distinct roles in *Lhb* regulation. At present, there is no evidence that FOXL2 regulates *Lhb* expression [417]. However, SMAD3 can potentiate the stimulatory effects of EGR1 (a mediator of GnRH action) on *Lhb* promoter activity [685] *in vitro* and this effect is dependent upon SMAD binding elements [684]. Therefore, the loss of SMAD3 binding to the *Lhb* promoter in *Smad3/4* cKO males may contribute to the reduction in *Lhb* mRNA expression. Unfortunately, we did not measure *Lhb* mRNA in mice lacking SMAD3-MH1 [408], so it is unclear whether the downregulation depends on coordinated loss of both SMAD3 and SMAD4 DNA binding activity. Moreover, it is not yet clear why *Lhb* levels are reduced in *Smad3/4* cKO males but elevated in females, though this may reflect sex specific endocrine effects.

Another major difference between *Smad3/4* and *Smad4/Foxl2* cKO models is in pituitary expression of *Gnrhr*, which is elevated in the former, but unchanged in the latter. The *Gnrhr* is similarly upregulated in pituitaries of mice lacking *Smad4* alone [409], but is unaltered in mice lacking *Foxl2* alone [417]. These data suggest that the loss of *Smad4* likely underlies the increase in *Gnrhr* in *Smad3/4* cKO mice. Though, we cannot rule out effects of the loss of SMAD3 DNA binding activity, as we did not measure *Gnrhr* in mice with the isolated *Smad3* deletion [408]. Interestingly, *Gnrhr* upregulation is not observed in pituitaries cultured from *Smad3/4* cKO males or females relative to controls (Figs. S2.3E,F). This suggests that endocrine factors (e.g., GnRH) contribute to the increase in gene expression observed *in vivo*. As such, neither SMAD4 nor the SMAD3-MH2 domain appears to regulate *Gnrhr* expression directly.

In conclusion, the data presented here indicate that: 1) SMAD3 regulates FSH synthesis by gonadotrope cells *in vivo*, 2) FSH production in *Smad4*-deficient mice is partly rescued by full-length SMAD3, 3) SMAD4 DNA binding activity plays a more essential role in transcriptional

regulation of *Fshb* than does SMAD3 DNA binding activity, 4) SMAD3/SMAD4 binding to the 8-bp SBE at -266/-259 of the murine *Fshb* promoter (SBE1) may be dispensable for transcription regulation of the gene, and 5) SMAD3 and SMAD4 play essential roles in *Fshb* expression *in vivo*. The gene deletions do not appear to markedly perturb gonadotrope cell development as expression of other gonadotrope-specific genes such as *Gnrhr*, *Lhb*, and *Cga* is largely intact. As SMAD3 and SMAD4 are mediators of TGFβ superfamily signaling and have not previously been implicated in GnRH action, these data indicate that activins play a dominant role over GnRH in the regulation of FSH and/or that GnRH action is somehow dependent upon a functional activin signaling cascade.

Experimental procedures

Materials

Pregnant mare's serum gonadotropin (PMSG or eCG, G4877), human chorionic gonadotropin (hCG, C1063), hyaluronidase (H3884), BSA (A4378), M199 medium with Hank's salt (M7653), avertin (2,2,2-Tri bromoethanol, T48402), paraformaldehyde (PFA, P6148) were from Sigma-Aldrich (St. Louis, MO). EvaGreen (AC814737H) was from Applied Biological Materials (ABM) Inc. (Richmond, BC). Polyethylenimine (PEI, 23966) was from Polysciences Inc (Warrington, PA). RNasin (0000183771), moloney murine leukemia virus reverse transcriptase (MMLV RT, 0000172807), DNase (0000156360), and Random hexamer primers (0000184865) were from Promega Corporation (Madison, WI). TRIzol® reagent (15596026), Fetal Bovine Serum (FBS, 10438026), Alexa Fluor 488 donkey anti-goat (A11055), Alexa Fluor 594 donkey anti-rabbit antibodies (A21207), ProLong® Gold Antifade reagent with DAPI (1266174), o-Phenylenediamine (OPD, 002003) were from Life Technologies (Eugene, OR).

Deoxynucleotide triphosphates (dNTPs, 800-401-TL), HBSS (311-511-CL), and DMEM (319-005-CL) were from Wisent Inc. (St-Bruno, QC). Rabbit anti-FSHβ antibody (AFP7798_1289P) was from NIDDK (Bethesda, MD). Goat anti-Lutropin β (LHβ) antibody (sc-7824) used in immunofluorescence was from Santa Cruz Biotechnologies Inc. (Dallas, TX). Tissu-Tek® optimal cutting temperature (OCT, 4583) compound was from Sakura Finetek Europe B.V. (Alphen aan den Rijn, Netherlands). Recombinant activin A (338-AC-050) was from R&D Systems (Minneapolis, MN). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). Total RNA Mini Kit (FA32808-PS) was from Geneaid (New Taipei City, Taiwan). Adenoviruses expressing GFP (Ad-GFP) and Cre-IRES-GFP (Ad-Cre) were from Baylor College of Medicine Vector Development Laboratory (Houston, TX). C57BL/6 mice were ordered from Charles River (Montreal, QC). Isoflurane (CP0406V2) was purchased from Fresenius Kabi (Homburg, Germany). Mouse LH reference (AFP5306A), rabbit anti-LH hormone (AFP240580Rb) used in the LH ELISA was provided by Dr. Parlow (NHPP). Monoclonal anti-bovine LH mAb (51887) used in the LH ELISA was provided by Dr. Roser (University of California-Los Angeles). Donkey anti-rabbit horseradish peroxidase (HRP) (P0448) was purchased from Dako Canada ULC (Mississauga, ON).

Constructs

The full-length and SMAD3-MH2 expression constructs were described previously [408]. To circumvent expression differences between full-length and truncated SMAD3 constructs, we introduced a mutation in the start codon in exon 1 of the latter to bias translation initiation toward the AUG in exon 4. We used the QuikChange site-directed mutagenesis protocol and the following primer set on the MH2 expression vector (forward: CTT CGG TGC CAG CCT TGT CGT CCA

TCC TG, reverse: CAG GAT GGA CGA CAA GGC TGG CAC CGA AG). The constitutively active ALK4 expression vector (T206D) and -1990/+1 m*Fshb*-luc reporter were described previously [389, 396].

Cell culture, transfection, promoter-reporter assays

SW480.7 (human SMAD4-deficient colon carcinoma; gift from Dr Fang Liu, Rutgers University, USA) [673, 674] were cultured in DMEM with 10% FBS (v/v) at 37oC and 5% CO2. For promoter-reporter experiments, cells were seeded in 48-well plates at a density of 70,000 cells/well. Approximately 24 h after seeding, cells were transfected with 225 ng/well of the -1990/+1 murine *Fshb*-luciferase reporter plasmid, along with 4.15 ng of FOXL2 and/or 25 ng of the indicated SMAD expression constructs using PEI in serum-free medium for 2 h. Where indicated, ALK4-TD was co-transfected at 10 ng/well. After transfection, serum-free medium was changed to complete medium. Forty-eight hours later, cells were rinsed with PBS and lysates were prepared as previously described [426, 686]. All conditions were performed in duplicate wells, in three independent experiments. Luciferase assays were performed as described previously [687] on an Orion II microplate luminometer (Berthold detection systems, Oak Ridge, TN).

Animals

The *Smad3*$^{fx/fx}$, *Smad4*$^{fx/fx}$ and *Gnrhr*$^{IRES\text{-}Cre/IRES\text{-}Cre}$ (hereafter GRIC) mice were described previously [648, 688, 689]. *Smad3*$^{fx/fx}$ were crossed with *Smad4*$^{fx/fx}$ animals to generate *Smad3*$^{fx/+}$;*Smad4*$^{fx/+}$ progeny, which were then crossed to produce *Smad3*$^{fx/fx}$;*Smad4*$^{fx/fx}$ animals. GRIC females were crossed to *Smad3*$^{fx/fx}$;*Smad4*$^{fx/fx}$ males. Their female GRIC/+;*Smad3*$^{fx/+}$;*Smad4*$^{fx/+}$ progeny were again crossed to *Smad3*$^{fx/fx}$;*Smad4*$^{fx/fx}$ males to

produce GRIC/+;*Smad3*$^{fx/fx}$;*Smad4*$^{fx/+}$ females. Conditional *Smad3/4* double knockouts (GRIC/+;*Smad3*$^{fx/fx}$;*Smad4*$^{fx/fx}$ or cKO) and control (*Smad3*$^{fx/fx}$;*Smad4*$^{fx/fx}$) littermates were finally generated by crossing *Smad3*$^{fx/fx}$;*Smad4*$^{fx/fx}$ males with GRIC/+;*Smad3*$^{fx/fx}$;*Smad4*$^{fx/+}$ females. To enable gonadotrope purification, *Gt[690]26Sor*$^{tm1(EYFP)Cos}$/J males (hereafter *ROSA26*$^{YFP/YFP}$) were crossed with GRIC/+;*Smad3*$^{fx/fx}$;*Smad4*$^{fx/+}$ females mice to generate GRIC/+;*Smad3*$^{fx/+}$; *Smad4*$^{fx/+}$;*ROSA26*$^{YFP/+}$ mice [691]. GRIC/+;*Smad3*$^{fx/+}$;*Smad4*$^{fx/+}$;*ROSA26*$^{YFP/+}$ females were then crossed with *Smad3*$^{fx/fx}$;*Smad4*$^{fx/fx}$ males to obtain experimental GRIC/+;*Smad3*$^{fx/fx}$;*Smad4*$^{fx/fx}$;*ROSA26*$^{YFP/+}$ mice, in which gonadotropes deficient in SMAD3 and SMAD4 expressed enhanced YFP (because of Cre activity). Genotyping primers are listed in Table 2.2. All animal experiments were performed in accordance with institutional and federal guidelines and were approved by the McGill University and Goodman Cancer Centre Facility Animal Care Committee (protocol 5204).

FACS

Cell sorting was performed at the Cell Vision Core Facility for flow cytometry and single cell analysis of the Life Science Complex at Rosalind & Morris Goodman Cancer Research Centre. Pituitary cell suspensions were prepared from 14 GRIC/+;*Smad3*$^{fx/fx}$;*Smad4*$^{fx/fx}$;*ROSA26*$^{YFP/+}$ male and female animals as described previously [417] and single cells' suspensions were prepared in PBS. Fourteen GRIC/+;*ROSA26*$^{YFP/+}$ male and female mice were used as control. Cells were then passed through a 70 µm nozzle at 70 pounds per square inch (psi) into a Becton Dickinson FACSAria software (v.8.0.1). The pituitary cells were then run and gated to sort YFP+ (gonadotropes) and YFP- (non-gonadotropes) cells from both control and experimental pituitaries. We total obtained 1.7×10^4 YFP+ and 7.1×10^5 YFP- cells for the controls; 1.4×10^4 YFP+ and

6.8×10^5 YFP- cells for the experimental animals. Sorting purity was assessed by culturing 5 µl of both YFP+ and YFP- cell from control and cKO pituitaries in one well of a 96-well plate with M199 media. Twelve hours later, YFP+/-ratio was calculated in each group. Total RNA were extracted from YFP+ and YFP- cells using Total RNA Mini Kit according to manufacturer's instructions. Pituitary selective gene expression and recombination was assessed by RT-qPCR.

Hormone analyses

Blood was collected by cardiac puncture and allowed to clot at room temperature for 20 min. Serum was obtained following centrifugation at 3000 x g and stored at -20°C until analysis. To measure pituitary FSH and LH content, pituitaries were isolated and homogenized in 300 µL PBS, sonicated, and centrifuged for 10 min at 16,000 g at 4°C. Supernatant was removed and kept at -20°C until assayed. All hormone assays were performed at the Ligand Assay and Analysis Core of the Center for Research in Reproduction at the University of Virginia (Charlottesville, Virginia) except for pituitary LH content (below). Serum FSH and LH levels were measured by multiplex ELISA. The reportable ranges were 2.40-300.0 ng/ml and 0.24-30.0 ng/ml for FSH and LH, respectively. Intra-assay CVs were < 5.0%. Testosterone was measured by ELISA. The reportable range is 10-1600.0 ng/ml. Intra-assay CVs is > 20%. For pituitary FSH content, all samples were diluted 50 times and then measured by radioimmunoassay. The reportable range was 2.9-40.0 ng/ml. For pituitary LH content, all homogenized samples were diluted 10^6 times in 0.05% PBST supplemented with 0.2% BSA and measured with an in-house sandwich ELISA as described previously [55].

Immunofluorescence

Ten to twelve week-old male and female animals were anesthetized with 200 mg/kg Avertin by intra-peritoneal (IP) injection followed by an overdose of isoflurane through a nose cone. The animals were transcardially perfused with PBS followed by freshly made 4% paraformaldehyde (PFA) in PBS. Pituitaries were isolated and post-fixed in 4% PFA for 3 h at room temperature. After a brief rinse with PBS, pituitaries were immersed in 30% sucrose overnight at 4°C and then embedded in OCT on dry ice. Pituitaries were sectioned (5 µm) on a cryostat and mounted on pre-chilled Micro slides. The slides were then warmed up to room temperature and sections blocked in 1.5% donkey serum in PBS for 1 h. Sections were washed 3 times in 0.2% PBST and then incubated with rabbit anti-rat FSHβ (1:500) and goat anti-LHβ (1:500) overnight at room temperature. After 3 washes with PBST, sections were incubated with Alexa fluor 594-conjugated donkey anti-rabbit (1:600) and Alexa fluor 488-conjugated donkey anti-goat (1:600) secondary antibodies for 1 h at room temperature. After another 3 washes with PBST, coverslips were mounted with ProLong Gold antifade reagent with (DAPI) and dried at 37°C for 15 min in the dark. Images were acquired on a Zeiss Axio Images were acquired on a Zeiss Axio Imager M2 microscope and were acquired with Axiocam 506 color camera (Zeiss) using Zeiss blue edition (Zen 2 lite) software.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Pituitaries from 10-week-old mice were extracted and immediately frozen in liquid nitrogen and stored at -80°C. Control females were euthanized at 7 am on the morning of estrus (as assessed by vaginal cytology). cKO females, which did not cycle, were euthanized the same day as control littermates. Samples were homogenized in 500 µl TriZol reagent and total RNA

extracted according to the manufacturer's protocol. RNA concentration was determined by NanoDrop. Two μg of RNA per sample were then reverse transcribed into cDNA using MMLV reverse transcriptase and random hexamer primers at a final volume of 40 μl. Two μl of cDNA were then used as template for qPCR analysis as described previously [396] on a Corbett Rotorgene 600 instrument (Corbett Life Science) using EvaGreen reagent and primers listed in Table 2.2. mRNA levels of target genes were determined using the $2^{-\Delta\Delta Ct}$ method. Ribosomal protein L19 (*Rpl19*) was used as a control for normalization.

Puberty onset, estrous cyclicity, and fertility assessment

To assess puberty onset, females were monitored daily for vaginal opening since post-natal 24 [692]. Starting at 8 weeks of age, estrous cyclicity was assessed daily for 2 to 3 weeks in the afternoon (~3 pm) by collecting vaginal cells with a cotton swab wet with sterile saline. The cells were smeared on a glass slide, stained with 0.1% methyl blue, and examined by light microscopy. Staging was assessed according to published guidelines [692]. One cycle was defined as the sequential appearance of all estrous cycle stages, regardless of the number of days spent in each stage. To assess fertility, 8-week-old males (n=6/genotype) and females (n=4/genotype) were paired with age-matched, wild-type C57BL/6 mice for 4 or 6 months respectively. Breeding cages were monitored daily and the date of birth and number of newborn pups was recorded. Pups were removed from the breeding couple at postnatal day 15. At the end of the mating trials, control and cKO animals were dissected; blood samples were collected as described above. Pituitaries were isolated, snap frozen, and kept at -80°C until processed for RNA extraction using TriZol. One side of ovaries or testes were collected in a pre-measured Eppendorf tube, weighed, and fixed in 10% Formalin (ovaries) or Bouin's buffer (testes).

Sperm counting

Epididymal sperm heads were counted using a hemacytometer as described previously [417]. Briefly, the left epididymis of the animal (n=9 per genotype) was immediately snap frozen in liquid nitrogen, weighed, and then homogenized (2 x 15 sec, separated by 30 sec) on ice in 2 ml 0.9% NaCl, 0.1% Thimerosal, and 0.5% Triton X-100 with a Polytron on power setting 4. Samples were then diluted 20 times in PBS. Ten µl of each sample were loaded and counted on each side of hemacytometer.

Sperm motility analysis

Sperm motility of 10 to 12-week-old male control (n=8) and cKO (n=6) mice was analyzed using computer-assisted sperm analysis (CASA), as described previously [693]. All materials and tools were pre-warmed and kept at 37ºC before and during all steps. The left side cauda epididymis of each animal was clamped off and briefly rinsed in pre-warmed Hank's salt M199 media supplemented with 0.5% (w/v) BSA and adjusted to pH 7.4. The tissue was cut 10 times with fine-point scissor and sperm were allowed to disperse in fresh M199 media with BSA for 5 min in a 35-mm Petri dish. The sperm suspension was then diluted 4 times before loading into a 2X-CEL 80-µm deep sperm analysis chamber. Subsequently, 20 µl of diluted sperm sample was analyzed using a Hamilton-Thorne IVOS automated semen analyzer (Hamilton-Thorne Biosciences, Beverly MA). IVOS parameters were set according to the standards determined by The Jackson Laboratory (courtesy of Hamilton-Thorne). Two slides were analyzed per sample, and 5 views from each slide were analysed.

Reproductive organ histology

Ovaries and testes were isolated from 10-week-old control and cKO females and males, respectively, weighed on a precision balance and fixed in 10% formalin or Bouin's buffer at room temperature. Tissues were then paraffin embedded and cut into 5 μm sections. Ovarian and testicular sections were stained with H&E. The images were acquired with Leica DFC310 FX 1.4-megapixel digital color camera under high-Quality transmitted light microscopy (Leica DM1000 LED) using Leica Application Suite Version 4.0.0 software.

Superovulation

Superovulation was performed in juvenile (post-natal day 23-28) females as described previously [417]. Control and cKO females were injected intraperitoneally (i.p.) with 5 IU PMSG at 5 pm. Forty-eight hours later, mice were treated i.p. with 5 IU hCG. After 14 h, mice were killed and cumulus-oocyte complexes (COCs) were harvested in PBS from ampullae of the oviduct. COCs were enzymatically dissociated using 0.5 mg/ml hyaluronidase for 10 min at 37°C. Oocyte number was then counted under an inverted microscope.

Primary pituitary cultures

Primary pituitary cultures were prepared as previously described [694]. Briefly, pituitaries were isolated from adult control and cKO animals, single suspended cells were seeded at a density of 300,000 cells/well in 48-well plates. Cells were settled for 16 hours with M199 culture media supplement with 10% FBS. The media were then replaced with 2% FBS-containing media in the presence or absence of 1 nM activin A. Note that cultures from males and females were prepared

and processed separately. Culture media were removed and cells were harvested and RNA was extracted using Total RNA Mini Kit following the manufacturer's instructions.

Statistical analysis

All data were analyzed by unpaired Student's t tests except pituitary gene expression in the primary culture experiments, which was analyzed by multiple t tests. For the reporter assay, two-way ANOVA was conducted, followed by a Bonferroni-corrected multiple comparison test. Statistical analyses were performed using GraphPad Prism 6 (GraphPad). Values of $p<0.05$ were considered statistically significant.

Acknowledgements

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McGill Life Science Complex was supported by funding from the Canadian Foundation for Innovation.

Footnotes

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Author contributions: Y.L. and D.J.B. were responsible for the experimental design, data analyses, and manuscript preparation. Y.L. conducted the majority of the experiments. G.S. conducted the promoter-reporter assays, some qPCR experiments, and edited the manuscript. U.B., C.D., and J.G. generated the Cre and floxed *Smad* mouse strains and edited the manuscript. All authors reviewed the results and approved the final version of the manuscript.

The authors declare that they have no conflicts of interest with the contents of this article.

Table 2.1 Pituitary gene expression profiles in different conditional knockout models

Genotype	*Fshb*		*Lhb*		*Cga*		*Gnrhr*		Fertility	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Fshb KO	↓↓	↓↓	=	↑	N/D	N/D	N/D	N/D	?	−
Smad2/3 cKO	=	=	N/D	N/D	N/D	N/D	N/D	N/D	+++	+++
Foxl2 cKO	↓	↓	=	=	=	=	=	=	++	+
Smad4 cKO	↓	↓	=	=	↓	=	↑	↑	+++	++
Smad4/Foxl2 cKO	↓↓	↓↓	↑	↑	↓	=	=	=	+++	−
Smad3/Smad4 cKO	↓↓	↓↓	↓	↑	↓	↑	↑	↑	++	−

N/D: not determined
↑: increased in cKO relative to control (more arrows indicate larger effects)
↓: decreased in cKO relative to control
=: equivalent between cKO and control
+++: comparable litter sizes and frequency to controls
?: males reproduce, but their fertility was not systematically characterized
++: smaller litters than controls
+: smaller litters and at lower frequency than controls
−: sterile

Table 2.2: Genotyping and qPCR primers

Genotyping	
Smad3	5’ – 3’
Forward	CTCCAGATCGTGGGCATACAGC
Reverse	GGTCACAGGGTCCTCTGTGCC
Recombined	TCGTCGATCGACCTCGAATAAC
Smad4	
Forward	GGGCAGCGTAGCATATAAGA
Reverse	GACCCAAACGTCACCTTCAG
Recombined	AAGAGCCACAGGTCAAGCAG
GRIC	
Forward	GGACATGTTCAGGGATCGCCAGGC
Reverse	GCATAACCAGTGAAACAGCATTGCTG
ROSA26 eYFP	
Forward	AAAGTCGCTCTGAGTTGTTAT
WT reverse	GCGAAGAGTTTGTCCTCAACC
eYFP reverse	GGAGCGGGAGAAATGGATATG

qPCR	
Smad3exons	5’ – 3’
Forward	CATTCCATTCCCGAGAACAC
Reverse	ATGCTGTGGTTCATCTGGTG
Smad3exons	
Forward	CATCCGTATGAGCTTCGTCA
Reverse	CATCTGGGTGAGGACCTTGT
Smad4	
Forward	TCACAATGAGCTTGCATTCC
Reverse	CCATCCACAGTCACAACAGG
Fshb	
Forward	GTGCGGGCTACTGCTACACT
Reverse	CAGGCAATCTTACGGTCTCG
Rpl19	
Forward	CGGGAATCCAAGAAGATTGA
Reverse	TTCAGCTTGTGGATGTGCTC
Gnrhr	
Forward	CACGGGTTTAGGAAAGCAAA
Reverse	TTCGCTACCTCCTTTGTCGT
Lhb	
*F*orward	AGCAGCCGGCAGTACTCGGA
*R*everse	ACTGTGCCGGCCTGTCAACG
Cga	
*F*orward	TCCCTCAAAAAGTCCAQGAGC
*R*everse	GAAGAGAATGAAGAATATGCAG

Figure legends

Figure 2.1 Model of SMAD signaling to the *Fshb* promoter in murine gonadotrope cells of wild-type and *Smad4* knockout mice. Top: In wild-type mice, activins stimulate the formation of complexes of SMAD proteins and FOXL2, which act via at least three *cis*-elements in the proximal *Fshb* promoter. SMADs can bind DNA via SMAD binding elements (SBE) and FOXL2 binds via forkhead binding elements (FBE). FOXL2 binds the distal FBE1 site and recruits SMAD3 via protein-protein interaction. Complexes of SMAD3 and SMAD4 can bind SBE1, which contains binding sites for both proteins. More proximally, SMAD4 binds SBE2, FOXL2 binds FBE2, and the two proteins are linked through their mutual association with SMAD3. Bottom: In mice lacking SMAD4 in their gonadotropes [672], SMAD3/SMAD4 binding to SBE1 is lost. However, SMAD3/FOXL2 binding to FBE1 is intact. In the absence of SMAD4, SMAD3 can bind SBE2, enabling activins to stimulate *Fshb* production, though at reduced levels relative to wild-type. MH1: DNA binding domain. MH2: Protein-protein interaction domain.

Figure 2.2 SW480.7 cells were transfected with the −1990/+1 murine *Fshb*-luc reporter and different combinations of FOXL2, SMAD3, SMAD3-MH2, and/or SMAD4. Cells were co-transfected with a constitutively active activin type I receptor (ALK4-TD; red bars) or empty expression plasmid (pcDNA3.0, black bars). Bars represent the mean (+ SEM) of three independent experiments. Reporter activity was normalized to that of cells transfected with empty expression plasmids (first set of bars at left). Two-way ANOVA was conducted to compare SMAD3/FOXL2 against SMAD3-MH2/FOXL2, and SMAD3/SMAD4/FOXL2 against SMAD3-MH2/SMAD4/FOXL2. This was followed by a Bonferroni-corrected multiple comparison test.

Bars with different letters are significantly different from one another, while those without letters were not subject to statistical analysis.

Figure 2.3 Recombination of *Smad3* and *Smad4* alleles in mouse gonadotropes. A) Schematic represention of the of floxed *Smad3* and *Smad4* alleles pre- and post-recombination with Cre. Exons 2 and 3 of *Smad3* and exon 8 (dark grey boxes) of *Smad4* were flanked with *loxP* sites (light grey triangles). B) PCR detection of floxed and recombined *Smad3* and *Smad4* alleles from the indicated tissues of control and cKO mice. C-E) RT-qPCR analysis of mRNA levels of *Smad3* and *Smad4* in purified gonadotropes (YFP+) and non-gonadotropes (YFP-) from adult control (YFP/+;GRIC) and cKO (*Smad3*$^{fx/fx}$;*Smad4*$^{fx/fx}$;YFP/+;GRIC) mice. In panels C and E, primers were directed against the deleted region of the genes/transcripts. In panel D, primers were directed against non-targeted exons in *Smad3*.

Figure 2.4 cKO males and females are FSH deficient. A, B) FSH and E,F) LH levels in mouse serum were measured by multiplex ELISA. Individual data points are shown (n=12 or 14 for control and cKO, respectively) as are the group means (horizontal line). C,D) Pituitary FSH content was assessed by RIA (n=8 or 5 for control and cKO males; n=11 or 7 for control and cKO females). All samples from cKO females were below the detection limit of the assay. G,H) Pituitary LH content was measured by ELISA. Data were analyzed by Student's t-test in each panel.

Figure 2.5 FSH protein expression is greatly diminished in cKO pituitaries. Immunofluorescence staining for LHβ (green) and FSHβ (red) in pituitaries of adult control and

cKO males (left panels) and female (right panels) mice. Nuclei were labeled with DAPI (blue). Scale bar=100μm.

Figure 2.6 *Fshb* expression is abolished in pituitaries of cKO mice. Pituitaries were collected from 10-week-old control and cKO mice (n=11 or 15 for control and cKO males; n=12 per genotype for females). mRNA levels of gonadotropin subunits (*Fshb*, *Lhb*, and *Cga*), GnRH receptor (*Gnrhr*), *Smad3,* and *Smad4* were measured by RT-qPCR. mRNA levels were normalized to the housekeeping gene *Rpl19*. Bars reflect group means (+SEM). Data were analyzed by Student's t-test. ***$p<0.0001$. **$p=0.0004$. n.s. not statistically significant.

Figure 2.7 Fertility is impaired in cKO mice. Control and cKO males (n=6 or 4) and females (n=4) were paired with wild-type C57BL6 mice for 6 or 4 months, respectively. A) Average number of litters and B) litter size in control and cKO males. Different colors are used to indicate data from individual animals. C) Average litter size in control and cKO females.

Figure 2.8 cKO males are oligospermic. A) Testes and seminal vesicles from adult control and cKO males. B) Testicular weights of 10-week-old control and cKO males (n=13 per genotype). C) Sperm counts from snap frozen epididymides of 10-week-old control and cKO males (n=9 per genotype). D) Sperm motility assessment from fresh cauda epididymides in adult control and cKO males (n=8 or 6 for control and cKO, respectively). E) Seminal vesicle weights of 10-week-old control and cKO males (n=13 per genotype). F) H&E staining of testicular sections from representative control and cKO males. Scale bar=100μm.

Figure 2.9 cKO females do not ovulate. A) Morphology of ovaries and uteri from adult control and cKO females. B and C) Ovary and uterus weights from 10-week-old control and cKO females (n=12 per genotype). D) Day of vaginal opening in control and cKO females (n=6 per genotype). E) H&E stained ovaries from representative 10-week-old control and cKO females. Corpora lutea (CL) are labeled. Scale bars=100 µm. F) Cumulus-oocyte complexes (COC) collected after exogenous gonadotropin stimulation in juvenile control and cKO females (n=18 or 7 for control and cKO, respectively).

Figure 2.10 Basal and activin A-stimulated *Fshb* expression were abolished in cKO pituitary cells. Primary pituitary cells were prepared from adult A) male or B) female control or cKO mice and treated with 1 nM activin A (red) or with vehicle (black). *Fshb* mRNA levels were measured by RT-qPCR. Bars represent the means (+ SEM) of 3 independent experiments. Bars with different symbols (a, b, c and d) differ significantly.

Supplementary figure legends

Figure S2.1 10-week-old control and cKO mice have comparable body weights. A) Body weight (in g) of 10-week-old control and cKO males (n=13 or 11). B) Body weights of 10-week-old control and cKO females (n=13 or 12).

Figure S2.2 Serum testosterone levels do not differ significantly between 10-week-old control and cKO males.

Figure S2.3 Gene expression in primary pituitary cells. A,B) *Lhb*, C,D) *Cga*, and E,F) *Gnrhr* mRNA levels in primary pituitary cell cultures from female (left column) and male (right column) control and cKO mice. Bars represent mean (+ SEM) from 3 independent experiments. Bars with different symbols ($ and ¢) differ significantly. The *Fshb* data from the same cultures are presented in Fig. 10.

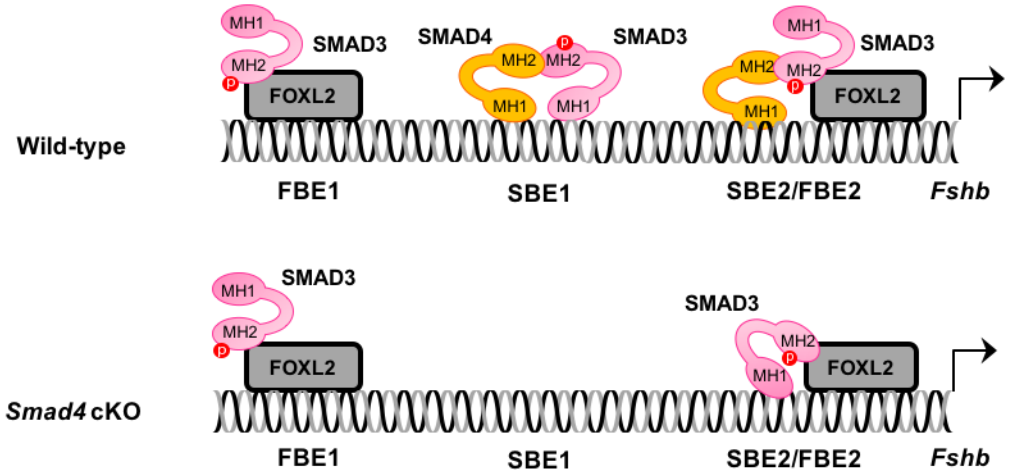
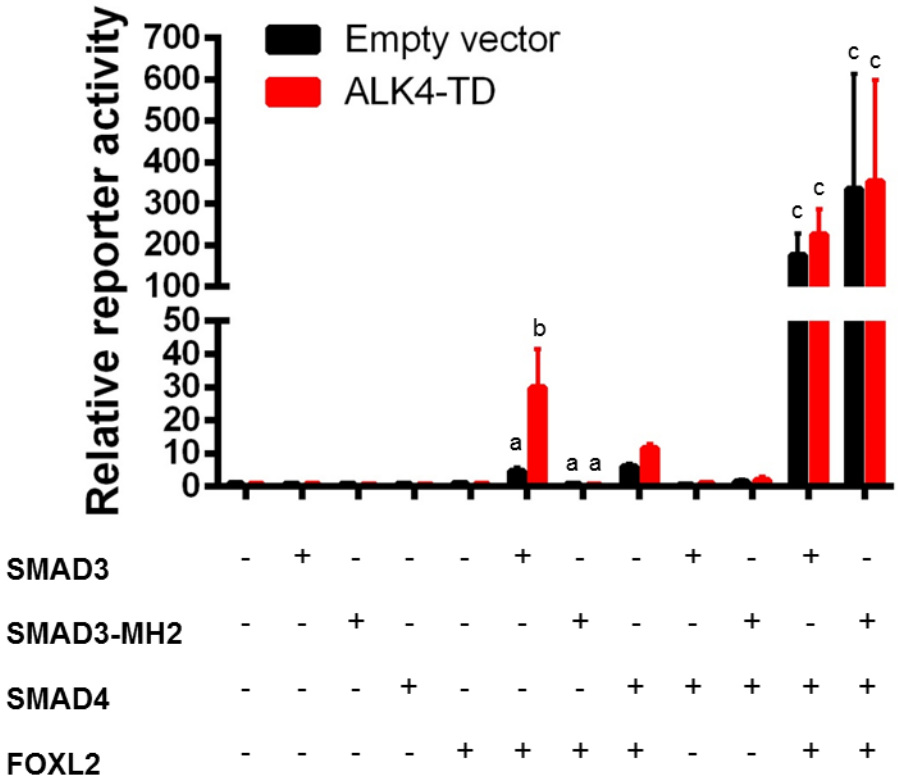

Wild-type
MH1
MH2
SMAD3
FOXL2
SMAD4
MH2
MH2
MH1
MH1
SMAD3
MH1
MH2
MH2
MH1
SMAD3
FOXL2
FBE1
SBE1
SBE2/FBE2
Fshb
Smad4 cKO
MH1
MH2
SMAD3
FOXL2
SMAD3
MH2
MH1
FOXL2
FBE1
SBE1
SBE2/FBE2
Fshb

Figure 2.1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600
500
400
300
200
100
50
40
30
20
10
0
Empty vector
ALK4-TD
a
b
a a
c
c
c
c
SMAD3 - + - - - + - - + - + -
SMAD3-MH2 - - + - - - + - - + - +
SMAD4 - - - + - - - + + + + +
FOXL2 - - - - + + + + - - + +

Figure 2.2

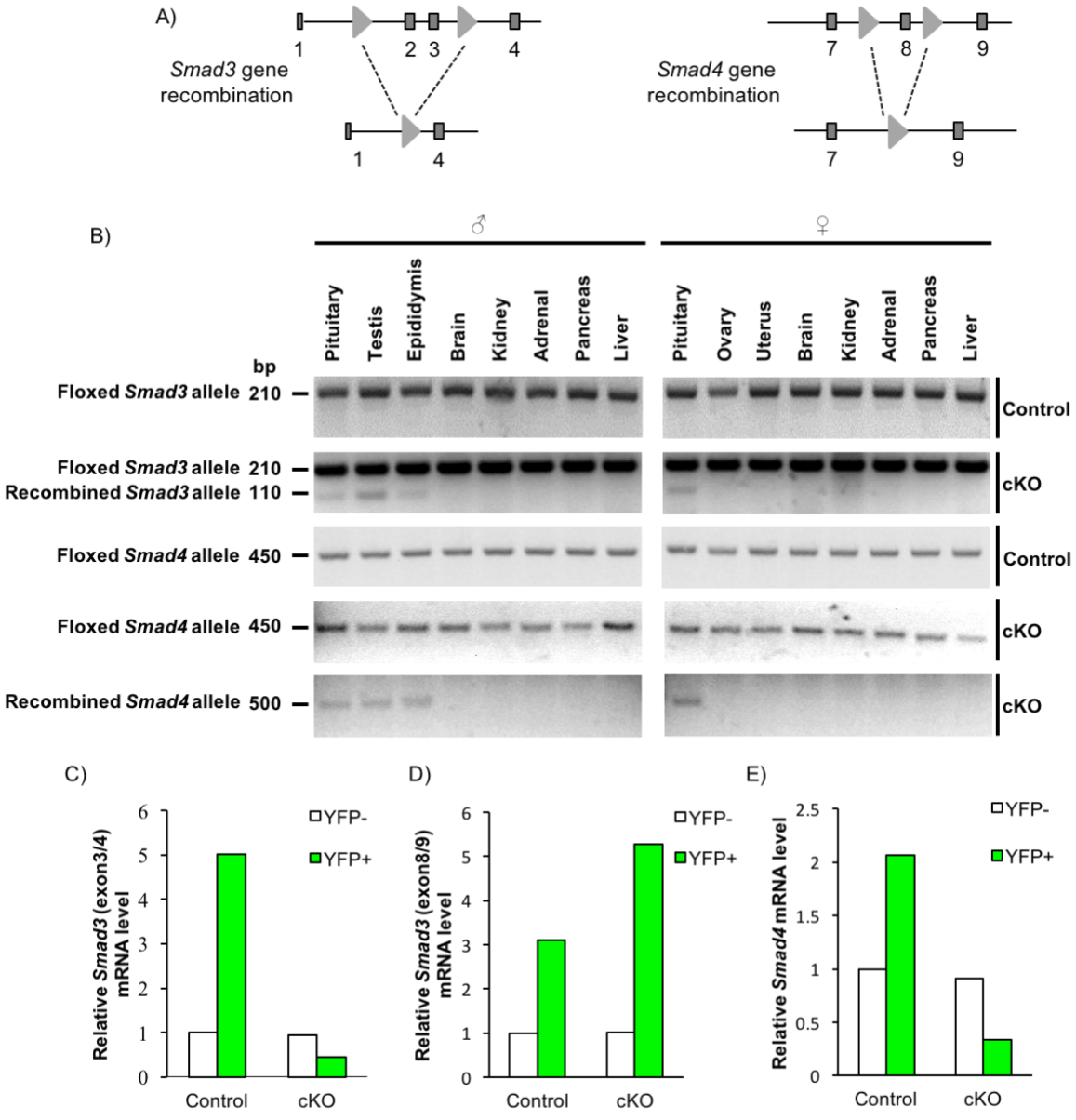

A)
Smad3 gene recombination
1
2 3
4
1
4
Smad4 gene recombination
7
8
9
7
9
B)
♂
♀
Pituitary
Testis
Epididymis
Brain
Kidney
Adrenal
Pancreas
Liver
Pituitary
Ovary
Uterus
Brain
Kidney
Adrenal
Pancreas
Liver
bp
Floxed Smad3 allele 210
Floxed Smad3 allele 210
Recombined Smad3 allele 110
Floxed Smad4 allele 450
Floxed Smad4 allele 450
Recombined Smad4 allele 500
Control
cKO
Control
cKO
cKO
C)
Relative Smad3 (exon3/4) mRNA level
YFP-
YFP+
Control
cKO
D)
Relative Smad3 (exon8/9) mRNA level
YFP-
YFP+
Control
cKO
E)
Relative Smad4 mRNA level
YFP-
YFP+
Control
cKO

Figure 2.3

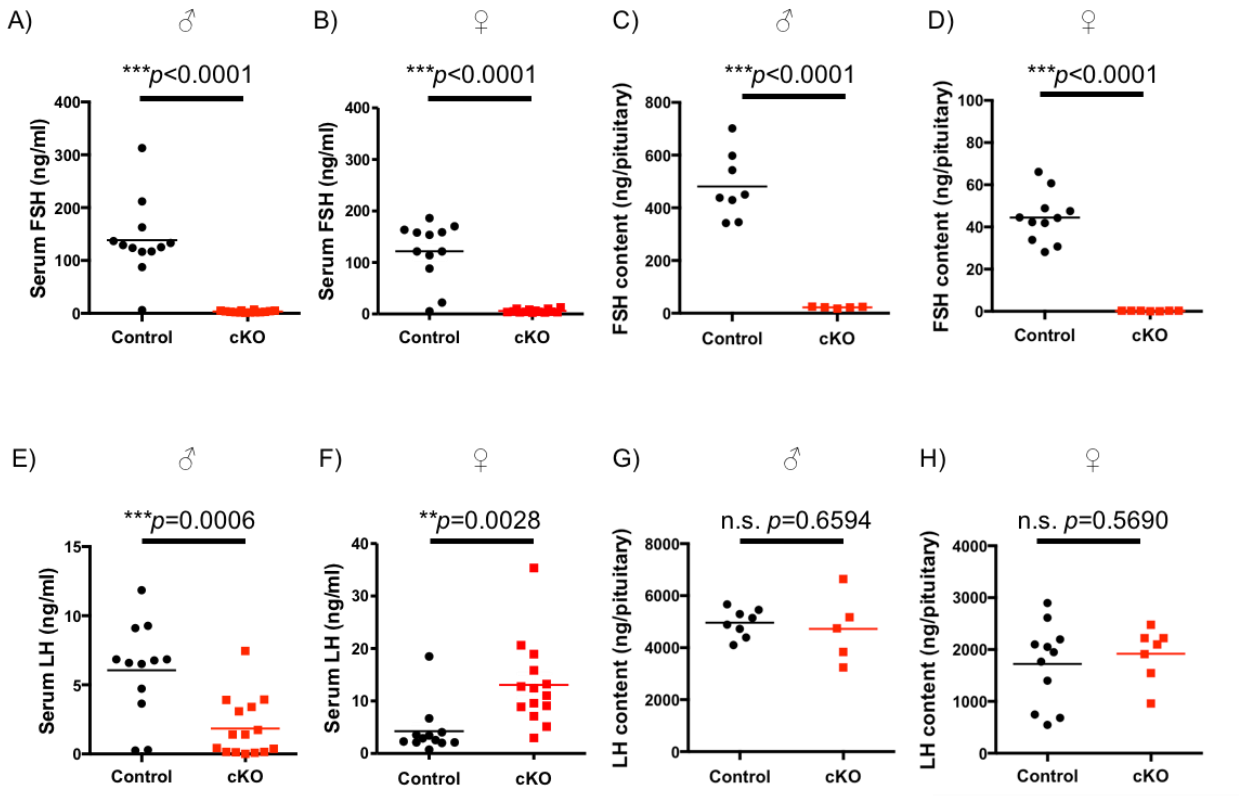

A)
♂
***p<0.0001
Serum FSH (ng/ml)
400
300
200
100
0
Control
cKO
B)
♀
***p<0.0001
Serum FSH (ng/ml)
400
300
200
100
0
Control
cKO
C)
♂
***p<0.0001
FSH content (ng/pituitary)
800
600
400
200
0
Control
cKO
D)
♀
***p<0.0001
FSH content (ng/pituitary)
100
80
60
40
20
0
Control
cKO
E)
♂
***p=0.0006
Serum LH (ng/ml)
15
10
5
0
Control
cKO
F)
♀
**p=0.0028
Serum LH (ng/ml)
40
30
20
10
0
Control
cKO
G)
♂
n.s. p=0.6594
LH content (ng/pituitary)
8000
6000
4000
2000
0
Control
cKO
H)
♀
n.s. p=0.5690
LH content (ng/pituitary)
4000
3000
2000
1000
0
Control
cKO

Figure 2.4

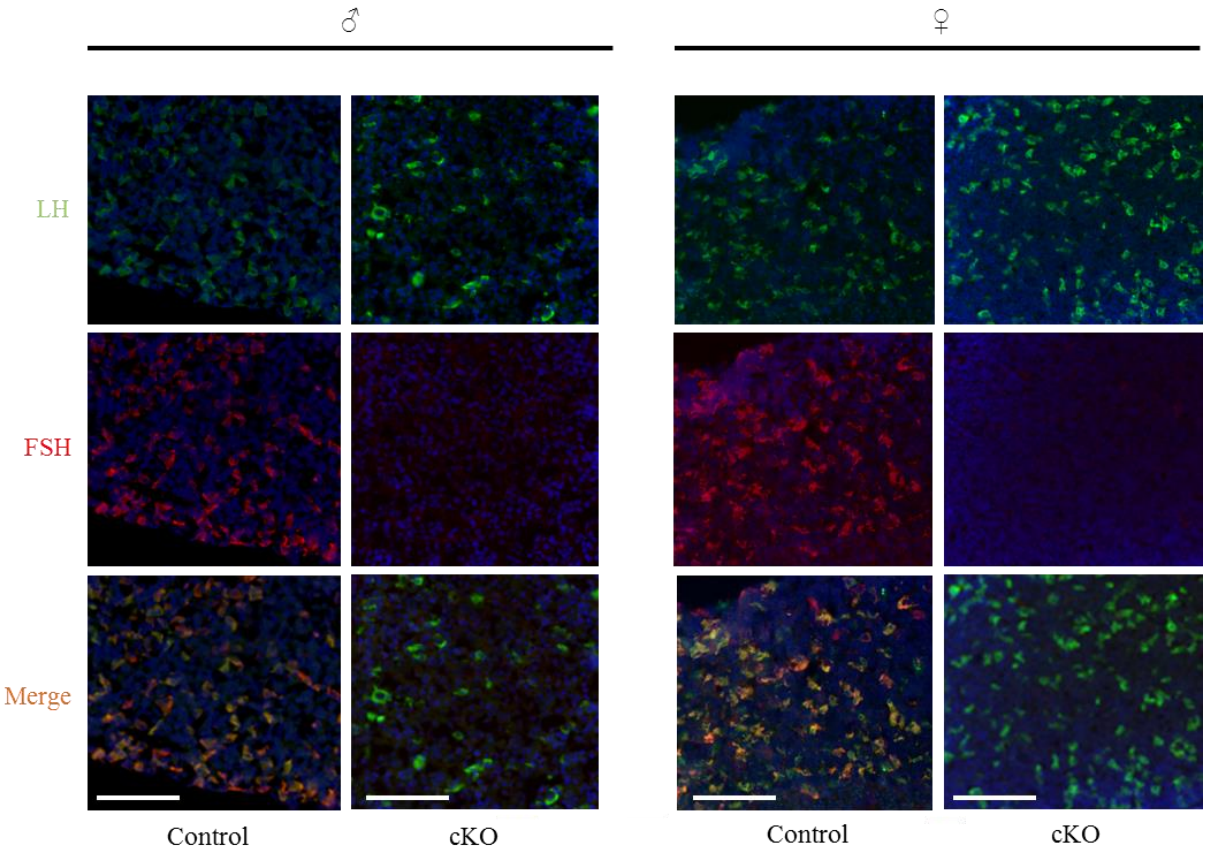


Figure 2.5

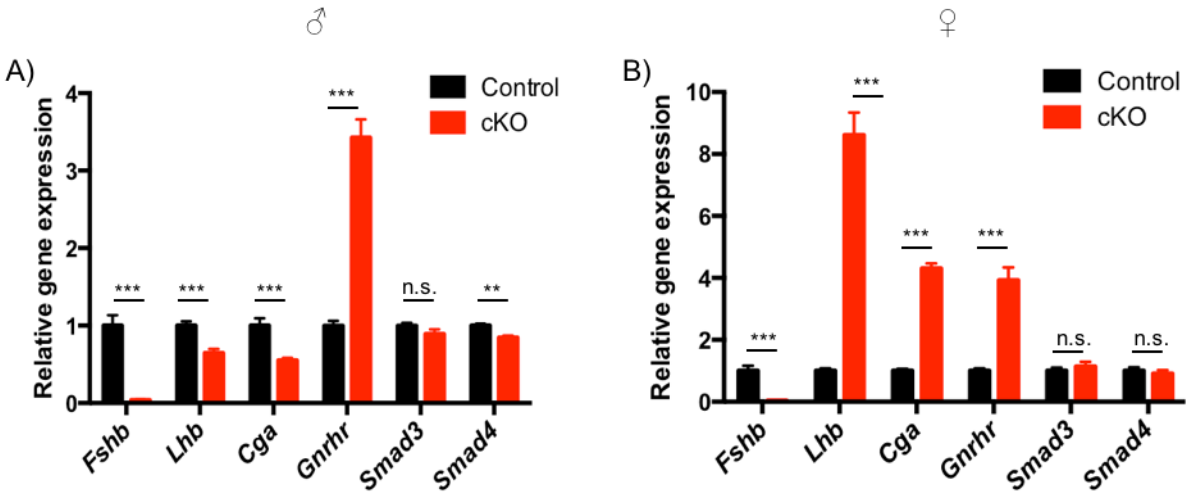

♂
A)
Relative gene expression
Control
cKO
***
***
***
***
n.s.
**
Fshb
Lhb
Cga
Gnrhr
Smad3
Smad4
♀
B)
Relative gene expression
Control
cKO
***
***
***
***
n.s.
n.s.
Fshb
Lhb
Cga
Gnrhr
Smad3
Smad4

Figure 2.6

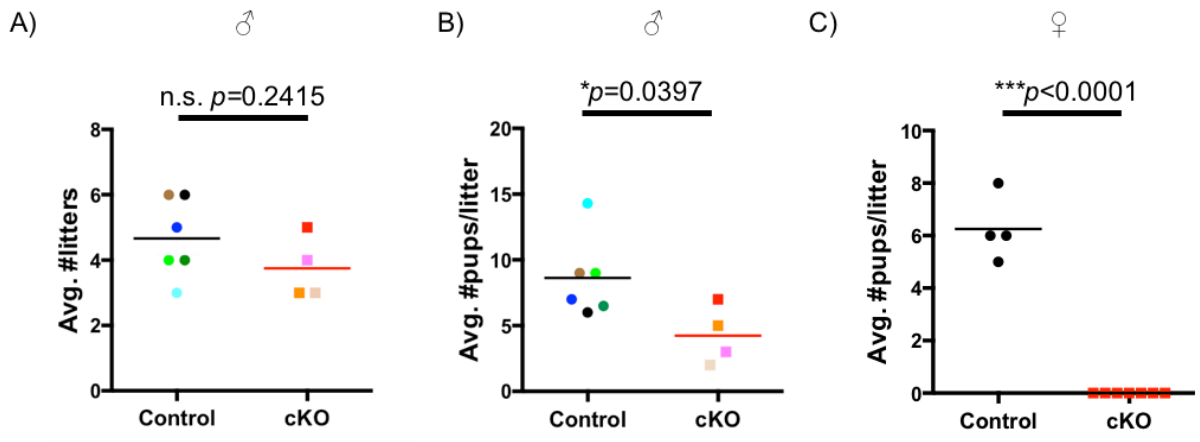
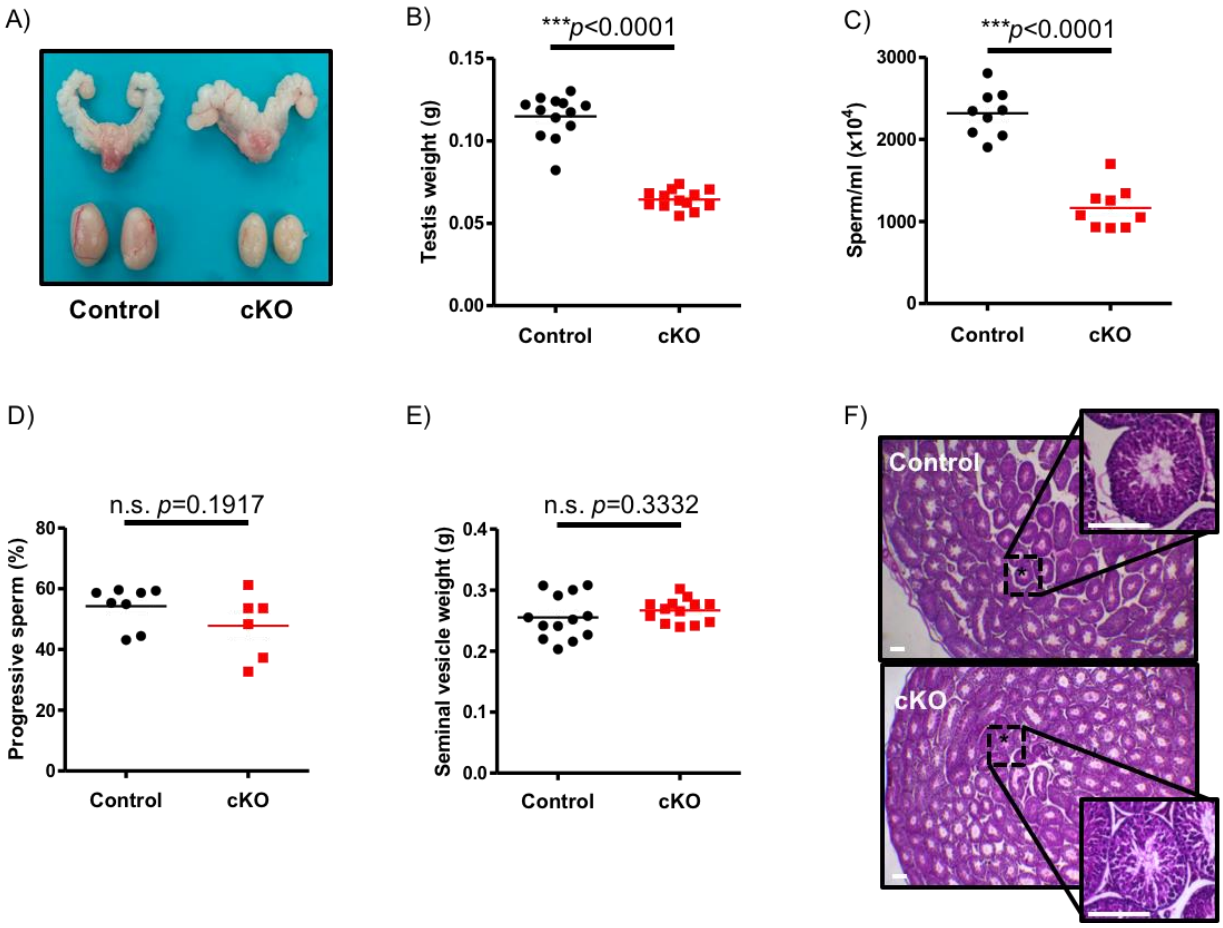

A)
♂
n.s. p=0.2415
Avg. #litters
Control
cKO
B)
♂
*p=0.0397
Avg. #pups/litter
Control
cKO
C)
♀
***p<0.0001
Avg. #pups/litter
Control
cKO

Figure 2.7


A)
Control
cKO
B)
***p<0.0001
Testis weight (g)
0.15
0.10
0.05
0.00
Control
cKO
C)
***p<0.0001
Sperm/ml (x10^4)
3000
2000
1000
0
Control
cKO
D)
n.s. p=0.1917
Progressive sperm (%)
80
60
40
20
0
Control
cKO
E)
n.s. p=0.3332
Seminal vesicle weight (g)
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0.3
0.2
0.1
0.0
Control
cKO
F)
Control
cKO

Figure 2.8

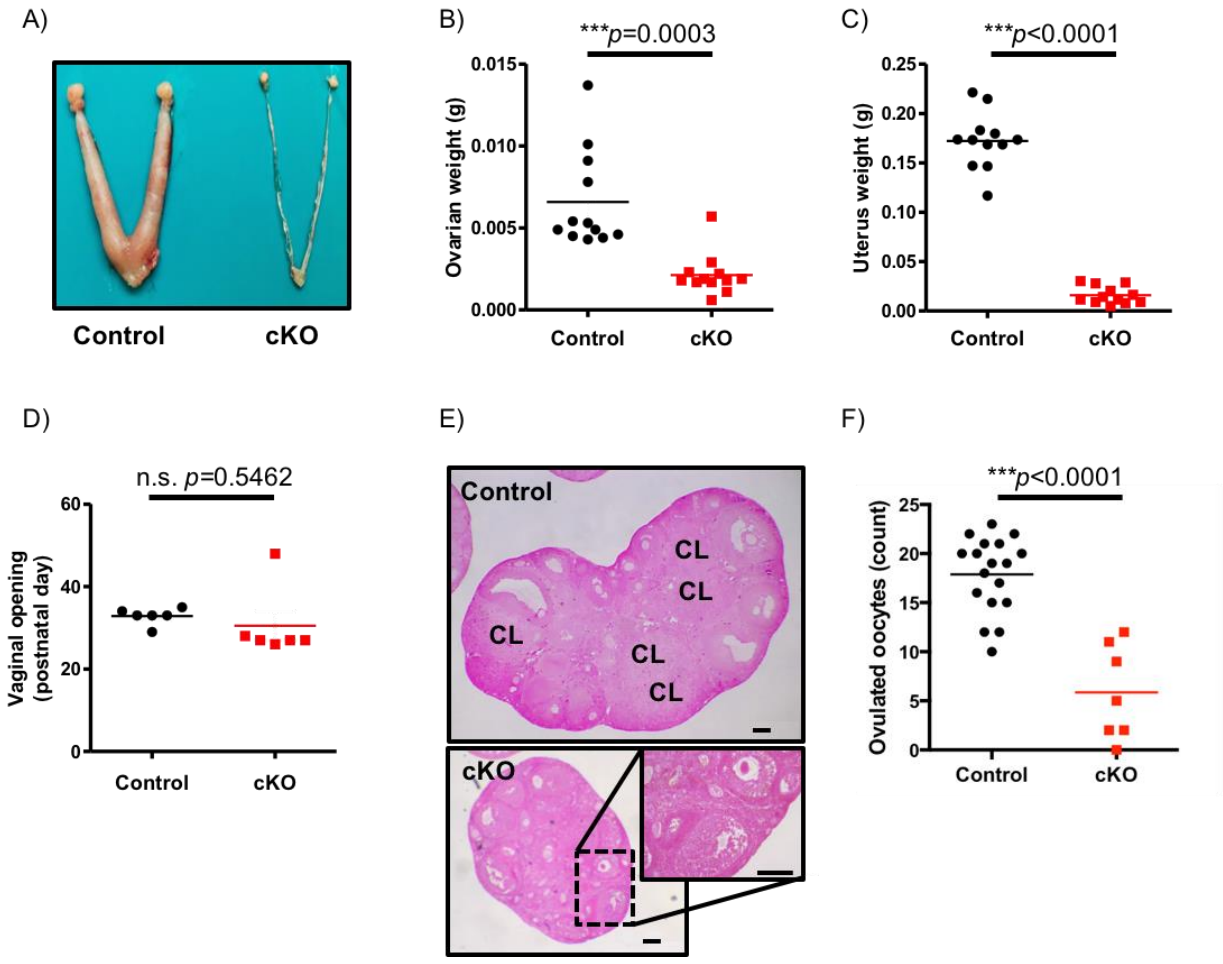
A)
Control
cKO
B)
***p=0.0003
Ovarian weight (g)
0.015
0.010
0.005
0.000
Control
cKO
C)
***p<0.0001
Uterus weight (g)
0.25
0.20
0.15
0.10
0.05
0.00
Control
cKO
D)
n.s. p=0.5462
Vaginal opening (postnatal day)
60
40
20
0
Control
cKO
E)
Control
CL
CL
CL
CL
CL
cKO
F)
***p<0.0001
Ovulated oocytes (count)
25
20
15
10
5
0
Control
cKO

Figure 2.9

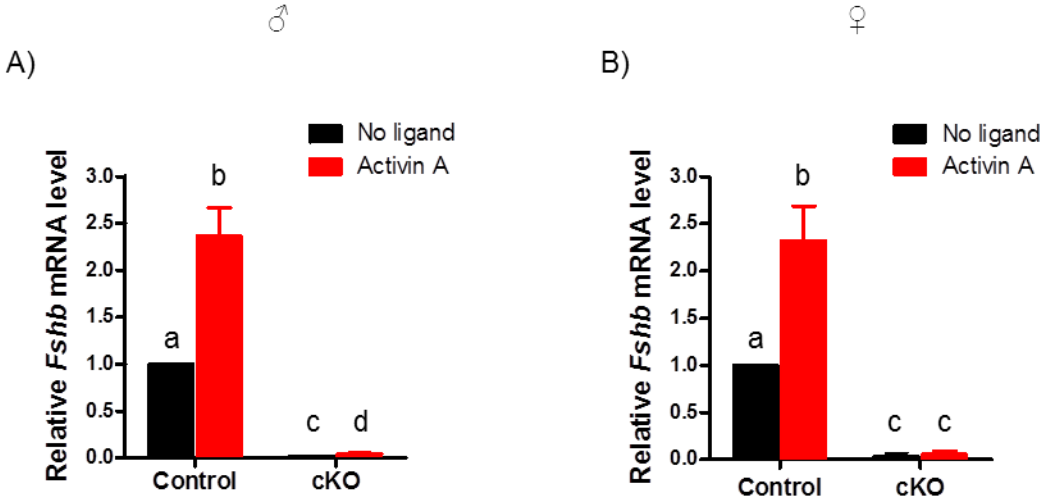
♂
A)
No ligand
Activin A
Relative Fshb mRNA level
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
c
d
Control
cKO
♀
B)
No ligand
Activin A
Relative Fshb mRNA level
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
c
c
Control
cKO

Figure 2.10

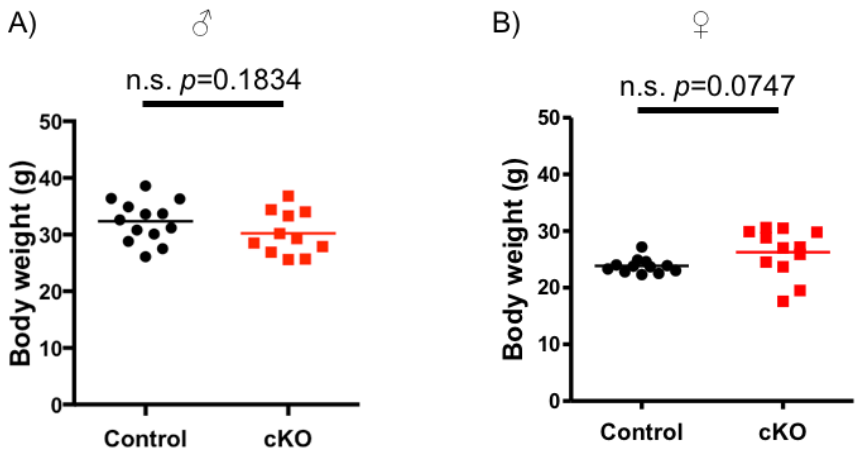
A)
♂
n.s. p=0.1834
Body weight (g)
50
40
30
20
10
0
Control
cKO
B)
♀
n.s. p=0.0747
Body weight (g)
50
40
30
20
10
0
Control
cKO

Figure S2.1

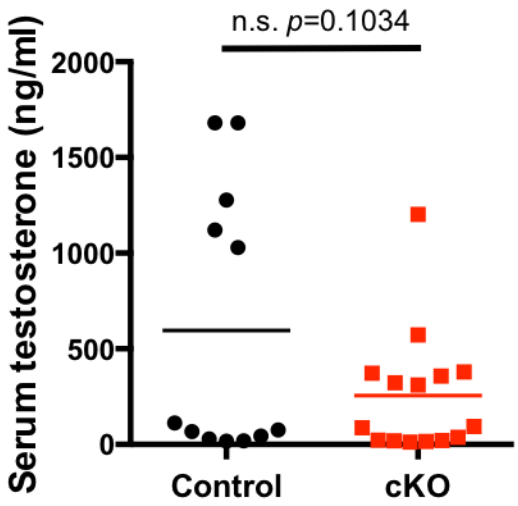
n.s. p=0.1034
Serum testosterone (ng/ml)
2000
1500
1000
500
0
Control
cKO

Figure S2.2

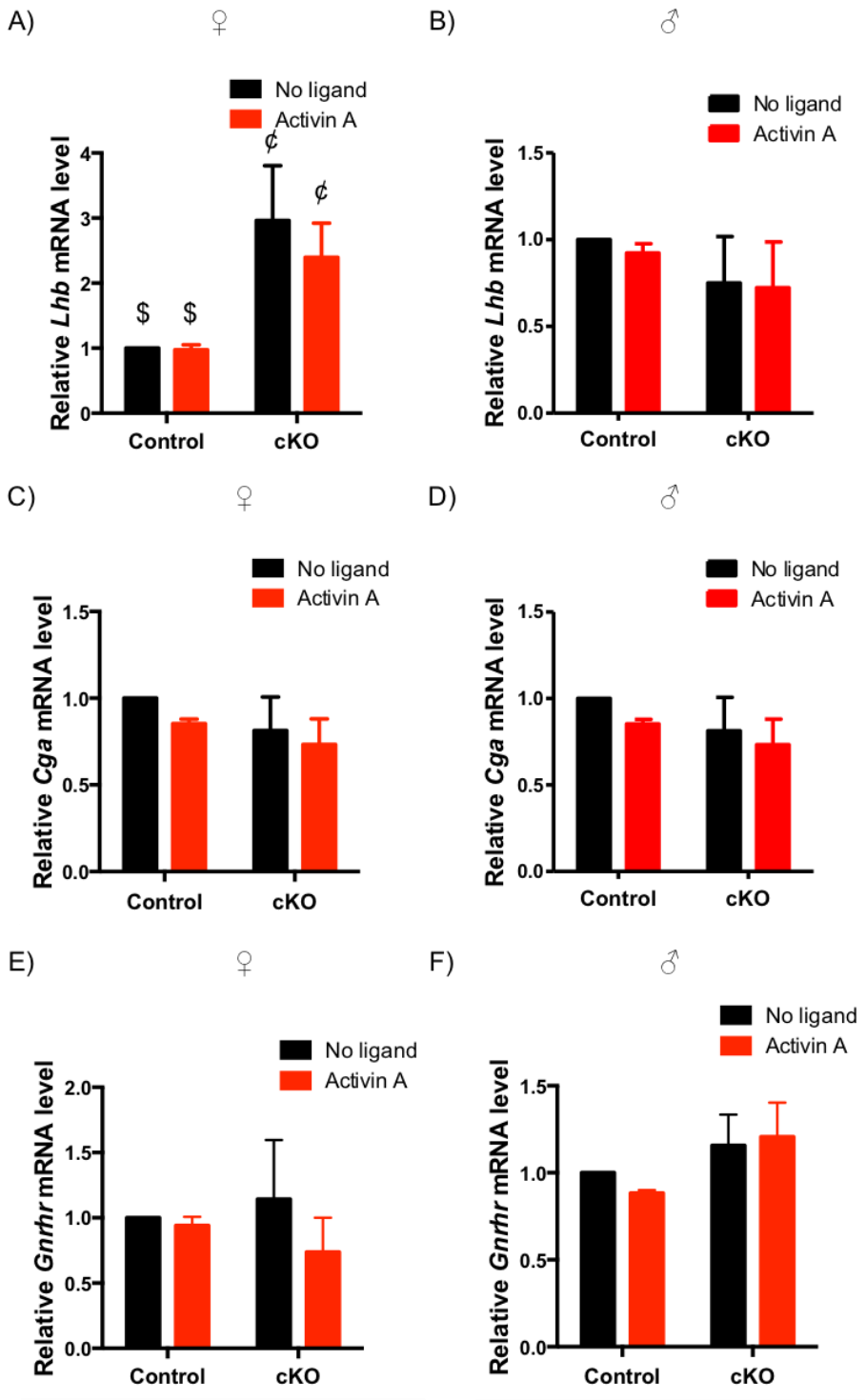
A)
♀
No ligand
Activin A
Relative Lhb mRNA level
4
3
2
1
0
$
$
¢
¢
Control
cKO
B)
♂
No ligand
Activin A
Relative Lhb mRNA level
1.5
1.0
0.5
0.0
Control
cKO
C)
♀
No ligand
Activin A
Relative Cga mRNA level
1.5
1.0
0.5
0.0
Control
cKO
D)
♂
No ligand
Activin A
Relative Cga mRNA level
1.5
1.0
0.5
0.0
Control
cKO
E)
♀
No ligand
Activin A
Relative Gnrhr mRNA level
2.0
1.5
1.0
0.5
0.0
Control
cKO
F)
♂
No ligand
Activin A
Relative Gnrhr mRNA level
1.5
1.0
0.5
0.0
Control
cKO

Figure S2.3

Chapter 3

In **Chapter 2**, I showed the requirement of SMAD3 in gonadotropes for FSH production and female fertility. The phenotypes of S3/4 cKO mice were consistent with those of mice lacking SMAD4 and FOXL2 in their gonadotropes [409]. In both models, the Cre driver used (GRIC) is first active during pituitary development, at E12.75 [649]. Thus, it was unclear whether the diminished FSH synthesis in these mice reflected impairments in normal gonadotrope development or the necessity for activin-signaling molecules (FOXL2/SMAD4 or SMAD3/SMAD4) in adult gonadotropes. To discriminate between these possibilities, here, we generated a new mouse line that enabled the inducible deletion of *Smad4* and *Foxl2* in gonadotropes of adult mice.

Follicle-stimulating hormone regulates ovarian follicle development but not spermatogenesis in adult mice

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Abbreviated title: FSH regulates gonadal function in adult female, but not male mice

Keywords: FSH, SMAD4, FOXL2, gonadotrope, inducible knockout, spermatogenesis

Abstract

The glycoprotein follicle-stimulating hormone (FSH), a product of pituitary gonadotrope cells, regulates ovarian follicle development in females and spermatogenesis in males. In humans, loss of function mutations in the FSHβ subunit gene (*FSHB*) cause primary amenorrhea in women and azoospermia in men. In *Fshb* knockout mice, females are similarly acyclic and sterile; however, males are oligozoospermic and fertile. It is presently unclear if FSH plays a more essential role in human than murine spermatogenesis or if mice develop a compensatory mechanism in the lifetime absence of FSH. Here, we generated an inducible Cre-driver line for the murine gonadotrope, which enabled us to assess the effects of FSH loss later in life. Specifically, we ablated FOXL2 and SMAD4, two transcription factors that selectively regulate *Fshb* transcription. Upon tamoxifen-induced gene deletion in animals with proven fertility, females exhibited a profound reduction in FSH levels, arrested ovarian follicle development, and sterility. FSH levels were comparably reduced in males; however, spermatogenesis and reproductive organ weights were completely unaffected. These data indicate that: 1) FOXL2 and SMAD4 are required to maintain FSH synthesis in gonadotrope cells of adult mice, 2) FSH is essential for female reproduction, but is dispensable for the maintenance of spermatogenesis in adult male mice, and 3) the inducible Cre-driver line developed here provides a powerful new tool to interrogate gene function in gonadotrope cells of adult mice.

Significance Statement

Follicle-stimulating hormone (FSH) is secreted by the pituitary gland and acts on cells in the ovary and testes. Here, we investigated the effects of impairing FSH production on reproductive physiology in adult mice. Using a newly developed mouse model, we disrupted the function of two genes known to regulate FSH synthesis in animals previously shown to have normal reproductive function. After the gene deletions, FSH levels were dramatically reduced. In females, this led to impaired fertility, because of a block in maturation of ovarian follicles; a necessary step prior to ovulation. In males, however, sperm production was completely normal. These results suggest that blocking FSH action may not be an effective contraceptive strategy in men.

Introduction

Follicle-stimulating hormone (FSH), a product of pituitary gonadotrope cells, plays fundamental roles in mammalian reproduction [221, 650]. In females, FSH acts on ovarian granulosa cells to regulate the latter stages of follicle development and estrogen biosynthesis prior to ovulation [695]. Purified and recombinant forms of the hormone are the foundation of ovarian stimulation protocols in assisted reproduction and *in vitro* fertilization [696]. Though FSH is also used to treat hypogonadism in men, its necessity in male reproduction is controversial. The hormone targets testicular Sertoli cells, stimulating their proliferation during development [245, 246]. In adulthood, FSH regulates androgen binding protein synthesis, and thereby intra-testicular testosterone concentrations [697]. Through these two mechanisms, FSH can regulate spermatogenesis. However, whether FSH is absolutely required for sperm production is less clear.

FSH is a dimeric protein, composed of the α gonadotropin subunit (CGA) and the hormone-specific FSHβ subunit (FSHB). Loss of function mutations in the *FSHB* gene are rare in humans, but uniformly lead to infertility in homozygous male and female carriers [650, 660, 698-703]. Affected males are normally virilized, but azoospermic. These data support the concept that antagonism of FSH action might provide an effective form of contraceptive in men. In contrast, however, male mice with inactivating mutations in the *Fshb* gene are oligozoospermic and fertile [221]. Though this might suggest an inter-species difference in FSH dependency for spermatogenesis, both men and male mice with loss of function mutations in the FSH receptor gene (*FSHR/Fshr*) are oligozoospermic and fertile [653, 654, 704]. These observations led to the near complete abandonment FSH-based male contraceptives. Nevertheless, FSH bioneutralization in adult non-human primates effectively decreases sperm production [705-707]. Moreover, in existing knockout mouse strains and in humans with ligand or receptor mutations, gene function

is eliminated from conception, allowing for the development of compensatory mechanisms. To our knowledge, no one has investigated the effects of impairing FSH production or action in adulthood on spermatogenesis in mice.

Here, we developed a novel mouse model, using the tamoxifen-inducible Cre-lox recombination system that, for the first time, enables the deletion of genes in adult murine gonadotropes. We previously reported that ablation of the *Foxl2* and *Smad4* genes in murine gonadotropes during development leads to a selective loss of *Fshb* expression, female sterility, and male oligozoospermia [409]. FOXL2 and SMAD4 mediate the actions of activins, members of transforming growth factor β superfamily, which are major drivers of *Fshb* transcription [393, 409, 417]. In the present study, we observed that deletion of *Foxl2* and *Smad4* in gonadotrope cells of adult mice similarly causes a selective reduction of *Fshb* expression. Females are rendered subfertile or, more often, sterile due to a block in ovarian follicle development at the early antral stage. In contrast to the case with developmental knockouts, testis size and spermatogenesis in males are unaffected in adult knockout mice, despite profound decreases in FSH levels. These data demonstrate that FSH is required for female fertility, but is completely dispensable for spermatogenesis in adult males.

Results

Generation of gonadotrope-specific tamoxifen-inducible Cre mice

Since 2008, GnRHR-IRES-Cre (GRIC) mice have been used to conditionally delete genes in the murine gonadotrope cell. The mice express the Cre recombinase enzyme from the endogenous *Gnrhr* locus beginning around embryonic day 13 [649]. Here, we modified the GRIC targeting vector by replacing the Cre expression cassette with Cre-ERT2 [625], which enables

tamoxifen-inducible Cre activity (Supplementary Fig. S3.1). Mice (hereafter inducible GRIC or iGRIC) were generated by conventional gene targeting in ES cells. We then crossed the mice to a reporter strain (*Rosa26*^{eYFP}) to determine the specificity and efficiency of tamoxifen-induced recombination [691]. iGRIC/+;*Rosa26*$^{eYFP/+}$ mice exhibited eYFP in luteinizing hormone β subunit (LHβ)-expressing pituitary gonadotropes following tamoxifen but not oil vehicle treatment (Fig. 3.1A). After testing a variety of treatment conditions, we found that four injections of 2 mg tamoxifen delivered every other day was highly efficient, leading to eYFP in 55 and 75% of LHβ+ gonadotropes two or four weeks, respectively, following the final injection (Fig. 3.1B). In contrast, nearly all LHβ+ gonadotropes expressed eYFP when using the original GRIC allele (GRIC/+;*Rosa26*$^{eYFP/+}$ mice; Fig. S3.2). No other cell lineages expressed eYFP in either the GRIC or iGRIC model, and no eYFP was observed in the latter without prior tamoxifen treatment. Thus, the new iGRIC model conferred both specific and efficient recombination in gonadotropes, though slightly less efficient than with the original GRIC model.

Tamoxifen efficiently induces recombination of floxed *Foxl2* and *Smad4* alleles in gonadotropes of adult mice

We previously showed that the GRIC model can efficiently recombine floxed *Foxl2* and *Smad4* alleles in murine gonadotropes, leading to severe FSH deficiency, female sterility, and male oligozoospermia [409]. Here, we examined the efficiency of recombination of these alleles using the new iGRIC model in adult mice. One month following the 4th tamoxifen injection, FOXL2 protein expression was ablated in essentially all LHβ+ gonadotropes (Fig. 3.1C). The remaining FOXL2+ cells were mostly thyrotropes (Fig. S3.3). The depletion of pituitary *Foxl2* mRNA expression was roughly equivalent to that seen with the GRIC model [409, 417] (see Fig. 3.4E,F).

SMAD4 (pending). These data indicate that iGRIC mice can be used to recombine several different loci (at least four at a time) in adult mice; though the efficiency of recombination differed somewhat between floxed alleles.

Impaired fertility and arrested follicular development in induced *Smad4/Foxl2* knockout females

A powerful feature of the inducible system is that each animal can serve as its own control. We therefore assessed fertility in experimental (iGRIC/+;*Foxl2*$^{fx/fx}$;*Smad4*$^{fx/fx}$) and control (*Foxl2*$^{fx/fx}$;*Smad4*$^{fx/fx}$) females before and after tamoxifen injections. Eight-week-old animals were paired with wild-type males and allowed to produce up to two litters. All females, regardless of genotype, showed normal puberty onset (data not shown) and fertility, indicating that there was no precocious Cre activity under physiologic conditions (Fig. 3.2A). One month after tamoxifen injections, experimental females (hereafter induced F2S4 cKO) became subfertile (4 of 9) or infertile (5 of 9; Fig. 3.2A). The four animals that maintained some fertility produced small litters of one to three pups, and three of four animals produced two or fewer litters in three months (Fig. 3.2A, right panels). In contrast, fertility remained intact in all the other groups, including tamoxifen-treated females with the control genotype (Fig. 3.2A, gray bars). Ovarian histology revealed an arrest in follicle development at the early antral stage and the absence of corpora lutea in most of the induced F2S4 cKO females (Fig. 3.2B). Indeed, many females that ovulated before tamoxifen-induced recombination failed to do so post-treatment (Fig. S3.4). Ovarian and uterine weights were comparable in all groups, though there was a non-significant reduction in ovarian weight in the induced F2S4 cKO mice (Figs. 3.2C and D).

Spermatogenesis is unaffected in induced F2S4 cKO mice

In contrast to the situation in females, induced recombination of the *Foxl2* and *Smad4* loci did not affect reproductive organs in adult males. Testicular, seminal vesicle weights (Figs. 3.3A-B) and Sertoli cell numbers (data not shown) were normal. Notably, sperm motility (Fig. 3.3C), sperm counts (Fig. 3.3D), and testicular morphology (Fig. 3.3E) in induced F2S4 cKO males were indistinguishable from controls two months following tamoxifen treatment. A complete spermatogenic cycle is estimated at 34.5 days in the mouse [708].

Induced knockout mice are FSH-deficient

The phenotype in females was consistent with FSH deficiency, which we investigated in detail. Before tamoxifen treatment, FSH levels were equivalent between genotypes in randomly cycling females (Fig. 3.4A, left). One month after the last tamoxifen injection, however, FSH was significantly reduced in induced F2S4 cKO females compared with control groups as well as to their own serum FSH levels before injection (~83% reduction; Fig. 3.4A, right). Similarly, pituitary FSH content was significantly reduced following tamoxifen treatment, as assessed by RIA (Fig. 3.5A) or immunofluorescence (Fig. 3.5E). Reductions in FSH protein derived from a significant decrease (~80%) in pituitary *Fshb* mRNA expression in induced F2S4 cKO mice (Fig. 3.4E). Despite the absence of a spermatogenesis phenotype, induced F2S4 cKO males similarly showed normal serum FSH before treatment (Fig. 3.4B, left), but significantly reduced serum FSH (~76%) (Fig. 3.4B, right) and pituitary FSH protein content (Fig. 3.5B) two months following the last tamoxifen injection. Pituitary *Fshb* mRNA levels were similarly reduced (~70%) one (Fig. S3.5) or two months (Fig. 3.4F) after treatment.

Induced F2S4 cKO females showed significantly increased serum LH compared with controls (Fig. 3.4C), which was correlated with increases in pituitary *Lhb* mRNA levels (Fig. 3.4E), but not pituitary LH content (Fig. 3.5C). In contrast, serum LH, pituitary *Lhb* mRNA, and pituitary LH content were comparable across all groups of males two months post-tamoxifen treatment (Figs. 3.4D-F and 3.5D). *Gnrhr* mRNA levels did not differ between genotypes, but *Cga* was reduced in induced F2S4 cKO males relative to the other groups (Figs. 3.4E-F).

In both sexes, pituitary *Foxl2* expression was significantly reduced (Figs. 3.4E-F). Note that, in addition to gonadotropes, FOXL2 is expressed in thyrotropes in the pituitary gland [709]. Therefore, the remaining *Foxl2* expression was likely contributed by thyrotropes (Fig. S3.3). In fact, the residual pituitary *Foxl2* expression levels in both males and females was comparable to that seen with the GRIC model (Figs. 3.4E-F) [417]. In contrast, *Smad4* mRNA expression did not differ between groups (Figs. 3.4E-F). This was not unexpected given the low abundance of gonadotropes in the pituitary (~5-10% of cells) and broad expression of *Smad4* in all lineages [133].

Tamoxifen effects on pituitary gene expression are transient

The absence of any effects in control mice treated with tamoxifen argues strongly that it was induced recombination rather than tamoxifen itself that produced the observed phenotypes in experimental mice. However, we treated adult wild-type males with one or four injections of tamoxifen and then collected their pituitaries 6 h, 1 day, 1 week, or 2 weeks later to assess the acute and lasting effects of the ligand. Tamoxifen suppressed *Gnrhr* mRNA maximally at 6 h (Fig. S3.6A). Levels increased, but still remained below control values at 1 day and 1 week. By 2 weeks

post-injection, *Gnrhr* mRNA levels had returned to baseline. In contrast, *Fshb*, *Foxl2*, *Lhb*, and *Cga* mRNA levels were not significantly impacted after tamoxifen injections (Figs. S3.6B-E).

Induced deletion of *Smad4* and *Foxl2* impairs activin-regulated *Fshb* expression in culture

FOXL2 and SMAD4 mediate the stimulatory actions of activins on FSH [393]. To determine whether activin regulation of *Fshb* was impaired in induced F2S4 cKO mice, we compared the behavior of their pituitaries in culture to those of the three control groups. Basal *Fshb* expression in cultured murine pituitaries depends on endogenous activins or related TGFβ ligands [378, 388, 409, 486, 710-712]. Pituitary cultures from control mice, of both sexes, showed equivalent levels of basal and activin A-stimulated *Fshb* mRNA expression (Figs. 3.6A-B). In contrast, basal and activin A-induced *Fshb* expression were reduced to nearly undetectable levels in pituitaries from induced F2S4 cKO mice. These effects were gene-specific, as expression of *Gnrhr*, *Lhb*, and *Cga* did not differ between the four groups (Fig. S3.7).

In a final experiment, we determined the ability of tamoxifen to induce gonadotrope-specific gene recombination *in vitro*. We cultured pituitary cells from GRIC/+;*Foxl2*$^{fx/fx}$;*Smad4*$^{fx/fx}$ mice that were previously untreated *in vivo*. We then added oil vehicle or the active metabolite 4-hydroxytamoxifen (4-OHT) to half of the wells. 4-OHT treatment led to a significant suppression of *Fshb* mRNA expression and a complete block of the activin A response (Fig. 3.6C). 4-OHT also reduced levels of *Foxl2* and *Gnrhr* mRNA, but not those of *Smad4*, *Lhb*, or *Cga* (Figs. S3.8A-E). It was 4-OHT-induced recombination in gonadotropes, and not 4-OHT itself, that led to decreases in *Fshb* expression, as 4-OHT did not affect the expression of *Fshb* or any of the other genes investigated, with the exception of *Gnrhr*, when applied to pituitary cultures from wild-type mice (Figs. 3.6D and S3.8F-J).

Discussion

Here, we describe the development of the first tamoxifen-inducible Cre-driver line for the murine gonadotrope cell lineage. Using these mice, we were able to selectively and efficiently delete two genes, *Foxl2* and *Smad4*, simultaneously in gonadotropes of adult mice. To our knowledge, this is the first example of the interrogation of gene function in adult gonadotropes of mice or any species. These animals display normal reproductive physiology prior to treatment with the inducing agent, tamoxifen. One month following treatment, both female and male mice show a profound suppression of FSH levels, secondary to a selective reduction of pituitary *Fshb* mRNA expression. This FSH deficiency leads to subfertility (reduced litter size), but more often sterility, in female mice that were previously fertile. The principle defect appears to be in FSH-stimulated ovarian follicle development, which is arrested at the early antral stage in most cases. This phenotype is comparable to that of mice lacking a functional *Fshb* subunit gene from conception or of mice with gonadotrope-specific deletions of *Foxl2* and *Smad4* or *Smad4* and *Smad3* beginning around embryonic day 13 [221, 409, 712]. Males similarly show profound FSH deficiency post-tamoxifen treatment. However, in contrast to global *Fshb* or conditional *Foxl2/ Smad4* and *Smad4/Smad3* knockout mice, which show profound oligozoospermia [409, 712], reduction of FSH in adulthood had no measureable effect on spermatogenesis.

The results of this work provide important new insights into the control of FSH synthesis as well as FSH actions in the reproductive axis. First, they show that FOXL2 and SMAD4 are essential for *Fshb* expression in gonadotropes of adult mice. Using GRIC mice to ablate these genes during embryonic life leads to a similar FSH deficiency as described here [409]. Despite the fact that LH production was largely intact in these animals, it was at least formally possible that the gene deletions somehow affected gonadotrope development, indirectly causing FSH deficiency

[409]. The results with the inducible GRIC (iGRIC) strain put this possibility to rest, conclusively demonstrating a necessity for FOXL2 and SMAD4 to maintain *Fshb* expression in adult gonadotropes. Second, the data show that FSH is fundamentally required for the later stages of ovarian follicle development. Regardless of whether FSH is eliminated during embryonic life or is reduced in adulthood, follicle development is arrested at the pre- or early antral stage. Third, the results suggest that FSH is not required for spermatogenesis in adult mice.

FSH regulation of spermatogenesis

It is well-known that FSH stimulates Sertoli cell proliferation during early postnatal development [245, 620, 713]. The number of Sertoli cells determines spermatogenic capacity post-pubertally. *Fshb* and *Fshr* knockout mice have reduced Sertoli cell numbers and lower sperm counts than wild-type mice [221, 653, 654, 697]. We did not previously measure Sertoli cell number in gonadotrope-specific *Foxl2/Smad4* or *Smad3/4* conditional knockout mice (using the GRIC Cre-driver), but these animals similarly show reduced testis size and sperm counts [409, 712]. Mice lacking *Foxl2* alone in gonadotropes exhibit decreases in FSH levels, testis size, sperm counts, and, importantly, Sertoli cell numbers [417]. Therefore, it seems likely that loss of both *Foxl2* and *Smad4* during gonadotrope development would lead to a similar reduction in Sertoli cell number. In contrast, the inducible knockout mice developed here have normal size testes and sperm counts. Therefore, reductions in FSH in adulthood do not appear to affect Sertoli cell number (given the normal sperm count), confirming that FSH regulates proliferation of these cells only during a critical developmental time window.

All of this said, impaired spermatogenesis in these mice might still have been anticipated given other actions of FSH in the testis, including stimulation of androgen binding protein (ABP) levels [234, 697, 714, 715]. ABP functions to increase intra-testicular testosterone (T) concentrations, which is important for spermatogenesis. That said, the absolute levels of T required are not defined and may differ across species. In hypogonadal (*hpg*; GnRH-deficient) mice, for example, exogenous T or DHT alone (in the absence of FSH) is sufficient to induce spermatogenesis, even when intra-testicular androgen levels remain very low [716, 717]. Thus, provided LH is available to stimulate T production above a needed threshold, reduced FSH stimulation of ABP may be of little consequence. In contrast, in humans and non-human primates, it is clear that FSH plays important roles in both the initiation and maintenance of spermatogenesis. For example, FSH can stimulate resumption of spermatogenesis in men rendered gonadotropin-deficient with T enanthate [718]. Similarly, in cynomolgus monkeys treated with a GnRH antagonist, exogenous FSH can stimulate spermatogenesis [719].

Though one could argue that the extent of FSH suppression achieved in our study may have been insufficient to affect spermatogenesis, this seems unlikely as FSH was reduced by ~76%. Also, an equivalent extent of FSH suppression was sufficient to profoundly impair ovarian function in females. It is also possible that FSH was not suppressed for an adequate period of time to affect spermatogenesis. However, animals were investigated two months following the initiation of gene recombination, which is roughly the length of two complete spermatogenic cycles [708]. Collectively, the data indicate that FSH does not regulate spermatogenesis in adult mice.

Differences in effects of *Foxl2/Smad4* deletion during development and in adulthood

Ablation of *Foxl2* and *Smad4* in developing (GRIC) and adult (iGRIC) gonadotropes produced very similar, though not identical phenotypes [409]. In addition to the differences in spermatogenesis discussed above, there are other notable discrepancies between the two models. In both males and females, the extent of FSH suppression was greater with GRIC than with iGRIC mice [409]. This likely reflects the extent of gene recombination, as inducible systems are inherently incomplete or mosaic [720, 721]. Another notable difference is in uterine weight, which was reduced in mice with the GRIC driver, but not with the iGRIC driver. The uterus is highly sensitive to estrogens [690, 722, 723]. It is therefore possible that estrogen suppression is greater with the GRIC than iGRIC model. Given the role of FSH in estrogen biosynthesis and the somewhat higher FSH levels in the iGRIC mice, this seems a distinct possibility. Unfortunately, RIAs and ELISAs for 17β-estradiol in mice have proven unreliable in our experience (data not shown), so we are not able to address this possibility directly [724]. Finally, in males with the GRIC allele, serum LH and pituitary *Cga* mRNA levels were significantly reduced, despite a significant increase in pituitary *Lhb* mRNA levels [409]. With the iGRIC model, LH levels were normal in males two months post-tamoxifen treatment. Pituitary *Cga* levels were again reduced, but to a lesser extent than with the GRIC driver, whereas *Lhb* was unaffected. We currently lack a clear explanation for these differences between the two models.

Generation of the first tamoxifen-inducible Cre-driver for murine gonadotrope cells

The novel mouse model described here, iGRIC, will be of broad utility to investigators interested in interrogating gene function in adult gonadotropes. Thus far, we have demonstrated that with four 2 mg tamoxifen injections every other day, we achieve greater than 75%

recombination with three different floxed alleles after four weeks. We were also able to effectively recombine up to four floxed alleles in individual gonadotropes. Based on our experience, it seems likely that recombination efficiency will differ between alleles and that some optimization may be required. Indeed, after we noted that YFP labeling increases between two and four weeks after the last of four tamoxifen injections in our standard protocol and that *Gnrhr* expression is transiently reduced by tamoxifen (Fig. S3.6), we asked whether modifying the injection protocol might further improve recombination of the *Rosa26*^{eYFP} reporter allele. Recall that Cre is expressed from the *Gnrhr* locus in the iGRIC model, so it is possible, if not likely, that tamoxifen simultaneously induces Cre activity from existing Cre-ERT2 fusion proteins in the gonadotrope while attenuating production of new Cre-ERT2. Therefore, we injected iGRIC/+;*Rosa26*$^{eYFP/+}$ reporter mice with 2 mg tamoxifen twice (with one day between injections) and then waited one week before administering two more injections of tamoxifen. Four weeks after the last injection, we observed YFP in >87% of LHβ+ cells (Fig. S3.9). Thus, we are confident that with some optimization, other investigators can achieve high levels of recombination with their floxed genes of interest.

Though we demonstrated the high efficiency and specificity of this new Cre-driver line, some may be wary of using tamoxifen in the context of reproductive studies. However, our experience should allay such fears. As we show, the effects of tamoxifen itself on pituitary gene expression are both modest and short-lived. One month (4 weeks) following the final injection, control animals treated with tamoxifen are indistinguishable from control mice treated with oil vehicle. This amount of time is also sufficient for gene recombination and its downstream effects to occur. Also, because gonadotropes are a stable cell population, one can expect the effects of recombination to be durable without any recovery of function, as we observed over a three-month period in females.

Finally, others may wonder whether there is Cre activity beyond the gonadotrope cell in these mice. Though we did not investigate this systematically, one can assume that extra-pituitary sites of Cre-ERT2 expression would mirror those of Cre expression in GRIC mice, namely the male germline and some (still undefined) neurons [649, 725-728]. Induced recombination in these cells must still be determined, but given that tamoxifen can cross both the blood-brain [729] and blood-testis barriers [730], at least some recombination in these tissues should be expected. The effects in testis, though, should be short-lived, as Cre activity is only observed at the round spermatid stage in GRIC mice [649].

In summary, we developed a novel inducible Cre-driver line for murine gonadotrope cells. Deletion of the *Foxl2* and *Smad4* genes in adult gonadotropes leads to FSH deficiency and impaired female reproduction. Sperm counts and motility remain intact, indicating that FSH is not essential for the maintenance of spermatogenesis in adult male mice. Our studies provide a valuable new mouse model as well as a road map for investigations of gene function in adult gonadotropes.

Materials and Methods

Generation of control and experimental animals

The *Foxl2*$^{fx/fx}$;*Smad4*$^{fx/fx}$ mice were described previously [409]. iGRIC mice (*Gnrhr*$^{tm1.1(cre/ERT2)Djb}$; MGI:5908151; iGRIC for simplicity) were generated as described in Supplemental Fig. S1. *Foxl2*$^{fx/fx}$;*Smad4*$^{fx/fx}$ mice were crossed with iGRIC/iGRIC animals to generate iGRIC/+;*Foxl2*$^{fx/+}$;*Smad4*$^{fx/+}$ progeny, which were then crossed to *Foxl2*$^{fx/fx}$;*Smad4*$^{fx/fx}$ mice to produce inducible knockouts (iGRIC/+;*Foxl2*$^{fx/fx}$;*Smad4*$^{fx/fx}$) and controls (*Foxl2*$^{fx/fx}$;*Smad4*$^{fx/fx}$). *Gt[690]26Sor*$^{tm1(EYFP)Cos}$/J (*Rosa26*$^{eYFP/eYFP}$) mice were ordered from

Jackson Labs (004077) and were crossed with iGRIC/iGRIC mice to generate iGRIC/+;*Rosa26*$^{eYFP/+}$ animals. Mice were genotyped by PCR of genomic DNA using the primers listed in Table 3.1. All animal experiments were performed in accordance with institutional and federal guidelines and were approved by the McGill University and Goodman Cancer Centre Facility Animal Care Committee (protocol 5204).

Tamoxifen injection

All adult animals received either 2 mg of tamoxifen (Sigma T5648; prepared in 100 μl of corn oil) or oil vehicle once daily i.p., every other day, four times in total unless specified in Fig. 3.1B and Fig. S3.9.

Immunofluorescence

Pituitary sections were prepared as described previously [712]. Sections were then blocked with normal serum and incubated with primary antibody rabbit anti-GFP (1:500, Bioss, bs-2194R), rabbit anti-FSHβ (1:500, NIDDK, AFP7798_1289P) or guinea pig anti-FSHβ (1:1000, NIDDK, AFP-1760191), rabbit anti-FOXL2 (1:8000, Dr. Dagmar Wilhelm) with goat anti-LHβ (1:500, Santa Cruz, sc-7824) overnight at 4°C. After three washes with PBS, sections were incubated with Alexa fluor 488-conjugated donkey anti-goat (1:600, Life Technologies, A11055), Alexa fluor 594-conjugated donkey anti-rabbit (1:600, Life Technologies, A21207), Alexa fluor 647-conjugated donkey anti-rabbit (1:200, Jackson Laboratories, 706-605-152) and Alexa fluor 594-conjugated donkey anti-guinea pig (1:200, Jackson Laboratories, 706-605-148) secondary antibodies for 1 h at room temperature. After another three washes with PBS, coverslips were mounted with ProLong Gold antifade reagent DAPI (Life Technologies, 1266174) and dried at

37°C for 15 min in the dark. Slides were analyzed on a Zeiss Axio Imager M2 microscope or an LSM710 confocal microscope. Images were acquired with an Axiocam 506 mono camera (Zeiss) using Zen 2.3 pro (Zeiss) software.

Cell counting

Total number of LHβ+/YFP+ and LHβ+/eYFP- cells were counted in each field of merged images. On average, a total of 80 LHβ+ cells were counted per field. Each data point represents the mean of 3-5 fields from one section. Samples were collected from 2-4 animals per genotype per treatment.

Immunohistology

Testes were isolated from male mice at least one-month after the last injection, fixed overnight in Bouin's buffer at room temperature then dehydrate in 70% ethanol for 3 days. Tissues were paraffin-embedding and cut into 4 μm sections. After deparaffinization, antigen unmasking was performed by submerging the sections in boiling 10 mM sodium citrate (pH 6.0) for 22 min. After 2 times wash by 0.025% PBS-Triton (Bioshop, TRX777), endogenous peroxidase activity was quenched in 3% hydrogen peroxide for 10 min. After 3 washes with PBST, nonspecific binding is blocked using 5% normal donkey serum (Vector Laboratories, S-1000) prepared in PBST for 1 hour at room temperature, then incubated with primary antibody rabbit anti-SOX9 (1:250, Cell Signaling, 82630) overnight at 4°C. After 3 washes with PBST, sections were incubated with biotinylated secondary antibody (goat anti-rabbit, 1:200, Vector Laboratories, PK-6101) for 1 h at room temperature. The signals were then amplified using avidin-biotin complex (ABC) method (Vector, PK-6101). according to the manufacturer instruction. After another 3

washes with PBST, peroxidase substrate solution (DAB, Vector, SK-4100 plus 1% hydrogen peroxide) is added and let it react for 4 min at room temperature. Sections were counter stained with Hematoxylin solution (Sigma, GHS232) and dehydrate in successive bath of ethanol then toluene, and mounted with Permount^{TM} (Fisher, SP15-100).

Sertoli cell count

Sertoli cell counting were performed by blinded observer. Tubules that appeared either round or nearly round in transverse sections were selected randomly. SOX9-positive cells (i.e., Sertoli cells) were counted at 400 × magnification using ZEN software (Zen 2012 blue edition; Carl Zeiss Microscopy GmbH 2011). Data are from counts of 30 seminiferous tubules (two counts of 15 seminiferous tubules per slide separated by 50 µm).

Reproductive organ histology

Ovarian histology was performed as described previously [712]. Images were acquired with a Leica DFC310 FX 1.4-megapixel digital color camera with a Leica DM1000 LED light microscope using Leica Application Suite Version 4.0.0 software or with a Zeiss Axio Imager M2 microscope as described above.

Fertility assessment

To assess fertility, females of both genotypes were first paired with wild type C57BL6 males (Charles River, 000664) until they produced two litters. We removed male partners upon visual observation of the second pregnancy. One week after the second litter was born, females were randomly assigned to one of the two treatment groups. Oil vehicle or tamoxifen was injected

once daily, every other day, four times in total in each female. Four weeks after the last injection, they were re-paired with fertility-proven, wild-type males for a period of three months or until they produced three litters. Date of birth and litter size were recorded, as described previously [712]. Pups were removed from the breeding couples at postnatal day 15.

Sperm counting and motility analyses

Epididymal sperm were counted using a hemocytometer as described previously [417, 712]. Sperm motility was analyzed using computer-assisted sperm analysis [650], as described previously [693, 712].

Hormone analyses

Blood was collected from the submandibular vein or by cardiac puncture, and serum obtained as described previously [712]. Serum FSH levels were assessed by Luminex assay (Millipore, MPTMAG-49K) following the manufacturer's instructions. Serum LH was measured with an in-house sandwich ELISA, as described previously [55]. To measure pituitary FSH and LH content, pituitary lysates were prepared as described previously [712]. Pituitary total protein concentration was measured by BCA protein assay kit (Thermo Fisher, 23225). Pituitary FSH content was measured by radioimmunoassay (RIA) at the Ligand Assay and Analysis Core of the Center for Research in Reproduction at the University of Virginia (Charlottesville, Virginia). The reportable ranges were 2.3-60.0 ng/ml or 1.8-60 ng/ml for females and males, respectively; intra-assay CVs were <20%. Female samples were diluted 10 times and male samples were diluted 40 times for assessment. Pituitary LH content was measured using the in-house sandwich ELISA, as described previously [731]. Samples were diluted 10,000 times for analysis.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Pituitaries were extracted, immediately frozen in liquid nitrogen, and stored at -80°C. Control females were euthanized in the morning of random days of the estrous cycle. Induced S4F2 cKO females, most of which did not cycle, were euthanized the same day as control littermates. Total RNA was extracted as described previously [712]. Reverse transcription and qPCR analyses were performed as described previously [396]. Primers used in qPCR analysis are listed in Table 3.1.

Primary pituitary cultures

Primary pituitary cultures were prepared from control and induced F2S4 cKO animals as previously described [694]. In the *in vitro* induced gene recombination experiment, cells were treated with 1.5 μM 4-OHT (Sigma, H7904) prepared in M199 culture media supplement with 10% FBS for 24 hours. Cells were then treated with activing A or non-treated as described previously [712].

Statistical analysis

Reproductive organ weights, sperm counts, sperm motility, serum hormonal levels, pituitary gene expression, and pituitary protein levels were analyzed by one-way ANOVA, followed by Bonferroni correction. Primary culture experiments were analyzed by one-way ANOVA as indicated above, Student's *t* test, or two-way ANOVA followed by Tukey *post-hoc* tests, where applicable. All statistical analyses were performed with Prism6 (GraphPad) software.

Data are presented as mean (scatter graph) or mean + SEM (bar graph). $p < 0.05$ was considered statistically significant. Statistical significance is indicated with symbols.

Supplemental methods

Tamoxifen preparation

Tamoxifen (Sigma, T5648) was dissolved in a small volume of 100% ethanol (pre-warmed to 55°C) and then re-suspend in corn oil. The mixture was rotated overnight at 37°C. The working concentration of tamoxifen was 2 mg/100 µl. 4-hydroxytamoxifen (4-OHT, Sigma, T176) was dissolved in 100% ethanol, then filter-sterilized (22 µm). The working concentration of 4-OHT was 1.5 µM.

Targeting vector construction

The original GnRHR-IRES-Cre (GRIC) targeting vector described in [648] was digested with *Asc*I. The 3.5 kb *Asc*I fragment, which contained the IRES, Cre, and PGK-Neo cassettes was ligated into the *Asc*I site of pNEB193 (New England Biolabs). The stop codon in Cre was then mutated to an *Xho*I restriction site using the QuikChange protocol (Agilent) and the following primers:

GGTGCGCCTGCTGGAAGATGGCGATCTCGAGTTAACGAAGTTCCTATTCCGAAGTT

and

AACTTCGGAATAGGAACTTCGTTAACTCGAGATCGCCATCTTCCAAGCAGGCGCACC.

The ERT2 coding sequence was PCR amplified from the vector pCAG-CreERT2 (Addgene clone # 14797) using Go Tag Flexi DNA polymerase (Promega M8291) and the following primers: AAGATGGCGATCTCGAGCCATC and CCGCTCGAGTCAAGCTGTGGCAGGGAAAC,

which added *Xho*I sites to both the 5' and 3' ends. The resulting PCR amplicon and pNEB193 vector with modified *Asc*I fragment were digested with *Xho*I and ligated together using T4 DNA ligase. The resulting plasmid contained an in-frame fusion of Cre and ERT2, as confirmed by DNA sequencing (GenomeQuebec). The *Asc*I fragment, now containing IRES, Cre-ERT2, and PGK-Neo, was then isolated by restriction digest and gel purification, and ligated into the parental GRIC vector from which the original *Asc*I fragment had been removed. Recombinant clones were screened by *Asc*I digest and sequenced to determine fragment orientation. The resulting inducible GRIC targeting vector (iGRIC; supplementary Fig. S3.1A) was linearized with *Not*I.

Generation of iGRIC mice

Linearized iGRIC vector was electroporated into R1 ES cells, which were cultured under selection in 400 µg/ml G418 (Invitrogen, Burlington, ON, Canada) for 8-9 days. Surviving colonies were selected and cultured in triplicate. DNA was extracted from two replicates and analyzed by Southern blotting. 5' and 3' probe templates (Fig. S3.1A) were generated by PCR from murine genomic DNA using the following primer sets: 5' probe, TTCAGGCAAAGACACATAACT and CTCCCCGTACCCCCAACT; 3' probe, CGGGGGAGGAAAAACATCTTA and ATTTGACCCCACTCAGCACT. Targeting efficiency was >40%, as indicated with both probes (Fig. S3.1B and data not shown). Two clones (D2 and B12) were expanded and injected into C57BL/6 blastocysts in the transgenic core facility at McGill University using standard approaches. Resulting chimeric males (at least 75% by coat color) were crossed to C57BL/6 females and tail DNA from brown-coated pups screened by Southern blot (*Eco*RV digest and 5' probe) for transmission of the targeted allele (data not shown). Both clones contributed to the male germline. iGRIC-Neo/+ mice were crossed to *Flp* deleter mice

(129S4/SvJaeSor-*Gt(ROSA)26Sor*$^{tm1(FLP1)Dym}$/J; stock no. 003946) to remove the *Frt*-flanked PGK-Neo selection cassette. Successful excision was confirmed by PCR with primers GTAACAAAGGCATGGAGCATC, CAAGCTTATCGATACCGTCTC, and CTGTTTACTTAGGACGTAGTC (Neo+, 327 bp; Neo-, 648 bp), and by Southern blotting of tail DNA (*Avr*II digest and 3' probe; Fig. S3.1C). iGRIC/+ animals were crossed to wild-type C57BL/6 mice to segregate the recombined iGRIC (now Neo-) and *Flp* alleles. Moving forward, only mice derived from clone D2 were used and the presence of the iGRIC allele was confirmed by PCR of genomic DNA using the primers: TCAATACCGGAGATCATGCAAG and GGTAGGATCATACTCGGAATAG. iGRIC/+ mice were intercrossed to generate iGRIC/iGRIC homozygotes, which were fertile. Here and below, all animal work was conducted in accordance with provincial and federal guidelines, and was approved in Animal Use Protocol 5204 by the Goodman Cancer Centre Facility Animal Care Committee at McGill University.

Immunofluorescence

Male GRIC/+;Rosa26eYFP/+ pituitary sections were prepared as described in the text Methods section. Sections were incubated in 1:500 dilutions of the following primary antibodies: rabbit anti-GFP and goat anti-LHβ, or with rabbit anti-rat FSHβ and goat anti-GFP (Rockland, 600-101-215) overnight at 4°C. Control and induced F2S4 cKO pituitary sections were prepared as described in the text Methods section. Sections were incubated with rabbit anti-FOXL2 (1:8000) and goat anti-TSH (1:250, Santa Cruz, sc7815) overnight at 4°C.

RNA extraction and RT-qPCR

To determine the acute or long-term impacts of tamoxifen treatment on pituitary gene expression, we i.p. injected wild-type adult males (aged between 10-15 weeks) on a mixed genetic background once only or four times (once daily, every other day) with 2 mg tamoxifen in 100 µl corn oil or equivalent amount of oil alone. Pituitaries were isolated either 6 hours (n=7), 1 day (n=6-7), 1 week (n=8) or 2 weeks (n=5-6) after the last injection.

To determine pituitary gonadotropin subunit (*Fshb*, *Lhb* and *Cga*), *Gnrhr*, and *Foxl2* expression in males 1 month after oil vehicle or tamoxifen injection, 8 to 9-week-old males of each genotype were injected with oil vehicle or tamoxifen once daily, every other day, up to four injections. One month after the last injection, animals were euthanized with isoflurane and CO_2, and the pituitaries were isolated. RNA extraction and RT-qPCR analysis were performed as described in the text Methods.

Female reproductive organ histology

The right ovary was removed from females of both genotypes. After recovering from surgery, females were injected once daily, every other day, for four injections of 100µl of oil alone or containing 2 mg tamoxifen. One month after the last injection, the animals were euthanized and the left ovary was isolated. Ovarian sections were prepared and analyzed as described in text Methods.

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Footnotes

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Author contributions: Y.L. and D.J.B designed the studies; Y.L., G.S., Y.W., X.Z., and A.B. performed experiments; C.D. and M.T. generated floxed mouse strains; D.B. and A.B. performed the gene targeting in ES cells. U.B. provided the GRIC targeting vector from which the iGRIC vector was generated; Y.L., G.S., and D.J.B. analyzed data; and Y.L. and D.J.B. wrote the paper.

The authors declare that they have no conflicts of interest with the contents of this article.

Figure legends

Figure 3.1 Validation of the efficiency and specificity of tamoxifen-induced recombination in gonadotropes. A) Male iGRIC/+;*Rosa*26$^{eYFP/+}$ male mice were treated with oil (vehicle) or tamoxifen [376] with one of five different treatment protocols (see schematic in panel B). Cells in pituitary sections were labeled for YFP (green) and LHβ (red). Arrows indicate LHβ+/YFP- cells. B) Top: Quantification of LHβ-positive cells co-labeled with YFP following different vehicle or tamoxifen treatments. Bottom: Schematic representation of the different tamoxifen treatment protocols. C) Immunofluorescence staining for FOXL2 (green) and LHβ (red) in pituitary sections from males in the four treatment groups. Scale bars = 50 μm

Figure 3.2 Impaired fertility and ovarian follicular development in induced F2S4 cKO females. A) Fertility assessment before and after oil or tamoxifen injections in control and induced F2S4 cKO females. The blow ups at the right indicate individual fertility data (litter size) for sterile (red dots) and subfertile females (cyan, royal blue, brown and pink dots). The number of litters produced by subfertile mice is also shown. B) Representative ovarian tissue sections in control and induced F2S4 cKO mice. CL, corpus luteum. Scale bar = 200 μm. Ovarian C) and uterine D) weights of control and induced F2S4 cKO females one month after oil or tamoxifen injections. Horizontal lines reflect group means (+ SEM). Each data point represents one animal. Fertility of each group before injection was analyzed by one-way ANOVA. Fertility before and after treatment in iGRIC/+;TAM group was analyzed by student's *t*-test. *$p < 0.05$.

Figure 3.3 Intact testicular weights and spermatogenesis in induced F2S4 cKO males. Testicular A) and seminal vesicle B) weights from control and induced F2S4 cKO males two

months after oil or tamoxifen injections. C) Progressive sperm motility from caudal epididymides. D) Sperm counts from snap frozen epididymides. E) Representative of immunohistology staining for SOX9 as Sertoli cell marker in testicular tissue sections from control and induced F2S4 cKO mice. Scale bar = 100 µm. There were no significant differences between groups.

Figure 3.4 Reduced FSH production and secretion in induced F2S4 cKO mice. Serum FSH levels in females A) and males B) before (left) and after [237] tamoxifen or oil treatment. Individual serum FSH levels after injection are shown in the boxes at the right. Serum LH levels in females C) and males D) post-treatment. E, F) RT-qPCR analysis of relative mRNA levels of pituitary *Fshb*, *Foxl2*, *Smad4*, *Cga*, *Lhb*, and *Gnrhr* in females and males after treatment with oil or tamoxifen. All gene expression was normalized to the housekeeping gene *Rpl19*. Dashed lines represent mRNA levels in mice lacking the *Foxl2* and *Smad4* genes during development (Fortin et al., 2014). Relative gene expression, and serum FSH levels before and after treatment were analyzed separated by one-way ANOVAs followed by Bonferroni correction. Serum FSH levels before and after treatment in the same group were analyzed by student's *t*-test. There were no significant differences between the control groups. $*p < 0.05$.

Figure 3.5 Reduced pituitary FSH protein content in induced F2S4 cKO mice. Pituitary FSH content in females A) and males B) after oil or tamoxifen treatment. Control female pituitaries were collected at random estrous cycle stages; cKO pituitaries were collected at the same time as their control littermates. C, D) Pituitary LH content in females and males post-treatment. E) Immunofluorescence staining for LHβ (green) and FSHβ (red) in pituitaries of male control and

induced F2S4 cKO male mice. Data were analyzed by one-way ANOVA followed by Bonferroni correction Scale bar = 50 µm. *$p < 0.05$.

Figure 3.6 Impaired basal and activin A-stimulated *Fshb* expression in cultured pituitary cells of induced F2S4 cKO mice. Primary pituitary cultures were prepared from control and induced F2S4 cKO females A) and males B) at least one month after oil or tamoxifen injection. Cells were treated with vehicle or 1 nM activin A. C) Primary pituitary cultures were prepared from male and female (combined) iGRIC/+;*Foxl2*$^{fx/fx}$;*Smad4*$^{fx/fx}$ mice or from D) wild-type mice. Cells were then treated with 1.5 µM of 4-OH tamoxifen (4-OHT) or vehicle. Twenty-four hours later, cells were treated with vehicle or 1 nM of activin A for 24 hours. In all panels, RNA was extracted and gene expression was assessed by RT-qPCR. Bars represent the means (+ SEM) of 3 independent experiments. In panels A and B, basal *Fshb* expression levels (black bars) were analyzed by one-way ANOVA followed by Bonferroni correction. #, significantly different from the other groups; Activin A stimulation on *Fshb* expression in each group was analyzed by Student's *t*-test (red vs. black bars). *$p < 0.05$. Data in panels C and D were analyzed by two-way ANOVA followed by Tukey *post-hoc* tests. Bars with different symbols differ significantly.

Supplemental figure legends

Figure S3.1 Generation of iGRIC mice. A) Schematic representation of the targeting strategy. The wild-type allele is shown at the top. The internal ribosomal entry site (IRES), Cre-ERT2, and PGK-Neo cassette was introduced after the translation terminal codon in exon 3 of the *Gnrhr* locus (see targeted allele, Neo+). The bottom schematic reflects the targeted allele after Flp-mediated deletion of the PGK-Neo cassette (Neo-). The location of *Eco*RV (E) and *Avr*II (A) restriction sites

used for Southern blot screening of ES cells (B) and mice (C) are shown, as are the predicted lengths of the restriction fragments as detected with the indicated 5' and 3' probes (hatched boxes at the bottom of panel A). Exons 2 (Ex2) and 3 (Ex3) are shown as black (coding sequence) or white (3' UTR) boxes. B) Southern blot of ES cell clone genomic DNA screened with the *Eco*RV digest and both the 5' (top) and 3' probes (bottom). C) Southern blot of tail DNA from mice following the cross of iGRIC/Neo+ mice with Flp-deleter mice using the *Avr*II digest and 3' probe. The Neo- reflects the successful deletion of the Neo cassette. These represent the iGRIC mice. iGRIC, inducible GnRH receptor IRES Cre-ERT2.

Figure S3.2 Co-expression of YFP and LHβ in pituitaries of GRIC/+;*Rosa26*$^{eYFP/+}$ mice. Dual-label immunofluorescence for LHβ (red) and YFP (green). The asterisk indicates a rare LHβ+/YFP- cell. Scale bar = 50 μm.

Figure S3.3 Co-expression of FOXL2, FSHβ and TSHβ in male control and induced S4F2 cKO pituitary sections. Triple-label immunofluorescence for FOXL2 (violet), FSHβ (red) and TSHβ (green). Scale bar = 50 μm.

Figure S3.4 Ovarian histology before and after tamoxifen-induced recombination. One ovary was isolated from females of both genotypes at a random stages of the estrous cycle prior to oil or tamoxifen injection. Animals then received four oil vehicle or tamoxifen injections (once daily, every other day). One month after the final injection, the remaining ovary was isolated from the same animals. All ovaries were fixed with 10% formalin before sectioning. Ovarian histology was assessed by H&E staining. CL, corpus luteum. Scale bar = 200 μm.

Figure S3.5 Pituitary gonadotropin subunit (*Fshb*, *Lhb* and *Cga*), GnRH receptor (*Gnrhr*), and *Foxl2* gene expression one month after oil or tamoxifen injection in male mice. Pituitary total RNA was extracted from a second cohort of control and experimental males one rather than two months following the last of four oil or tamoxifen injections. Expression of the indicated genes was analyzed by RT-qPCR. The blue dashed lines indicate the gene expression levels in induced F2S4 cKO males two months post-injection (data from Fig. 4F). Bars reflect group means (+ SEM). Data for each gene were analyzed with separate one-way ANOVA followed by Bonferroni correction. $*p < 0.05$.

Figure S3.6 Gene expression in pituitaries of tamoxifen-treated wild-type males. Mice were injected i.p. once or four times (once daily, every other day) with 2 mg of tamoxifen or oil alone (vehicle). Pituitaries were isolated 6 hours (blue), 1 day (orange), 1 week (red) or 2 weeks (grey) after the last injection. Total RNA was extracted and expression of the indicated genes analyzed by RT-qPCR. Bars are means (+SEM) of 5-8 individuals. Data for each gene were analyzed separately by one-way ANOVA followed by Bonferroni correction. Bars with different symbols differ significantly.

Figure S3.7 Gene expression in pituitary cells cultured from control and induced F2S4 cKO mice. Primary pituitary cultures were prepared from control and induced F2S4 cKO females (left) and males [237] at least 1 month after the last injection. Cells were treated with vehicle (black bars) or 1 nM activin A (red). Relative mRNA expression was analyzed by RT-qPCR using primers listed in Table 1. Bars represent the means (+ SEM) of three independent experiments. Basal gene

expression levels (black bars) were analyzed by one-way ANOVA followed by Bonferroni correction. #, significantly different from the other groups.

Figure S3.8 Tamoxifen-regulated gene expression in cultures of iGRIC/+;*Foxl2*$^{fx/fx}$;*Smad4*$^{fx/fx}$ and wild-type pituitaries. Expression of the indicated genes was determined by qPCR using the cDNA samples in Fig. 3.6C (panels A-E) and Fig. 3.6D (panels F-J). Data were analyzed by two-way ANOVA followed by Tukey *post-hoc* tests. Bars with different symbols differ significantly.

Figure S3.9 Quantification of LHβ-positive cells co-expressing YFP following the indicated treatments. Male animals were treated and pituitaries processed essentially as in Fig. 3.1A,B, with the indicated differences in the timing of the injections.

Table 3.1: Genotyping and qPCR primers

Genotyping	
Foxl2	5' – 3'
Forward	GGACAGCTTCTGGATGCAGAGCC
Reverse	CAGCGGAGGCGACAAAGCGGAGTCGCAGG
Smad4	
Forward	GGGCAGCGTAGCATATAAGA
Reverse	GACCCAAACGTCACCTTCAG
iGRIC	
Forward	TCAATACCGGAGATCATGCAAG
Reverse	GGTAGGATCATACTCGGAATAG
Wildtype allele	
Forward	GAACTACAGCTGAATCAGTC
Reverse	CTAACAACAAACTCTGTACA
ROSA26 eYFP	
Forward	AAAGTCGCTCTGAGTTGTTAT
WT reverse	GCGAAGAGTTTGTCCTCAACC
eYFP reverse	GGAGCGGGAGAAATGGATATG
qPCR	
Rpl19	
Forward	CGGGAATCCAAGAAGATTGA
Reverse	TTCAGCTTGTGGATGTGCTC
Fshb	
Forward	GTGCGGGCTACTGCTACACT
Reverse	CAGGCAATCTTACGGTCTCG
Foxl2	
Forward	ACAACACCGGAGAAACCAGAC
Reverse	CGTAGAACGGGAACTTGGCTA
Smad4	
Forward	TCACAATGAGCTTGCATTCC
Reverse	CCATCCACAGTCACAACAGG
Cga	
Forward	TCCCTCAAAAAGTCCAQGAGC
Reverse	GAAGAGAATGAAGAATATGCAG
Lhb	
Forward	AGCAGCCGGCAGTACTCGGA
Reverse	ACTGTGCCGGCCTGTCAACG
Gnrhr	
Forward	CACGGGTTTAGGAAAGCAAA
Reverse	TTCGCTACCTCCTTTGTCGT

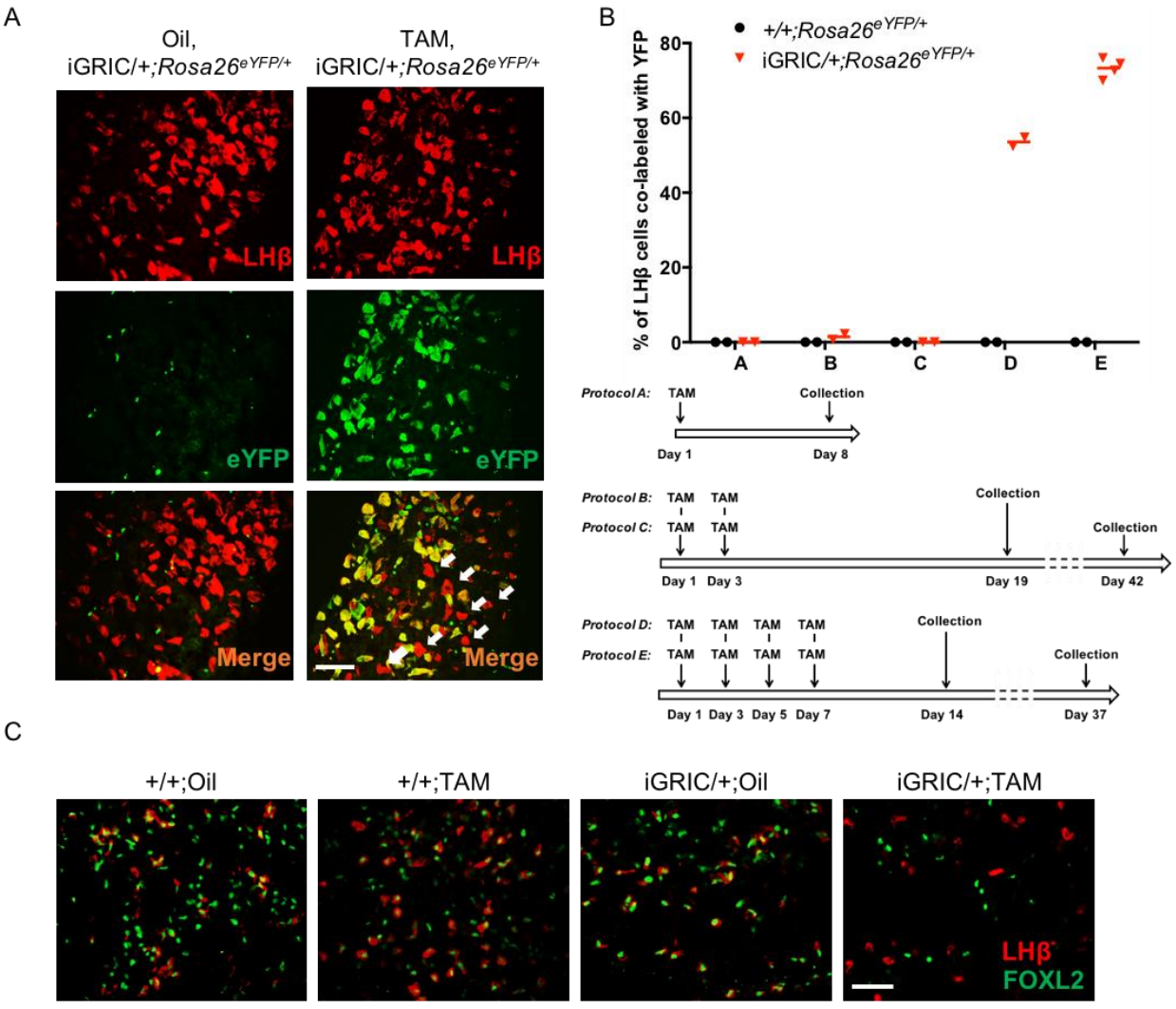
A
Oil,
iGRIC/+;Rosa26eYFP/+
TAM,
iGRIC/+;Rosa26eYFP/+
LHβ
LHβ
eYFP
eYFP
Merge
Merge
B
+/+;Rosa26eYFP/+
iGRIC/+;Rosa26eYFP/+
% of LHβ cells co-labeled with YFP
80
60
40
20
0
A
B
C
D
E
Protocol A:
TAM
Collection
Day 1
Day 8
Protocol B:
Protocol C:
TAM
TAM
TAM
TAM
Collection
Collection
Day 1
Day 3
Day 19
Day 42
Protocol D:
Protocol E:
TAM
TAM
TAM
TAM
TAM
TAM
TAM
TAM
Collection
Collection
Day 1
Day 3
Day 5
Day 7
Day 14
Day 37
C
+/+;Oil
+/+;TAM
iGRIC/+;Oil
iGRIC/+;TAM
LHβ-
FOXL2

Figure 3.1

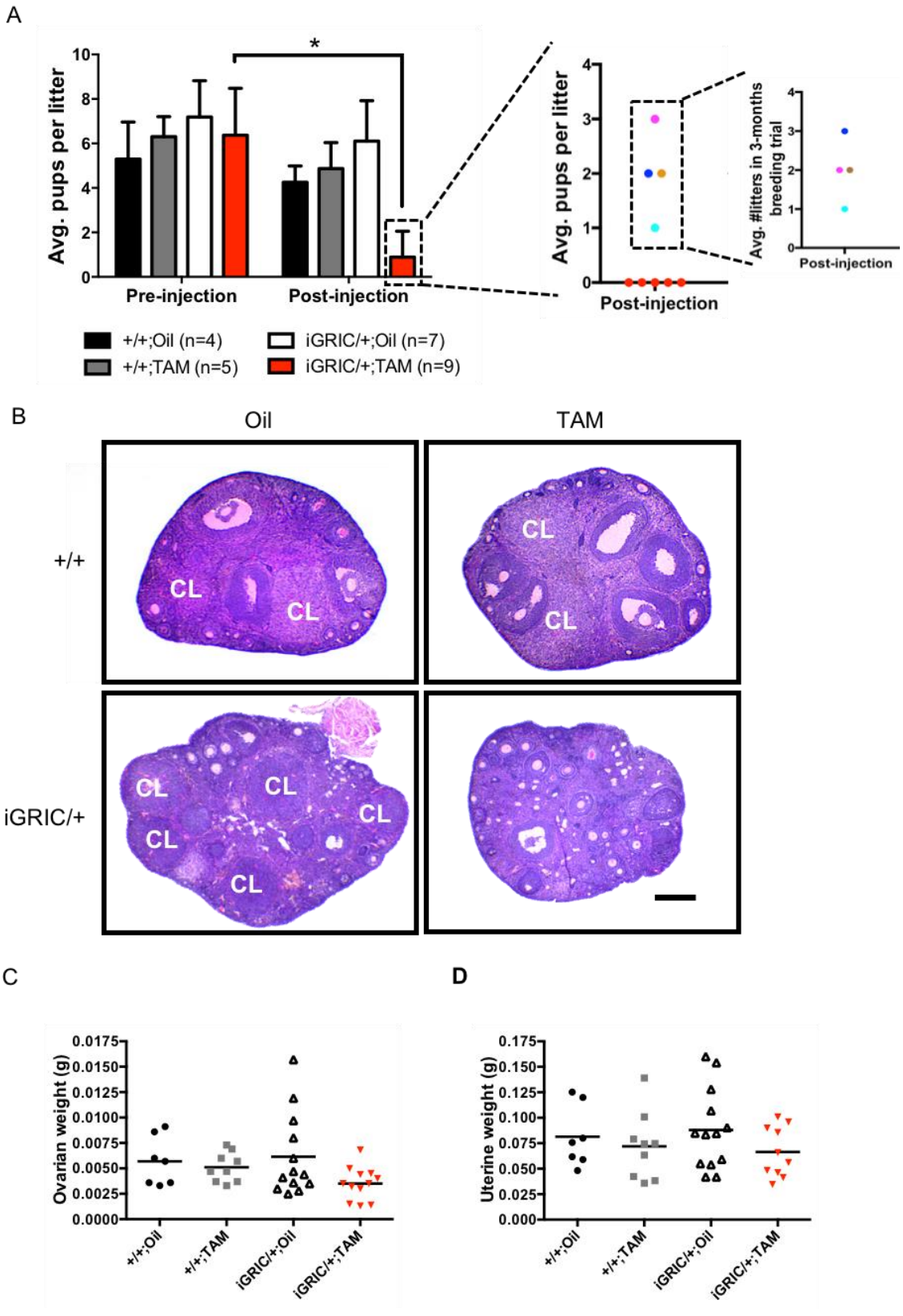
A
Avg. pups per litter
Pre-injection
Post-injection
*
+/+;Oil (n=4)
+/+;TAM (n=5)
iGRIC/+;Oil (n=7)
iGRIC/+;TAM (n=9)
Avg. #litters in 3-months breeding trial
B
Oil
TAM
+/+
iGRIC/+
CL
C
Ovarian weight (g)
D
Uterine weight (g)
+/+;Oil
+/+;TAM
iGRIC/+;Oil
iGRIC/+;TAM

Figure 3.2

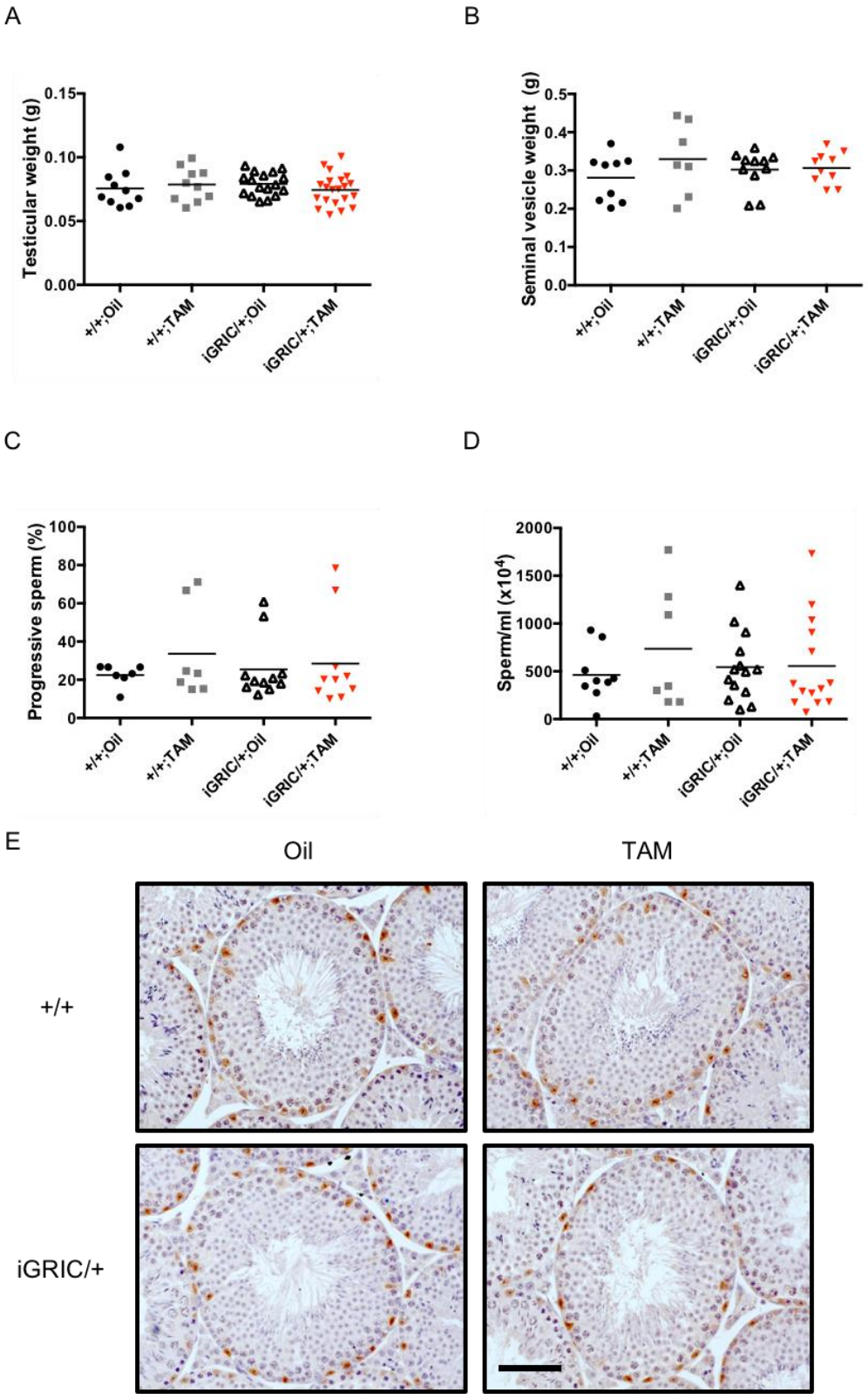
A
Testicular weight (g)
0.15
0.10
0.05
0.00
+/+;Oil
+/+;TAM
iGRIC/+;Oil
iGRIC/+;TAM
B
Seminal vesicle weight (g)
0.5
0.4
0.3
0.2
0.1
0.0
+/+;Oil
+/+;TAM
iGRIC/+;Oil
iGRIC/+;TAM
C
Progressive sperm (%)
100
80
60
40
20
0
+/+;Oil
+/+;TAM
iGRIC/+;Oil
iGRIC/+;TAM
D
Sperm/ml (x10^4)
2000
1500
1000
500
0
+/+;Oil
+/+;TAM
iGRIC/+;Oil
iGRIC/+;TAM
E
Oil
TAM
+/+
iGRIC/+

Figure 3.3

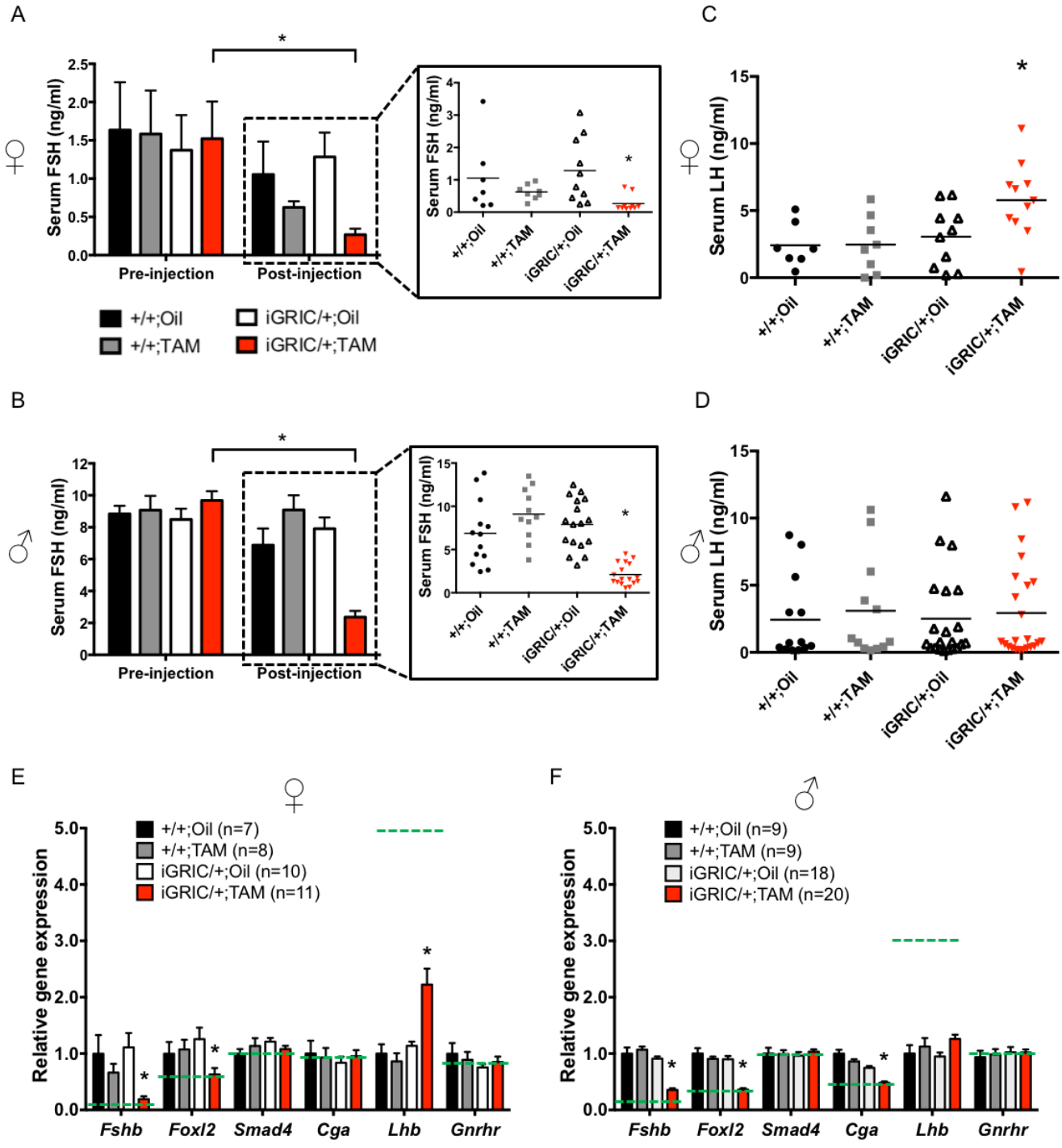
A
C
B
D
E
F
♀
♂
Serum FSH (ng/ml)
Serum LH (ng/ml)
Pre-injection
Post-injection
+/+;Oil
+/+;TAM
iGRIC/+;Oil
iGRIC/+;TAM
+/+;Oil (n=7)
+/+;TAM (n=8)
iGRIC/+;Oil (n=10)
iGRIC/+;TAM (n=11)
+/+;Oil (n=9)
+/+;TAM (n=9)
iGRIC/+;Oil (n=18)
iGRIC/+;TAM (n=20)
Relative gene expression
Fshb
Foxl2
Smad4
Cga
Lhb
Gnrhr

Figure 3.4

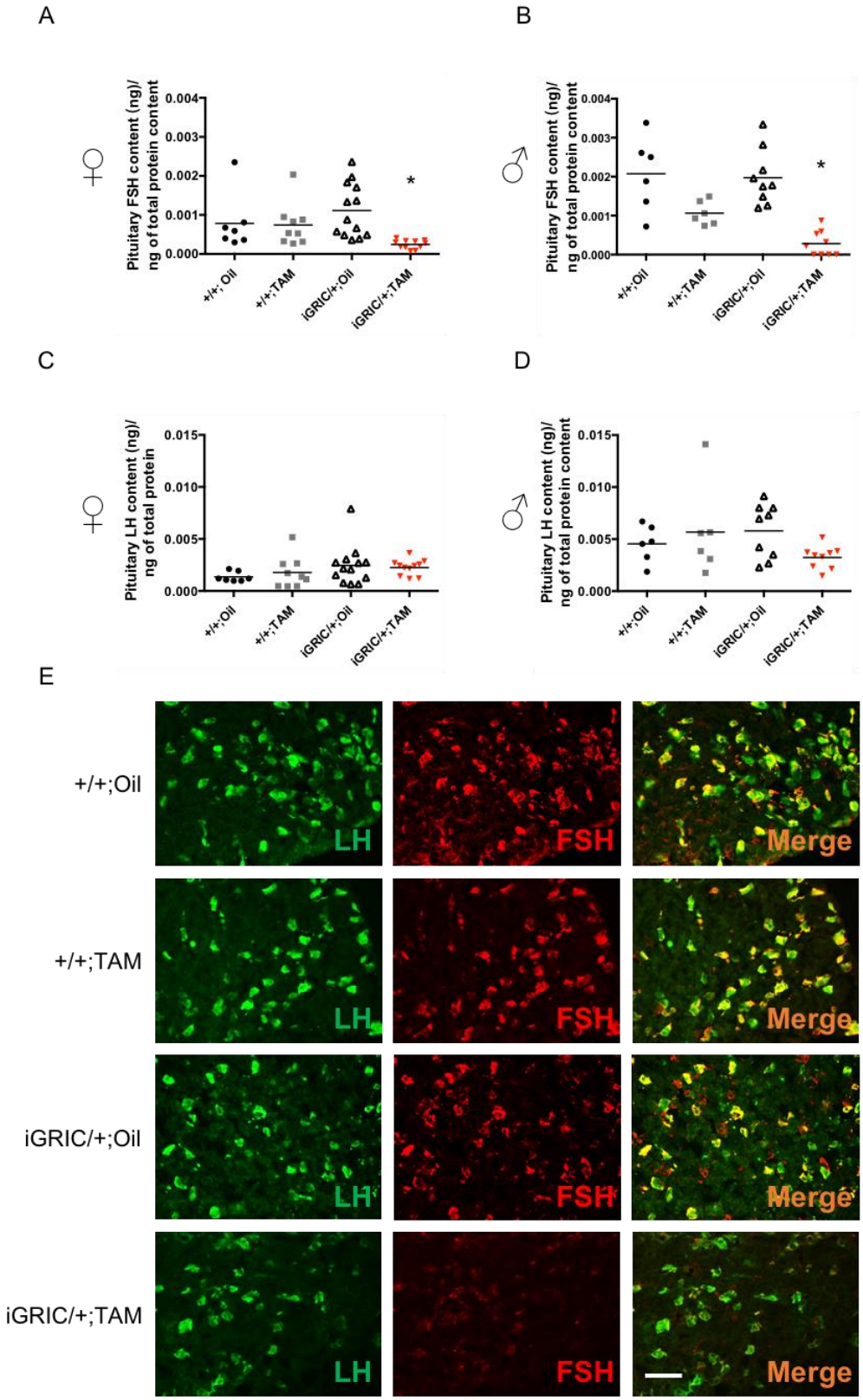
A
B
♀
♂
Pituitary FSH content (ng)/ ng of total protein content
0.004
0.003
0.002
0.001
0.000
*
+/+;Oil
+/+;TAM
iGRIC/+;Oil
iGRIC/+;TAM
C
D
Pituitary LH content (ng)/ ng of total protein
0.015
0.010
0.005
0.000
E
+/+;Oil
+/+;TAM
iGRIC/+;Oil
iGRIC/+;TAM
LH
FSH
Merge

Figure 3.5

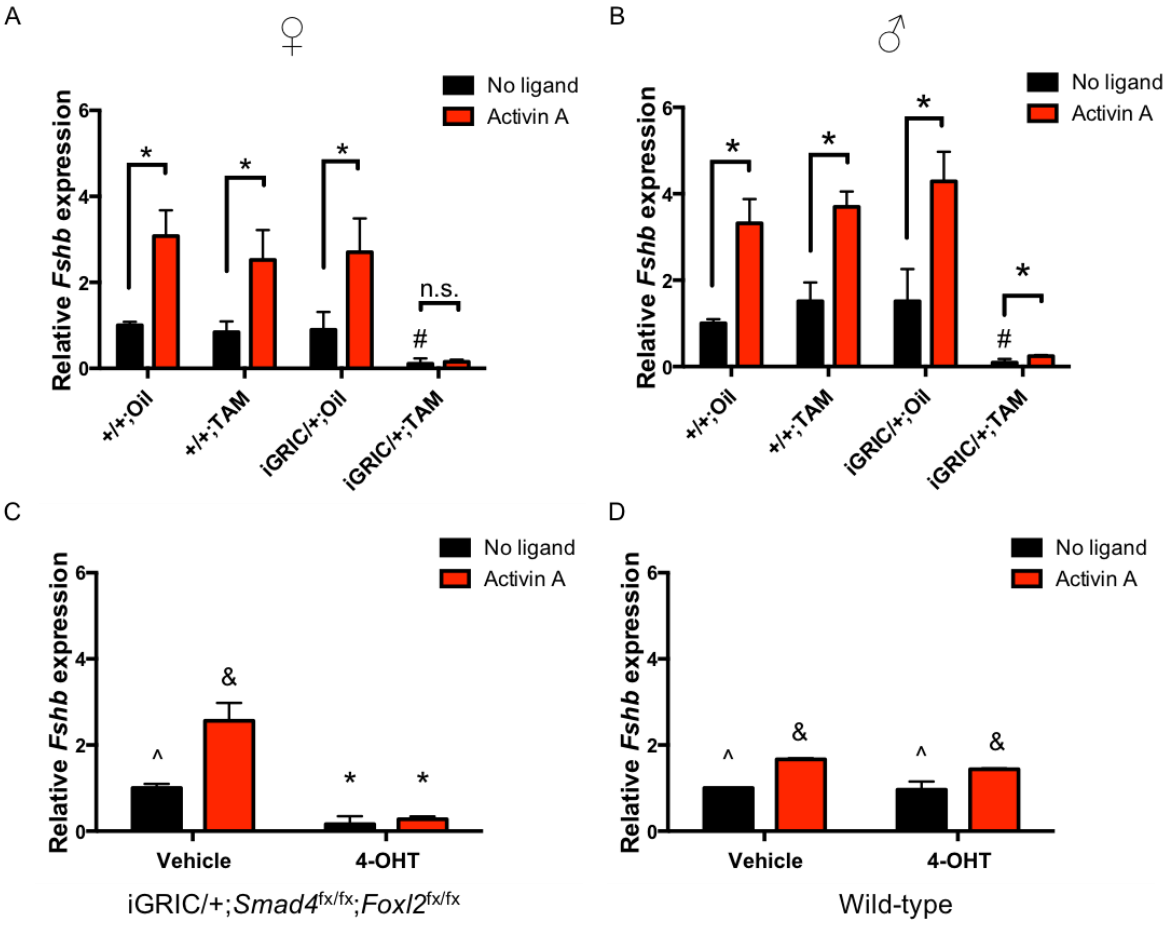
A
♀
No ligand
Activin A
Relative Fshb expression
+/+;Oil
+/+;TAM
iGRIC/+;Oil
iGRIC/+;TAM
n.s.
B
♂
C
Vehicle
4-OHT
iGRIC/+;Smad4fx/fx;Foxl2fx/fx
D
Wild-type

Figure 3.6

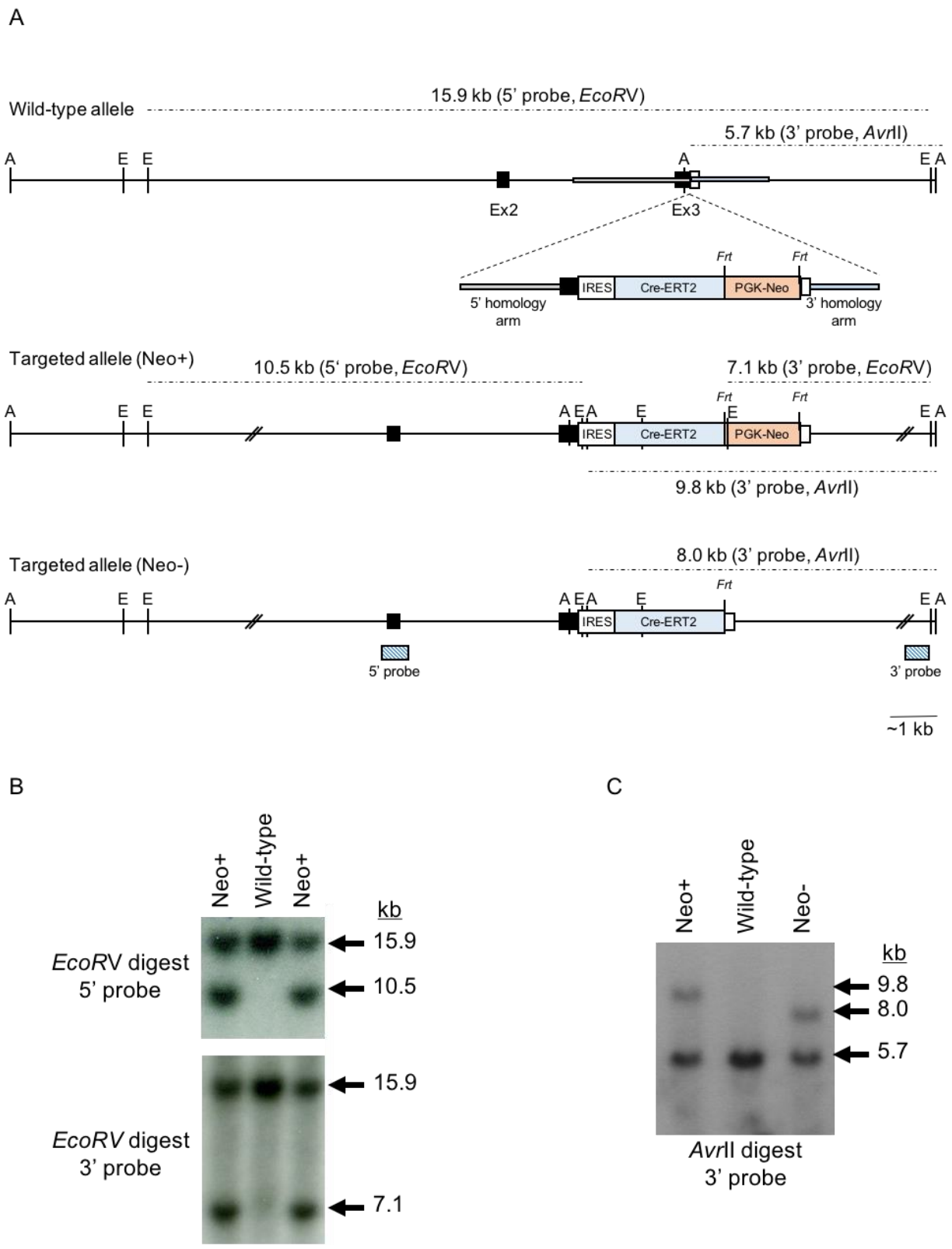
A
Wild-type allele
15.9 kb (5' probe, EcoRV)
5.7 kb (3' probe, AvrII)
A E E A E A
Ex2
Ex3
Frt Frt
IRES Cre-ERT2 PGK-Neo
5' homology arm
3' homology arm
Targeted allele (Neo+)
10.5 kb (5' probe, EcoRV)
7.1 kb (3' probe, EcoRV)
9.8 kb (3' probe, AvrII)
Targeted allele (Neo-)
8.0 kb (3' probe, AvrII)
5' probe
3' probe
~1 kb
B
Neo+ Wild-type Neo+
kb
EcoRV digest 5' probe
15.9
10.5
EcoRV digest 3' probe
15.9
7.1
C
Neo+ Wild-type Neo-
kb
9.8
8.0
5.7
AvrII digest 3' probe

Figure S3.1

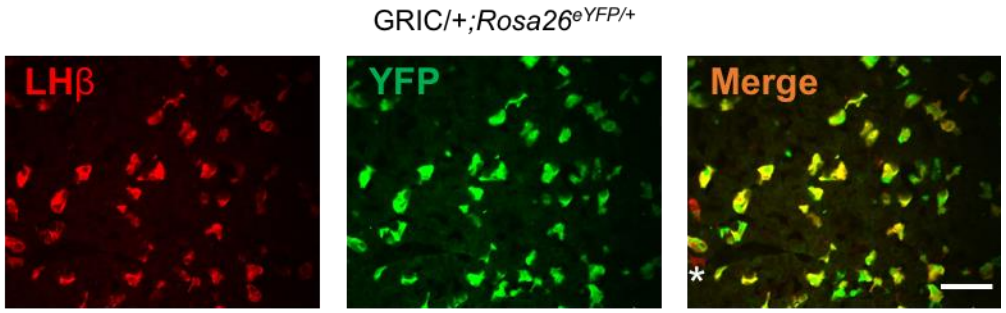

Figure S3.2

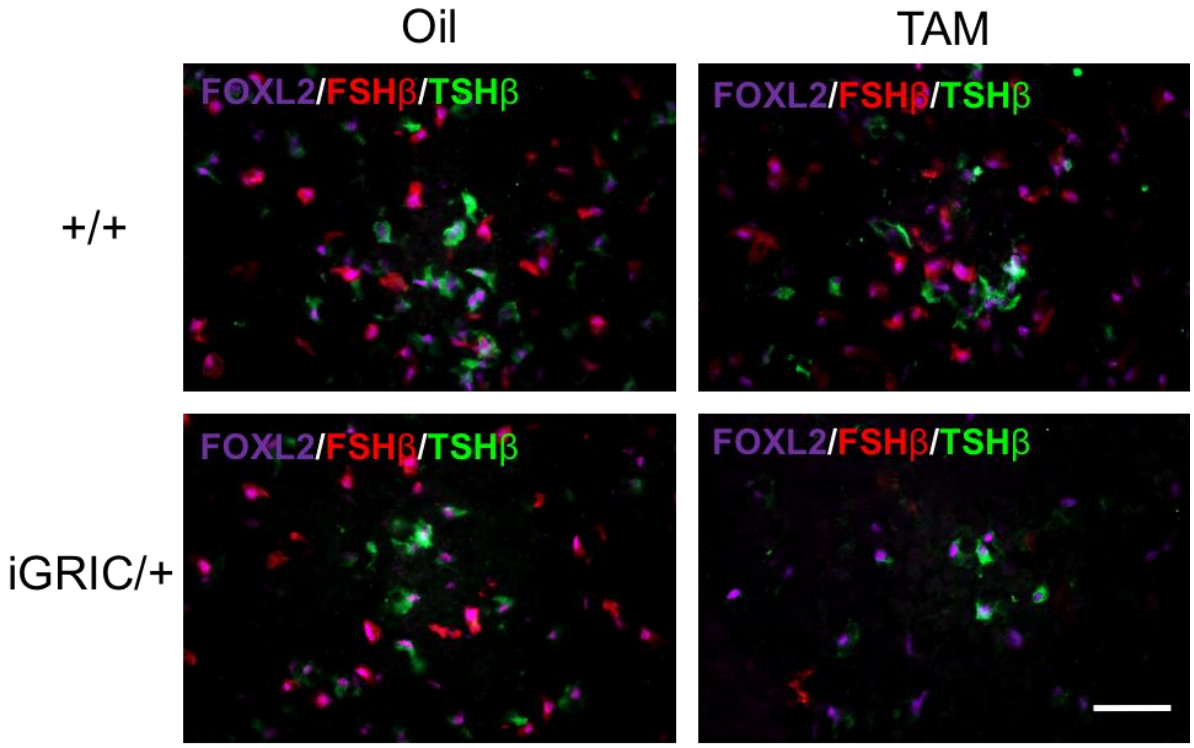
Oil
TAM
+/+
iGRIC/+
FOXL2/FSHβ/TSHβ
FOXL2/FSHβ/TSHβ
FOXL2/FSHβ/TSHβ
FOXL2/FSHβ/TSHβ

Figure S3.3

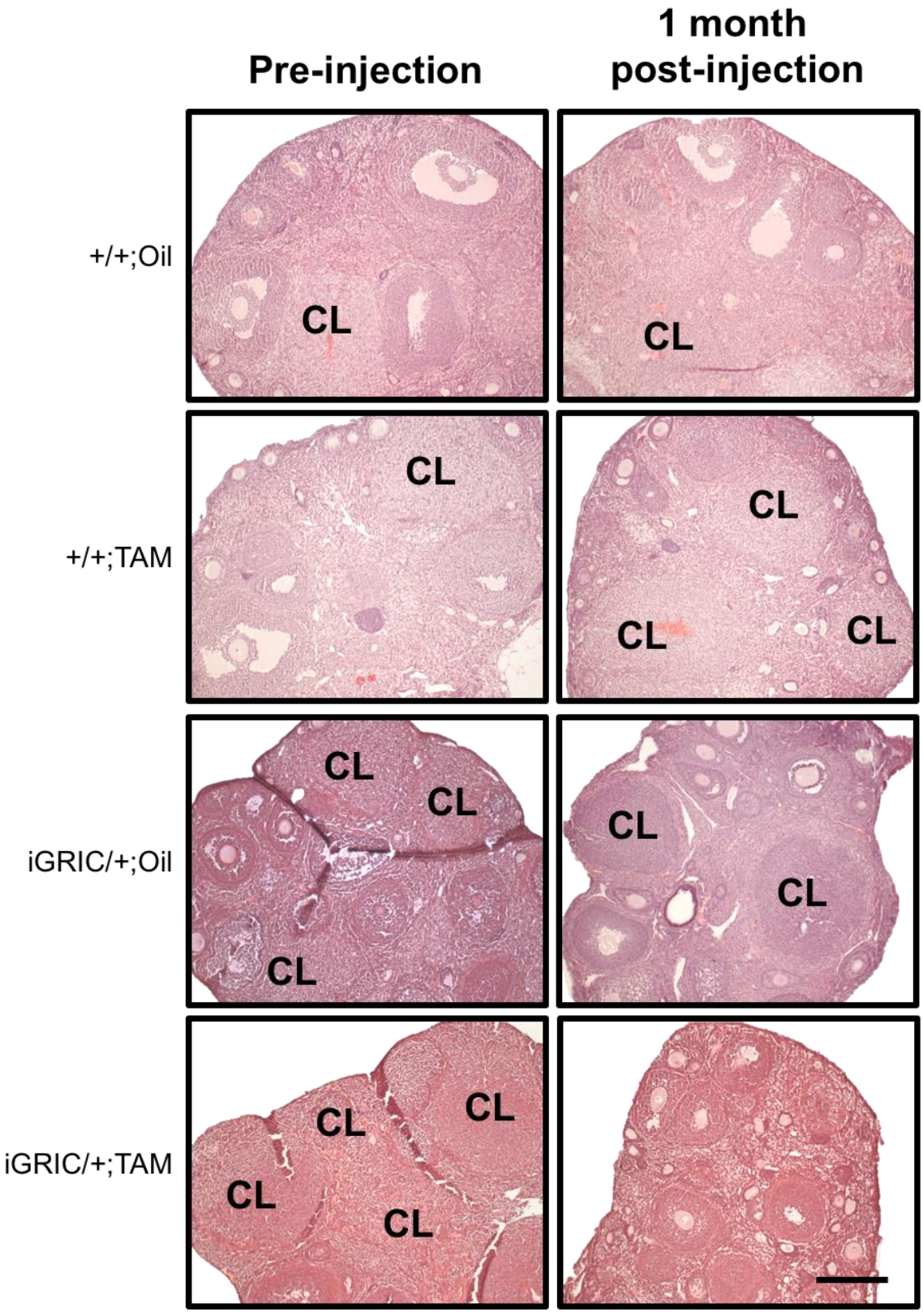
Pre-injection
1 month post-injection
+/+;Oil
+/+;TAM
iGRIC/+;Oil
iGRIC/+;TAM
CL

Figure S3.4

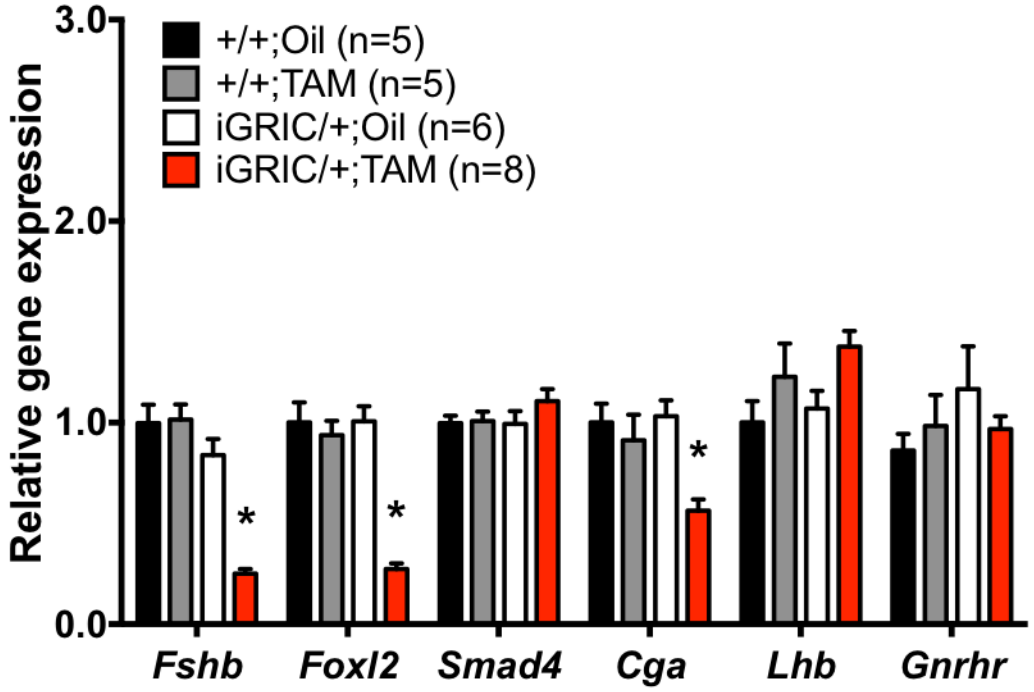
+/+;Oil (n=5)
+/+;TAM (n=5)
iGRIC/+;Oil (n=6)
iGRIC/+;TAM (n=8)
Relative gene expression
3.0
2.0
1.0
0.0
*
*
*
Fshb
Foxl2
Smad4
Cga
Lhb
Gnrhr

Figure S3.5

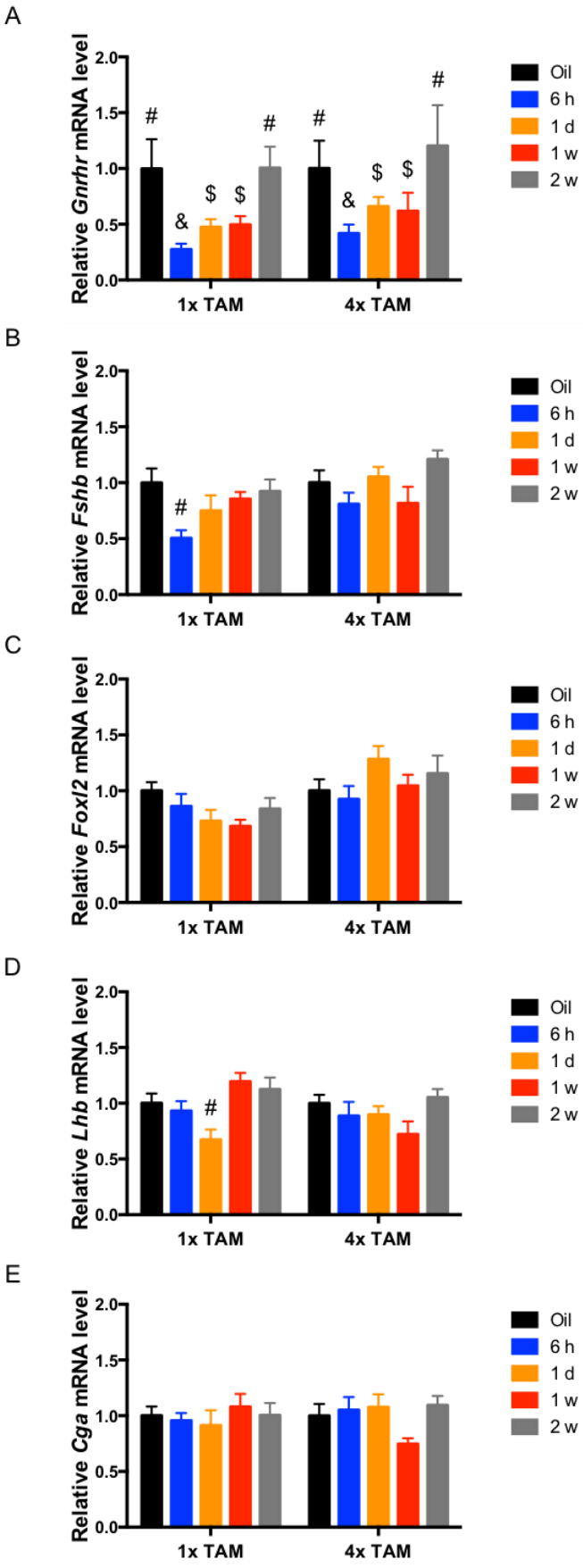
A
Relative Gnrhr mRNA level
2.0
1.5
1.0
0.5
0.0
#
&
$
$
#
#
&
$
$
#
1x TAM
4x TAM
Oil
6 h
1 d
1 w
2 w
B
Relative Fshb mRNA level
#
C
Relative Foxl2 mRNA level
D
Relative Lhb mRNA level
#
E
Relative Cga mRNA level

Figure S3.6

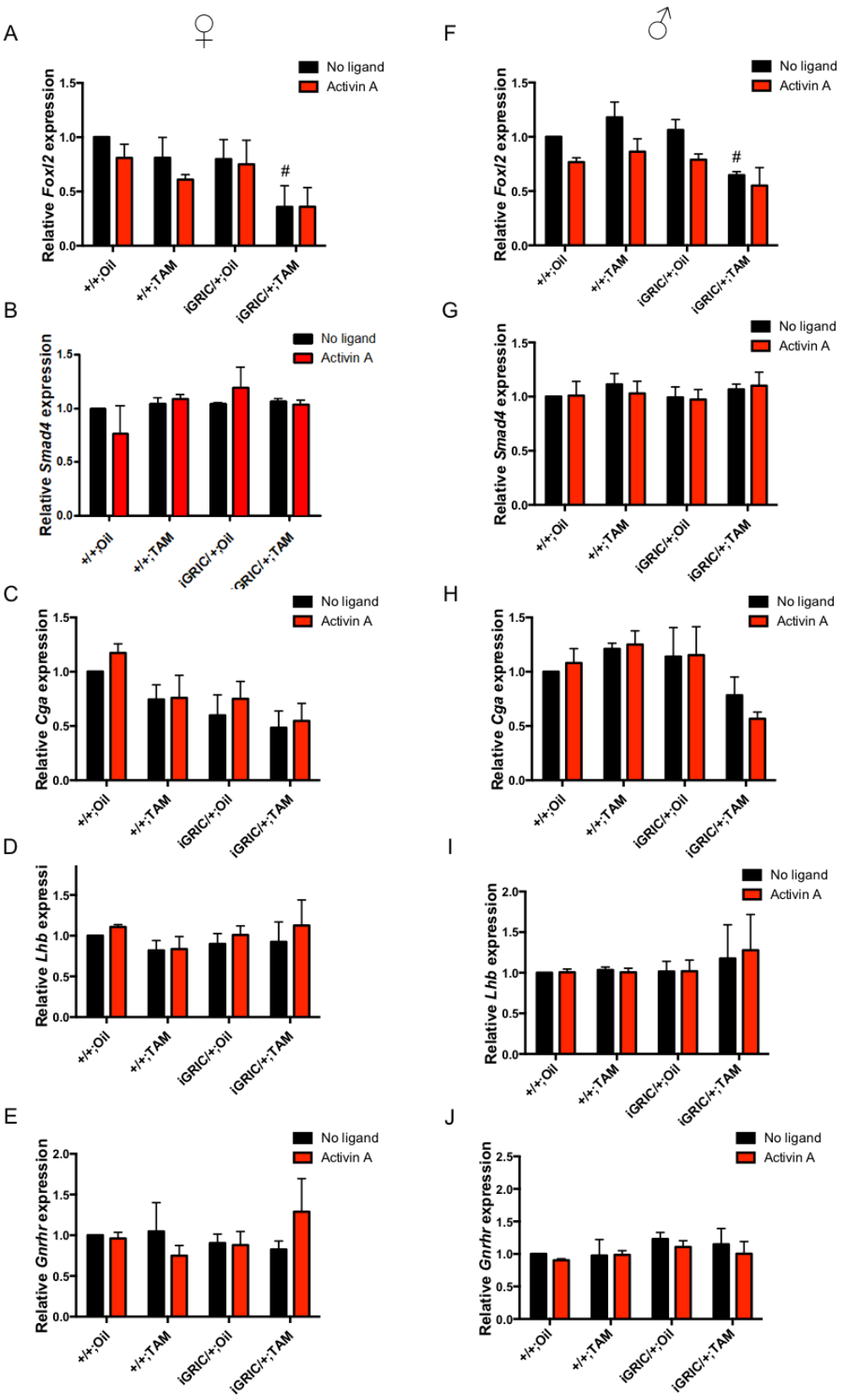
A
♀
F
♂
No ligand
Activin A
Relative Foxl2 expression
Relative Smad4 expression
Relative Cga expression
Relative Lhb expression
Relative Gnrhr expression
+/+;Oil
+/+;TAM
iGRIC/+;Oil
iGRIC/+;TAM
#

Figure S3.7

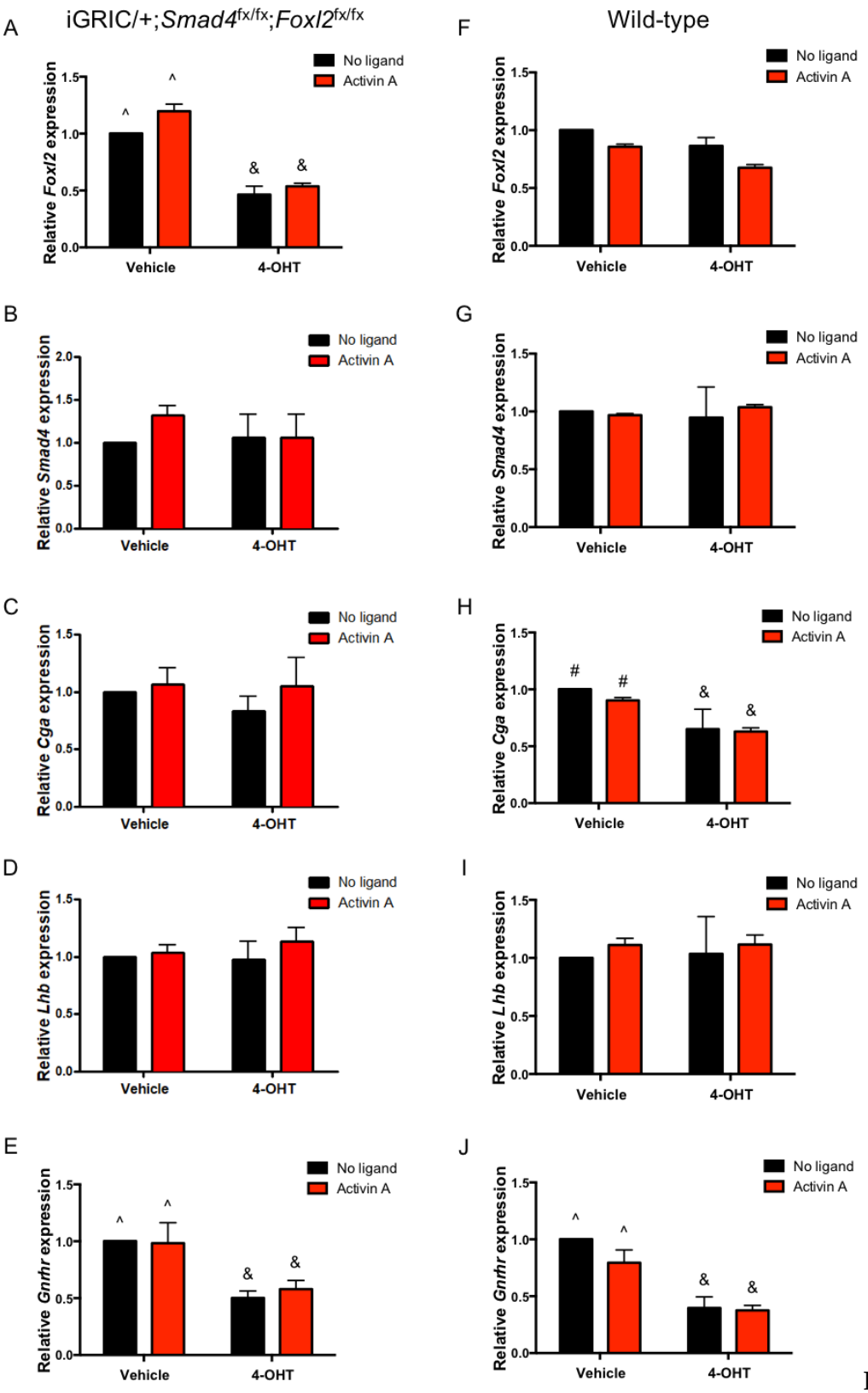
A
iGRIC/+;Smad4fx/fx;Foxl2fx/fx
F
Wild-type
No ligand
Activin A
Relative Foxl2 expression
Relative Smad4 expression
Relative Cga expression
Relative Lhb expression
Relative Gnrhr expression
Vehicle
4-OHT
B
G
C
H
D
I
E
J

Figure S3.8

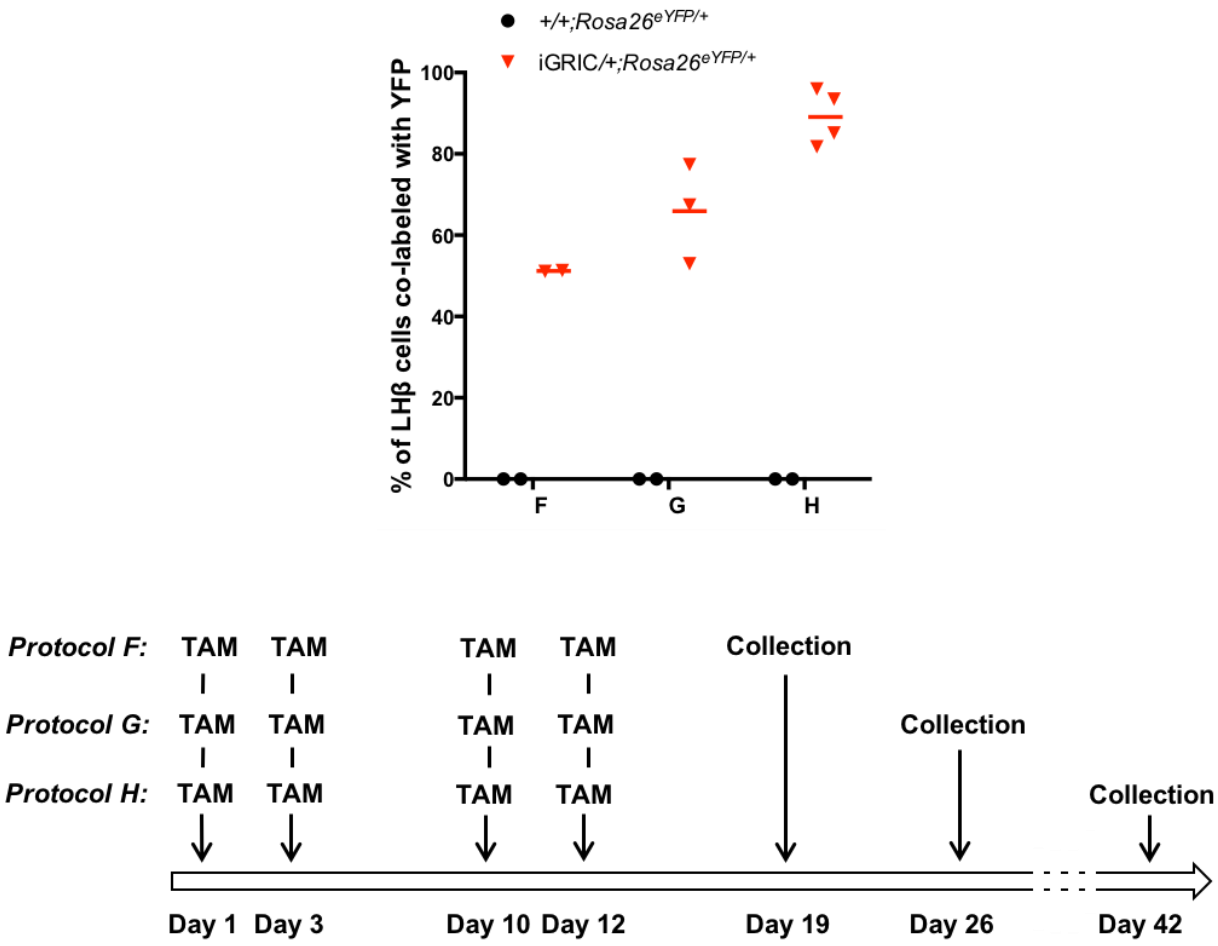
+/+;Rosa26eYFP/+
iGRIC/+;Rosa26eYFP/+
% of LHβ cells co-labeled with YFP
100
80
60
40
20
0
F
G
H
Protocol F:
Protocol G:
Protocol H:
TAM
TAM
TAM
TAM
TAM
TAM
TAM
TAM
TAM
TAM
Collection
Collection
Collection
Day 1
Day 3
Day 10
Day 12
Day 19
Day 26
Day 42

Figure S3.9

Chapter 4

In **Chapters 2 and 3**, I demonstrated the importance of activin-like signaling, via SMAD3, SMAD4, and FOXL2, in the regulation of FSH production *in vivo*. Here, in **Chapter 4**, I tested the current model of inhibin action on FSH synthesis *in vivo*. Previous *in vitro* studies indicated that inhibins act as competitive activin receptor antagonists, particularly in the presence of the co-receptor, TGFBR3 [429, 434]. However, the role of TGFBR3 in inhibin action and in FSH synthesis in gonadotropes has not been determined *in vivo*, in part, because *Tgfbr3* global knockout mice are embryonic lethal [454]. Here, we established the first 'floxed' *Tgfbr3* murine model, allowing us to ablate the gene specifically in gonadotropes *in vivo.*

Enhanced fertility, but not FSH secretion, in female mice with a gonadotrope-specific deletion of the inhibin co-receptor TGFBR3 (betaglycan)

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Abbreviated title: Inhibin antagonize FSH production requires TGFBR3

Keywords: FSH, inhibin, TGFBR3, gonadotropes

Abstract

Follicle-stimulating hormone (FSH) is an essential regulator of ovarian follicle development and fertility in females. FSH synthesis is stimulated by intra-pituitary activins and is suppressed by gonadal inhibins. Activins stimulate transcription of the FSHβ subunit gene (*Fshb*) in pituitary gonadotrope cells. According to *in vitro* observations, inhibins suppress FSH by acting as competitive activin receptor antagonists, particularly in the presence of the co-receptor, TGFBR3 (betaglycan). However, the role of TGFBR3 in inhibin action *in vivo* has not been determined, in part, because *Tgfbr3* knockout mice die during embryonic development. We therefore generated mice harboring 'floxed' alleles for *Tgfbr3*, allowing us to ablate the gene specifically in gonadotropes *in vivo* (hereafter, T3cKO mice). In primary culture, pituitaries from T3cKO mice were relatively insensitive to exogenous inhibin A-mediated antagonism for FSH production. Similarly, exogenous inhibin A suppressed FSH in control but not T3cKO males *in vivo*. T3cKO females were supra-fertile, ovulating more eggs per cycle, exhibiting more implantation sites in the uterus, and producing approximately two more pups per litter than controls. Additionally, T3cKO ovaries contained a greater number of large antral follicles and corpora lutea than controls. Though these phenotypes would be predicted to result from increases in FSH, we did not detect differences in the synthesis or secretion of the hormone between control and T3cKO animals of either sex. The mechanism of enhanced fertility in T3cKO females is presently unresolved. Collectively, the results indicate that TGFBR3's role in pituitary gonadotropes may be more complex than originally conceived.

Introduction

Follicle-stimulating hormone (FSH), a glycoprotein produced by pituitary gonadotrope cells, plays essential roles in mammalian reproduction [221]. In females, FSH stimulates ovarian granulosa cell proliferation and estrogen biosynthesis [732, 733]. In males, FSH stimulates testicular Sertoli cell proliferation early in life, regulating spermatogenesis in adulthood [221, 245, 246, 652, 697]. FSH receptor (FSHR)-deficient female mice exhibit arrested ovarian follicle development and infertility; in males, sperm production is greatly reduced [221, 409, 712, 734]. Isolated FSH deficiency in humans is rare but uniformly causes hypogonadism and infertility [650, 653, 654, 660, 698-703].

FSH is a dimeric glycoprotein composed of an α subunit (CGA) shared with the other glycoprotein hormones and a hormone-specific β subunit (FSHB) [162]. The rate-limiting step in FSH synthesis is the transcription of its β subunit, which is regulated by multiple factors within the hypothalamic-pituitary-gonadal (HPG) axis [161, 257, 436, 666, 735]. Both gonadotropin-releasing hormone (GnRH) from the hypothalamus and pituitary-derived activins (or related TGFβ superfamily ligands) stimulate FSH production [293, 409, 712]. In contrast, gonadally-derived inhibins selectively suppress FSH synthesis and secretion [432, 736, 737].

Inhibins are heterodimeric TGFβ superfamily ligands composed of the inhibin α subunit disulfide-linked to one of two inhibin β subunits (inhibin βA or inhibin βB) to form inhibin A or inhibin B, respectively. The structurally-related activins are homo- or hetero-dimers of inhibin β subunits: activin A (βA-βA), activin B (βB-βB), or activin AB (βA-βB). Like other TGFβ ligands, activins signal via complexes of type I and II serine/threonine kinase receptors [372]. Inhibins, in contrast, do not appear to signal, but rather antagonize activin signaling by competitively binding to their type II receptors [431, 432]. Because inhibins bind to these receptors with at least 10-fold

lower affinity than activins [433-435], effective antagonism should require inhibins to be in large excess relative to activins. However, inhibins robustly antagonize activin action when present at equimolar or even lower concentrations [436-438]. It was subsequently discovered that the transforming growth factor β (TGFβ) type III receptor (TGFBR3, also known as betaglycan) markedly increases the affinity of inhibins for activin type II receptors *in vitro* [434, 439]. In fact, in the presence of TGFBR3, inhibins block activin binding to type II receptors [429, 434, 447, 465], providing a candidate mechanism for inhibins to potently suppress FSH production by pituitary gonadotrope cells.

Nonetheless, it is presently unclear whether inhibins act via TGFBR3 to suppress FSH *in vivo*. *Tgfbr3* knockout mice die during embryonic development because of heart and lung defects, precluding their use for studies of inhibin action [454]. To circumvent this problem, we developed the first conditional (floxed) *Tgfbr3* mouse model, enabling us to delete the protein selectively in gonadotropes using the Cre/lox system. The resulting animals are viable and fertile, and females produce larger litters than controls. Though this phenotype and others are consistent with enhanced FSH levels, we failed to detect alterations in FSH synthesis or secretion in conditional knockout animals. Inhibin A antagonism of FSH production and secretion in pituitaries of these animals is, however, impaired. These data indicate that TGFBR3's role in FSH regulation *in vivo* may be more complex than predicted.

Results

Recombination of floxed *Tgfbr3* creates a loss of function allele

Previously, homozygous deletion of *Tgfbr3* exon 2 created a loss of gene function and embryonic lethality, due to heart and liver defects [454]. Here, we floxed exon 2 of *Tgfbr3* and

predicted that Cre-mediated recombination would similarly produce a loss of gene function. *Tgfbr3*$^{fx/fx}$ mice were crossed with *EIIa*-Cre deleter mice to recombine the allele in early development [738, 739]. Resulting *Tgfbr3*$^{+/\Delta ex2}$ males and females (Fig. 4.1B) were then intercrossed to generate wild-type (*Tgfbr3*$^{+/+}$), heterozygous (*Tgfbr3*$^{+/\Delta ex2}$), and homozygous progeny (*Tgfbr3* $^{\Delta ex2/\Delta ex2}$). Genotypes were determined by PCR of genomic DNA (Fig. 4.1C). Among the 134 live-born pups analyzed, 56 (41.8%) were wild-type and 78 (58.2%) were heterozygotes. We did not identify any live-born mice with the homozygous genotype (*Tgfbr3*$^{\Delta ex2/\Delta ex2}$). We next repeated the heterozygous crosses and isolated the embryos from pregnant females at 13.5, 15.5, 16.5, or 17.5 days post-coitus (dpc; Fig. 4.1D). Beginning as early as 13.5 dpc, homozygotes exhibited apparent hematopoietic defects relative to wild-type and heterozygous (not pictured) littermates, as reflected by their paler appearance, particularly in the liver. Resorption of dead homozygous mice was frequently observed by 17.5 dpc. These data indicate that recombination of the floxed *Tgfbr3* allele causes a loss of gene function.

Generation of gonadotrope-specific *Tgfbr3* knockout mice

To study TGFBR3 function in canonical inhibin target cells, we used a GnRH receptor-dependent Cre-driver (GRIC) [648] to ablate the protein specifically in pituitary gonadotropes. *Gnrhr*$^{GRIC/+}$;*Tgfbr3*$^{fx/fx}$ females were crossed with *Tgfbr3*$^{fx/fx}$ males to generate control (*Tgfbr3*$^{fx/fx}$) and conditional knockout mice (*Gnrhr*$^{GRIC/+}$;*Tgfbr3*$^{fx/fx}$; hereafter T3cKO). To confirm the specificity of gene recombination, we collected various tissues for analysis by PCR. Recombination was detected in pituitaries of T3cKO mice of both sexes, and in the testes and epididymides of males (data not shown), consistent with previous reports of Cre activity in gonadotropes and in the male germline of GRIC mice [649]. Recombination was not observed in

brain, heart, liver, kidney, adrenal, pancreas, lung, stomach, intestine, spleen, ovary, or uterus (data not shown). To determine the efficiency of recombination in gonadotropes, we performed double-label immunofluorescence for TGFBR3 and FSHβ (Fig. 4.2). TGFBR3 immunoreactivity was detected at the membrane of all FSHβ-immunoreactive cells as well as in some FSHβ-negative cells of control pituitaries. In contrast, TGFBR3 staining was lost in FSHβ-positive cells (examples labeled by asterisks in Fig. 4.2, right panels) but not in the other pituitary cell lineages of T3cKO mice (arrowheads in Fig. 4.2, right panels). These data demonstrate that TGFBR3 was selectively and effectively depleted in gonadotropes of T3cKO mice.

Impaired inhibin A antagonism of FSH synthesis and release in the absence of TGFBR3 *in vivo* and *in vitro*

To assess whether TGFBR3 is required for inhibin action in gonadotropes, we first performed two *in vitro* culture experiments. In the first, we acutely ablated *Tgfbr3* gene function in primary pituitary cultures from *Tgfbr3*$^{fx/fx}$ mice with a Cre-expressing adenovirus (ad-Cre). *Tgfbr3* mRNA expression was profoundly reduced in cells transduced with ad-Cre relative to a control adenovirus expressing GFP (ad-Cre) (Fig. 4.3A). In the second experiment, we cultured pituitaries from control and T3cKO mice. Here, the reduction in *Tgfbr3* mRNA expression reflected the deletion specific to gonadotrope cells (Fig. 4.3C), whereas in the first experiment, the *Tgfbr3* allele was recombined in all cells. Inhibin A potently and dose-dependently inhibited *Fshb* mRNA expression and FSH secretion in cultures from *Tgfbr3*$^{fx/fx}$ mice transduced with ad-GFP (Fig. 4.3B and S4.1A) and from control mice (Fig. 4.3D and S4.1B). In contrast, inhibin A action was abrogated in *Tgfbr3*-deficient cells in both experiments (Fig. 4.3B,D and S4.1). Notably, we

consistently observed a reduction in basal *Fshb* expression in *Tgfbr3*-deficient cells. The mechanism underlying this decrease is not yet clear (though see *Discussion*).

Next, we assessed inhibin action *in vivo* by injecting inhibin A or vehicle into the tail vein of 10-week-old control or T3cKO mice. Males were used because their FSH levels are relatively stable and higher than those of females. We measured serum FSH before and 6 h post-injection in all animals. FSH levels were significantly reduced in control but not in T3cKO males post-inhibin A injection. FSH levels were equally unaffected by vehicle treatment in the two genotypes (Fig. 4.3E). Thus, both *in vitro* and *in vivo*, depletion of *Tgfbr3* renders gonadotropes relatively insensitive to exogenous inhibin A. We have not yet assessed the effects of inhibin B *in vitro* or *in vivo*.

Enhanced folliculogenesis and fertility in female T3cKO mice

We next assessed fertility in T3cKO mice. We focused on females given that male mice do not require FSH for fertility [221]. Puberty onset (assessed by vaginal opening) and estrous cyclicity were comparable in control and T3cKO females (Fig. S4.2A-C). Reproductive organ weights were also similar between genotypes in adults of both sexes (Fig. S4.2D-F). Over the course of a 6-month breeding trial, T3cKO females reproduced on average two more pups per litter compared with their control littermates (9.6±0.5 vs. 7.5±0.5) (Fig. 4.4A). This enhanced fertility appeared to derive from increased folliculogenesis, as T3cKO ovaries contained more large antral follicles (11.4±1.8 vs. 6.0±0.8) and corpora lutea (18.1±1.6 vs. 13.2±1.7) than controls (Fig. 4.4B-D). Moreover, T3cKO females ovulated more eggs in natural cycles (9.8±0.7 vs. 6.8±0.9; Fig. 4.4E) and exhibited a greater number of embryo implantation sites in their uteri compared to controls (8.5±0.5 vs. 6.8±0.5; Fig. 4.4F).

FSH synthesis and secretion appear normal in intact and gonadectomized T3cKO mice

Enhanced folliculogenesis in T3cKO females would be predicted to derive from enhanced FSH secretion, which in turn would be expected in the context of impaired inhibin actions in gonadotrope cells. Surprisingly, however, serum FSH levels were indistinguishable between littermate control and T3cKO females assessed at different estrous cycle stages (Fig. 4.5A-B). Serum FSH levels were also equivalent between genotypes in adult males (Fig. 5C). In the pituitary, FSH content (Fig. 4.5D-E) and *Fshb* (Fig. 4.5F-G), LHβ subunit (*Lhb*), and common α subunit (*Cga*) mRNA levels were equivalent between genotypes in both sexes (data not shown). In contrast, *Gnrhr* mRNA expression was significantly increased in pituitaries of both male and female T3cKO mice (Fig. 4.5F-G).

We next examined changes in FSH secretion and synthesis following gonadectomy. In females, FSH levels were significantly increased 7 h post-ovariectomy, and were stably and maximally elevated by one week (Fig. 4.6A). Responses were equivalent between genotypes (Fig. 4.6A). In males, serum FSH levels increased 1.92-fold one month post-castration in control males (Fig. 4.6B). In T3cKO males, the fold post-castration increase in serum FSH was relatively blunted (1.38-fold) (Fig. 4.6B). Importantly, however, post-castration FSH levels were equivalent between genotypes, whereas FSH was somewhat higher in intact T3cKO relative to control mice in this experiment. This elevation was not observed consistently across cohorts of animals (e.g., Fig. 4.5C). Pituitary *Fshb* mRNA levels were significantly increased in control, but not T3cKO males post-castration (Fig. 4.6C). However, *Fshb* mRNA levels were statistically equivalent between the two genotypes after 2 weeks of ovariectomy (Fig. 4.6D) or castration (Fig. 4.6C). Additionally, serum LH levels was significantly increased in control, but not in T3cKO males, 1 month post-

castration when compare to the sham groups (Fig. S4.3A). Whereas, serum LH levels were not significantly different between the control and T3cKO female mice post-ovariectomy (Fig. S4.3B).

GnRH action is equivalent in control and T3cKO males

Gnrhr mRNA levels were elevated in pituitaries of T3cKO mice relative to controls (Fig. 4.5F-G). This raised the possibility that, perhaps in response to changes in the activin/inhibin system in the pituitary, T3cKO mice became more dependent upon GnRH for FSH regulation. To examine this idea, we first treated control and T3cKO males with vehicle or 1, 10, or 100 ng of GnRH (s.c.) and measured LH and FSH secretion before and 15, 30, or 60 min post-injection. GnRH rapidly and dose-dependently stimulated LH secretion and the responses were equivalent in the two genotypes (Fig. 4.7A-B). As has been reported previously [740-743], GnRH only weakly stimulated FSH secretion (Fig. 4.7C-D) [743, 744]. Again, there was no clear difference between genotypes.

We next examined the role of endogenous GnRH action in regulating FSH secretion. In a first experiment, we injected (s.c.) male control and T3cKO males with the GnRH antagonist, Antide, or with vehicle. Serum LH levels were significantly and equivalently reduced in both genotypes 24 h post-Antide treatment (Fig. 4.8A), confirming that GnRH action was completely blocked (Fig. 4.8A). FSH levels were also reduced in response to Antide and the effect appeared to be more robust in T3cKO than control males (Fig. 4.8B). At the pituitary level, *Fshb*, but not *Lhb* expression was inhibited by Antide (Fig. 4.8C-D). Here, the enhanced response to Antide in T3cKO mice was less robust than seen with secreted hormones. In a follow-up experiment, we treated control and T3cKO males with a second GnRH receptor antagonist (cetrorelix) and measured the effects on LH and FSH secretion 1, 3, 5, 7, 9, and 24 h post-injection (s.c.). LH levels

were completely suppressed within 1 h in both genotypes and only began to recover slightly by 24 h (Fig. 4.8E). In contrast, reductions in serum FSH showed slower kinetics, with values dropping gradually between 1 and 24 h (Fig. 4.8F). In this experiment, basal FSH levels (before injection) were lower in T3cKO than in control mice. Nonetheless, FSH secretion decreased at similar rates in the two genotypes. Collectively, the results of GnRH agonist and antagonist experiments suggest that GnRH action was unaltered in T3cKO mice, despite the upregulation of *Gnrhr* mRNA levels.

Control and T3cKO females respond similarly to exogenous gonadotropins

As there were no apparent differences in FSH synthesis and secretion, we next asked whether ovaries of T3cKO were somehow be more sensitive to gonadotropin stimulation than those of controls. We therefore treated juvenile females with PMSG (eCG) and hCG, and then counted ovulated cumulus-oocyte complexes in the oviduct. The responses of control and T3cKO mice were comparable (Fig. 4.9).

Discussion

Here, we developed the first 'floxed' *Tgfbr3* murine model, allowing us to conditionally ablate the *Tgfbr3* gene in gonadotropes. *A priori*, this manipulation was predicted to reduce gonadotrope sensitivity to inhibins, leading to increases in FSH secretion, folliculogenesis, and fertility. Though antral follicle development and litter sizes were, in fact, augmented, we failed to detect changes in FSH synthesis or secretion in either female or male conditional knockout mice *in vivo*. At the same time, the ovaries of conditional knockout females did not appear to be more sensitive to gonadotropins. Therefore, the mechanisms underlying enhanced fertility in this model

remain undefined, but likely involve changes at the pituitary level given that the genetic manipulation was specific to this tissue (and to gonadotropes in particular).

Although we did not observe changes in FSH *in vivo*, deletion of *Tgfbr3* in gonadotropes produced the predicted reduction in sensitivity to exogenous inhibin A both *in vitro* and *in vivo*. In cultured pituitary cells, Cre-mediated recombination of the floxed *Tgfbr3* gene completely blocked inhibin A suppression of *Fshb* mRNA expression. This was true when gene recombination occurred acutely (with an adenovirus encoding Cre) or chronically (with the GRIC Cre-driver *in vivo*). Moreover, FSH was suppressed in control but not T3cKO males treated with inhibin A *in vivo*. A limitation of the latter experiment, however, is that we only treated animals with a single dose of inhibin A and only sampled at one time point post-injection. Therefore, it is possible that inhibin sensitivity would be observed in knockout mice under different experimental conditions. It should also be noted that we did not assess the effects of inhibin B because the recombinant ligand was not available to us. There is some suggestion that inhibin B may act differently than inhibin A [737, 745]; it will therefore be critical to test inhibin B sensitivity in our model.

Two results of the pituitary culture experiments raise important, but currently unresolved questions. First, we observed a ~50% reduction in basal *Fshb* expression in knockout cultures. This was true whether recombination occurred acutely in culture or chronically *in vivo*. In these cultures, basal *Fshb* expression depends entirely upon the actions of a TGFβ ligand that signals through one (or more) of three type I receptors (ACVR1B, ACVR1C, or TGFBR1) and is antagonized by follistatins or inhibins [386, 487]. Though this ligand has long been assumed to be an activin, and most likely activin B, our and other results challenge this idea. First, the drop in basal *Fshb* expression suggests that the endogenous ligand may use TGFBR3 as a co-receptor. Activins do not bind TGFBR3 [434, 449]. Second, mice lacking the *Inhbb* subunit, which cannot

make activin B, have elevated (not reduced) FSH levels [746]. Together, these data suggest that a TGFβ ligand other than activin B may be the main driver of *Fshb* expression, at least in murine primary cultures. This ligand has yet to be identified, but is unlikely to be TGFβ1-3, as gonadotropes do not express the TGFβ type II receptor [710, 747] and these ligands are not inhibited by follistatins [466, 748].

A second question raised by our results is how FSH levels appear to be normal *in vivo*, when synthesis of the hormone is clearly impaired *in vitro* in conditional knockouts. It is possible that the loss of inhibin antagonism is offset by the impaired stimulation by the to-be-identified TGFβ ligand (see above). However, this seems unlikely given that control and conditional knockout mice responded equivalently to gonadectomy. In the absence of inhibin action, control mice should have shown higher FSH levels than the conditional knockouts if the TGFβ ligand used TGFBR3 as a co-receptor.

Another possible explanation for the difference between *in vitro* and *in vivo* results concerns GnRH, which is present in the latter but not former case. As a result, changes in GnRH actions might compensate for alterations in TGFβ superfamily signaling caused by TGFBR3 ablation. Consistent with this idea, we observed an upregulation of *Gnrhr* mRNA levels in pituitaries of both female and male conditional knockouts relative to controls. The best-known drivers of *Gnrhr* expression in the mouse are GnRH and estrogens [294, 296, 297, 749-753]. In females, the difference between genotypes was greatly reduced following ovariectomy (Fig. S4.3C), consistent with a role for estrogens in mediating the increase in receptor levels. In contrast, in males, enhanced *Gnrhr* expression in conditional knockouts was maintained following castration (Fig. S4.3D). The basis for this sex difference is presently unclear, but the enhanced *Gnrhr* mRNA levels in males does not appear to be explained by changes in GnRH signaling. The

GnRH receptor antagonist, Antide, reduced *Gnrhr* expression in both control and conditional knockout males, but did not abolish the genotype difference (Fig. S4.4). Finally, the increase in *Gnrhr* mRNA levels in conditional knockouts did not appear to be cell autonomous, as the genotype difference was lost in cultured pituitary cells (data not shown).

Regardless of the mechanism, it is unclear that increases in *Gnrhr* mRNA levels translate into alterations in GnRH signaling, as GnRH receptor agonists or antagonists produced similar effects on LH and FSH secretion in control and conditional knockout mice. There were also no apparent differences in pulsatile LH release (in response to endogenous GnRH release) in control and conditional knockout males (Fig. S4.5). Therefore, if there are changes in GnRH signaling that impact FSH (or LH) secretion, we have been unable to detect them. That said, there are two other variables known to affect FSH action that we have not yet examined: pulsatile release and post-translational modifications.

In general, given its relatively long half-life and predominant release via the constitutive secretory pathway, FSH is not generally considered a pulsatile hormone. However, folliculogenesis may be enhanced in murine ovaries in response to a more pulsatile FSH stimulus or at least to FSH released predominantly via the regulated secretory pathway [212]. At present, we lack an assay with sufficient sensitivity to measure FSH in serial blood samples (e.g., every 5-10 min). Therefore, we have been unable to assess pulsatile FSH release directly. Nevertheless, measurements every hour or two on estrous morning revealed relatively constant FSH levels. Finally, FSH is a glycoprotein and post-translation modifications of the protein are regulated across the reproductive cycle in women [159, 190, 196, 197]. According to recent data, human hypoglycosylated FSH is more active than hyperglycosylated FSH in ovarian granulosa cells [190]. Unfortunately, given the small size of mice, we are unable to directly measure FSH glycoforms

and activity in control and conditional knockout mice. However, different glycoforms have different serum-half lives in humans [169, 182-184, 754]. Here, FSH elimination rates were comparable following GnRH antagonist treatment in male control and conditional knockout mice. Therefore, any changes in FSH glycoforms are not obvious, at least not in males.

In summary, we ablated the putative inhibin co-receptor, TGFBR3, in pituitary gonadotropes and observed the expected increases in folliculogenesis and fertility in female mice. Unexpectedly, these phenotypes were not associated with the anticipated increases in FSH synthesis or secretion *in vivo*. It is conceivable that there are changes in the nature of FSH release (e.g., more pulsatile) or of the ligand itself (e.g., hypoglycosylation) that explain the ovarian and reproductive phenotypes, but we presently lack supporting evidence for either possibility. It also feasible that changes in FSH production or release occur transiently at a specific time of the estrous cycle that we did not sample in our studies. However, the absence of altered FSH in males argues against this possibility. Importantly, however, male mice make inhibin B, but not inhibin A. Therefore, we must be open to the possibility that ablation of TGFBR3 impairs inhibin A but not inhibin B actions in the pituitary. We are currently producing inhibin B in our lab to test this possibility. Finally, the novel floxed strain described here will allow us and others to interrogate TGFBR3's role in a variety of cell types and physiological contexts.

Materials and Methods

Generation of floxed *Tgfbr3* mice

Mice harboring a floxed *Tgfbr3* allele were produced by conventional gene targeting in murine embryonic stem (ES) cells using standard techniques. Briefly, a loxP site was introduced ~450 bp upstream of exon 2 (in the first intron) and a floxed neomycin (Neo) positive selection

cassette was introduced ~490 bp downstream of exon 2 (in intron 2; see Fig. 4.1A). The targeting construct was electroporated into J1 ES cells, and correctly targeted clones were injected into C57BL6 blastocysts. Resulting chimeric males where crossed to C57BL6 females and agouti pups were genotyped. Mice heterozygous for the modified allele (*Tgfbr3*$^{fx\text{-}Neo/+}$) were crossed with the *EIIa*-Cre deleter strain to excise the floxed Neo cassette. We identified mice in which Neo was removed, but the floxed exon 2 remained intact. These mice (*Tgfbr3*$^{tm1.1Hlin}$; hereafter *Tgfbr3*^{fx}) were then crossed with wild-type C57BL6 mice to segregate the floxed *Tgfbr3* and Cre alleles. *Tgfbr3*$^{fx/+}$ males and females were intercrossed to generate *Tgfbr3*$^{fx/fx}$ homozygotes. The latter are overtly normal and fertile, suggesting that introduction of the loxP sites does not affect gene function.

Global *Tgfbr3* knockout mice

To generate mice with a global deletion of *Tgfbr3* exon 2, *Tgfbr3*$^{fx/fx}$ animals were crossed with *EIIa*-Cre mice (FVB/N-Tg(EIIa-Cre) C5379Lmgd/J, The Jackson Laboratory, 003724) to obtain heterozygous (*Tgfbr3*$^{+/\Delta ex2}$;*EIIa*-Cre/+) progeny. *Tgfbr3*$^{+/\Delta ex2}$;*EIIa*-Cre/+ mice were then crossed with wild-type C57BL6 mice to obtain *Tgfbr3*$^{+/\Delta ex2}$ offspring without the *EIIa*-Cre transgene. Finally, *Tgfbr3*$^{+/\Delta ex2}$ males and females were crossed to generate the following three genotypes: *Tgfbr3*$^{+/+}$, *Tgfbr3*$^{+/\Delta ex2}$ and *Tgfbr3*$^{\Delta ex2/\Delta ex2}$. Genotyping was performed on genomic DNA using the primers in Table 1.

Conditional knockout mice

Gonadotrope-specific *Tgfbr3* knockout mice were generated using *Tgfbr3*$^{fx/fx}$ and *Gnrhr*$^{GRIC/GRIC}$ [648] mice. The *Gnrhr*^{GRIC} allele was always introduced from the female because

of Cre activity in the male germline in this strain [649]. First, *Tgfbr3*$^{fx/fx}$ males were crossed with *Gnrhr*$^{GRIC/GRIC}$ females, generating *Tgfbr3*$^{fx/+}$;*Gnrhr*$^{GRIC/+}$ pups. Because the *Tgfbr3* and *Gnrhr* genes are linked on chromosome 5, the *Tgfbr3*^{fx} and *Gnrhr*^{GRIC} alleles were in *trans* (on sister chromatids) in these mice. To generate conditional knockout mice (*Tgfbr3*$^{fx/fx}$;*Gnrhr*$^{GRIC/+}$) mice, the *Tgfbr3*^{fx} and *Gnrhr*^{GRIC} alleles first had to be positioned in *cis* (on the same chromatid). This was accomplished via meiotic recombination. *Tgfbr3*$^{fx/+}$;*Gnrhr*$^{GRIC/+}$ (*trans*) females were crossed to wild-type (*Tgfbr3*$^{+/+}$;*Gnrhr*$^{+/+}$) males. In offspring that inherited both the *Tgfbr3*^{fx} and *Gnrhr*^{GRIC} alleles (*Tgfbr3*$^{flox/+}$;*Gnrhr*$^{GRIC/+}$), *Tgfbr3*^{fx} and *Gnrhr*^{GRIC} were by definition in *cis*. We observed co-segregation of the modified or wild-type alleles in 7 out of 92 genotyped pups, indicating that the *Tgfbr3* and *Gnrhr* genes are separated by approximately 7.6 cM (close to the anticipated 8.34 cM). Subsequently, *Tgfbr3*$^{fx/+}$;*Gnrhr*$^{GRIC/+}$ (*cis*) females were crossed with *Tgfbr3*$^{fx/fx}$;*Gnrhr*$^{+/+}$ males to generate *Tgfbr3*$^{fx/fx}$;*Gnrhr*$^{GRIC/+}$ mice. *Tgfbr3*$^{fx/fx}$;*Gnrhr*$^{GRIC/+}$ females were then bred to *Tgfbr3*$^{fx/fx}$*;Gnrhr*$^{+/+}$ males to produce *Tgfbr3*$^{fx/fx}$*;Gnrhr*$^{+/+}$ (control) and *Tgfbr3*$^{fx/fx}$*;Gnrhr*$^{GRIC/+}$ (experimental or T3cKO) littermates in a 1:1 ratio. Genotypes were determined by PCR using primers listed in Table 4.1. All animal work was performed in accordance with institutional and federal guidelines and approved by the McGill University and Goodman Cancer Centre Facility Animal Care Committee (protocol 5204).

Southern blotting

Ten micrograms of tail genomic DNA was digested with *Eco*RI (Promega, R6018) and separated on an 0.8% agarose gel. DNA was denatured with an alkaline solution (pH 12.0) containing 1.5 M NaCl (Bioshop, SOD001) and 0.5 M NaOH (Bioshop, SHY700) at room temperature for 45 min, and then neutralized with 1.5 M NaCl in 1 M Tris (Bioshop, TRS001)

buffer (pH7.4) for 30 min to prevent re-hybridization. DNA was transferred onto a positively charged nylon membrane (Amersham, RN303B) for 18 hr by capillary action with 20X concentrated saline sodium citrate (SSC, Bioshop, SSC795) buffer. DNA was cross-linked to the membrane with ultraviolet light (Stratagene UV Stratalinker 800). A double-stranded probe was generated by PCR using primers GGGGCCCTTCTGTAGAAATC and CTGCATGCCTTTTCCTCATT and DNA from a wildtype *Tgbfr3* allele as template. The probe was denatured at 95°C for 2 min and immediately cooled on ice. Ten ng of probe was radioactively-labeled using the Prim-a-Gene® Labeling system (Promega, U115A and M220A) and [α^{32}P]dATP (PerkinElmer, BLU012H250UC), [α^{32}P]dCTP (PerkinElmer, NEG013100UC), dGTP, and dTTP (Promega, U1330) on a 37°C hot plate for 1 h. The membrane was pre-hybridized in 50% formamide (Fluka, 47670), 1 M NaCl, 1% SDS (Bioshop, SDS001), 10% dextran sulfate (EMD Millipore, S4030) and 2 mg ssDNA (Promega, M3011) at 65°C for 1 h. Hybridizaton was performed overnight in the same buffer with 50 ng probe at 42°C overnight. The membrane was washed with 1X SSC buffer plus 0.1% SDS at room temperature. The bands were visualized on X-ray film by autoradiography (3.5-day exposure at -80°C with intensifying screen). All radioactive work was performed under McGill Internal Radioisotope Permit (R-00314).

Immunofluorescence

Paraformaldehyde (PFA, Sigma, 158127)-fixed and paraffin-embedded pituitary sections were prepared and immunofluorescence was performed as previously described [409]. Primary antibodies used were: anti-FSHβ (NIDDK AFP7798-1289, 1:500, raised in rabbit) and anti-TGFBR3 (R&D, AF-242-PB, 5 μg/ml, raised in goat). Secondary antibodies used were: Alexa fluor 594-conjugated anti-rabbit (Invitrogen, A21-207, 1:500 raised in donkey), biotinylated anti-

goat (Vector, BA-9500, 1:150, raised in horse), and Alexa fluor 488-conjugated Streptavidin (Invitrogen, S-11223, 1:500). Images were acquired on a Zeiss LSM 510 confocal microscope.

Primary pituitary cultures

Primary pituitary cultures were prepared as previously described [408, 409]. In the *in vitro* recombination experiments, pituitary cells from an equal number of male and female *Tgfbr3*$^{fx/fx}$ mice were seeded at a density of 400,000 cells/well in 48-well plates. Cells were cultured for 24 h after plating and then infected with adenoviruses expressing GFP (Ad-GFP) or Cre-IRES-GFP (Ad-Cre) [Baylor College of Medicine Vector Development Laboratory (Houston, Texas)] at a multiplicity of infection (MOI) of 60 in 10% FBS (Life Technologies, 10438026)-containing media for 24 h. In a second set of experiments, pituitaries were collected from control and T3cKO females and seeded at the same density as described above in culture medium supplement with 10% FBS. In all culture experiments, the cells were further cultured for 24 h in medium with 2% FBS containing recombinant inhibin A (generously provided by Dr. Teresa Woodruff, Northwestern University, Chicago, IL) as indicated in Fig. 4.3A-D and Fig. S4.1. Cells were harvested with 0.25% trypsin and RNA was extracted using the Total RNA Mini Kit (Geneaid, FA32808-PS) following the manufacturer's instructions. Culture medium was collected for measurement of secreted FSH (see below).

Inhibin A preparation and injection

Recombinant inhibin A was prepared as described in [745] and was kept at -80°C before use. Inhibin A was diluted in 50 mM sodium acetate (pH 5.0) supplemented with 0.1% BSA to make a working dilution of 1 µg/100 µl. Ten-week old control and T3cKO males were i.v. (tail

vein) injected with 50 µg/kg body weight of inhibin A or equivalent volume of vehicle. Before injection, a small blood sample (~50 µl) was collected from each mouse via submandibular venipuncture. Six hours after inhibin A injection, terminal blood samples were collected via cardiac puncture. All blood samples were allowed to clot at room temperature and serum was collected following centrifugation. Serum FSH levels were compared before after inhibin A administration in the same animals.

Hormone analyses

Blood samples were collected through submandibular, tail vein, or cardiac puncture as specified in each experiment. Serum was obtained as described previously [712]. Serum FSH and LH levels were assessed by multiplex ELISA (performed at the Ligand Assay and Analysis Core of the Center for Research in Reproduction at the University of Virginia), Luminex assay (FSH only, Millipore, MPTMAG-49K) following manufacturer's instructions, or an in-house sandwich LH-ELISA as described previously [712]. The methods used are specified in the figure legends. Pituitary homogenates were prepared as described previously [409] from males and females (on estrous morning, ~7 am). Pituitary FSH content and secreted FSH levels in culture medium were measured by radioimmunoassay (RIA) at the Ligand Assay and Analysis Core at UVa.

Reproductive organ analyses

Reproductive organs were collected from 10-week-old males and females (metestrus/diestrus afternoon) and weighed on a precision balance. Reproductive organ weight was normalized to the body weight assessed after euthanasia.

Gonadectomy and blood sample collection

Gonadectomy was performed on 10-week-old control and T3cKO males and females (at 11 am on proestrus). The surgeries were performed following McGill Standard Operating Procedures (SOPs) 206 and 207. A small blood sample (~30 µl) was collected before the operation for both males and females by submandibular venipuncture. Similar blood samples were collected 7 h or 1 week after ovariectomy. One month after castration or 2 weeks after ovariectomy, terminal blood samples (by cardiac puncture) and pituitary glands were collected.

GnRH agonist and antagonist injection

GnRH (Sigma, L8008) was prepared in saline at a concentration as indicated in Fig. 4.7. Blood samples were collected through the tail vein before and after GnRH injections (s.c.). Antide (Sigma, A8802) was dissolved in sterile PBS containing 20% propylene glycol (Hemochem, P5900U). Three mg/kg body weight of Antide or equivalent volume of vehicle was injected (s.c.) in control and T3cKO males. Twenty-four hours after injection, blood samples were collected via cardiac puncture. Cetrorelix acetate (Sigma, C5249) was dissolved in sterile water. Five-hundreds µg/kg body weight of Cetrorelix acetate or equivalent amount of vehicle were s.c. injected in control and T3cKO males.

RT-qPCR analysis

Pituitaries were isolated from 10-week-old control and T3cKO mice, immediately frozen in liquid nitrogen, and stored at -80°C until analysis. Females were euthanized at the estrous cycle stages (and times) indicated in the figures. All pituitaries were homogenized in 500 µl TRIzol reagent (Life Technologies, 15596026), and total RNA was extracted following manufacturer's

instructions. RNA concentration was determined by NanoDrop. Two-hundred ng of RNA per sample were reverse transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Promega, 172807) and random hexamer primers (Promega, 184865) in a final volume of 40 µl. Two µl of cDNA were then used as template for qPCR analysis on a Corbett Rotorgene 600 instrument (Corbett Life Science) using EvaGreen reagent (Diamed, ABMMastermix-S) and primers listed in Table 4.1. Relative gene expression was normalized to the housekeeping gene, ribosomal protein L19 (*Rpl19*). All oligos were synthesized by Integrated DNA Technologies (IDT, Coralville, IA).

Puberty onset, estrous cyclicity, and fertility assessment

Starting at 7 weeks of age, estrous cyclicity was assessed daily in the morning (~10 a.m.), by collecting vaginal cells with a cotton swab dampened with sterile saline. The cells were smeared on a glass slide, stained with 0.1% methyl blue, and examined by light microscopy. Staging was assessed according to published guidelines [755, 756]. One cycle was defined as the sequential appearance of cells characteristic of all estrous cycle stages, regardless of the number of days spent in each stage. To assess fertility, 10-week-old female T3cKO and control mice were paired with C57BL6 male mice (Charles River, 000664), for a period of 6 months. Starting from 20 days after pairing, the cages were inspected daily for the presence of newborn mice. As soon as a litter was present, pups were carefully counted and put back into the cage. Pups were separated from the mother at postnatal day 15 to avoid interfering with the following pregnancy and/or delivery.

Natural ovulation

Ten-week old control and T3cKO females were paired with age-matched wild-type C57BL6 males. Females were inspected daily at 7 am until a vaginal plug was visualized. Females were then euthanized and cumulus-oocyte complexes (COCs) were harvested in PBS from ampullae of the oviducts on both sides. Cumulus cells were dissociated from the oocytes by incubating with 0.5 mg/ml hyaluronidase (Sigma, H3884) for 10 min at 37 ℃. Total oocyte numbers from each female were counted under an inverted microscope.

Superovulation

Superovulation was performed in juvenile (postnatal days 25-28) control and T3cKO females as described previously [712]. Briefly, 5 IU of pregnant mare's serum gonadotropin (PMSG or eCG, Sigma, G4877) were i.p. injected in females at 5 pm. Forty-eight hours later, the mice received 5 IU human chorionic gonadotropin (Sigma, C1063) i.p. Fourteen to 16 hr later, the mice were euthanized, and COCs retrieved and counted as described above.

Early implantation site assessment

Estrous cyclicity was recorded in both control and T3cKO females for 4-5 cycles. On the following proestrus afternoon, control and T3cKO females were paired with stud wild-type males. The following morning, plug formation was inspected as described above. The day of plug formation was considered 0.5-day post coitum (dpc). On 5.5 dpc, females were i.v. injected with 100 µl of 1% Evans blue dye (Sigma, E2129) prepared in saline. Five minutes after injection, females were euthanized, uteri were isolated, and implantation sites were counted.

Histology

Ovarian samples were isolated from 10-week old control and T3cKO females and fixed in 10% formalin at room temperature. Tissues were then paraffin-embedded and cut at 5-µm thickness. All sections were collected continuously and in order through the ovary. Every 5th section was stained with H&E for antral follicle and corpora lutea (CL) counting. Both ovaries were analyzed and the follicle or CL counts summed for statistical analysis.

Statistics

Female fertility, ovulation, CL and antral follicle counts, serum hormones, pituitary gene and protein levels, estrous cycle frequency, organ weights and puberty onset were compared using Student's t tests. Estrous cycle stages, serum FSH levels before and after GnRH antagonist or inhibin A injection or before and after gonadectomy were analyzed using 2-way ANOVA followed by Tukey post-hoc tests. Data were log transformed when variances were not equal between groups. Statistical analyses were performed using Prism 6 (GraphPad). $p<0.05$ was considered statistically significant.

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Footnotes

Author contributions: Y.L., J.F. and D.J.B designed the experiments; Y.L. and J.F. performed most of the experiments; L.O. participated in the natural ovulation and ovarian histology experiments; H.L. generated the floxed mouse strain; U.B. provided the GRIC mouse strain; Y.L., J.F., and D.J.B. analyzed data; and Y.L. and D.J.B. wrote the manuscript.

The authors declare that they have no conflicts of interest with the contents of this article.

Table 4.1: Genotyping and qPCR primers

Genotyping	
Tgfbr3	5' – 3'
Forward	TGATCTTAGTGGTAACCTCGCC
Reverse	CTAGCATGACAGGAATGTAC
Recombined	TTAGGTCGGTGCTGTCCTTGTT
Gnrhr^{GRIC}	
Forward	GGACATGTTCAGGGATCGCCAGGC
Reverse	GCATAACCAGTGAAACAGCATTGCTG
Wildtype allele	
Forward	GAACTACAGCTGAATCAGTC
Reverse	CTAACAACAAACTCTGTACA
qPCR	
Rpl19	
Forward	CGGGAATCCAAGAAGATTGA
Reverse	TTCAGCTTGTGGATGTGCTC
Fshb	
Forward	GTGCGGGCTACTGCTACACT
Reverse	CAGGCAATCTTACGGTCTCG
Cga	
Forward	TCCCTCAAAAAGTCCAQGAGC
Reverse	GAAGAGAATGAAGAATATGCAG
Lhb	
Forward	AGCAGCCGGCAGTACTCGGA
Reverse	ACTGTGCCGGCCTGTCAACG
Gnrhr	
Forward	CACGGGTTTAGGAAAGCAAA
Reverse	TTCGCTACCTCCTTTGTCGT

Figure legends

Figure 4.1 Generation and validation of floxed *Tgfbr3* mice. A) Schematic representation of the strategy used to generate the conditional *Tgfbr3* allele loxP sites are pictured as black triangles and exons are shown as boxes. Note that exon sizes are not drawn to scale. Neo, neomycin selection cassette. B) Southern blot analysis of wild-type (+) and recombined (Δex2) *Tgfbr3* alleles. C) Example of PCR genotyping of heterozygous (*Tgfbr3*$^{+/\Delta ex2}$), homozygous (*Tgfbr3*$^{\Delta ex2/\Delta ex2}$), or wild-type (*Tgfbr3*$^{+/+}$) mice. D) Examples of wild-type and homozygous knockout embryos at the indicated ages. dpc, days post-coitus.

Figure 4.2 Gonadotrope-specific knockout of TGFBR3. Immunofluorescence of pituitary sections from adult control (left) and T3KO [237] males using antibodies against TGFBR3 (green, top) and FSHβ (red, middle). Overlays of the two images are shown at the bottom. Arrowheads, cells expressing TGFBR3, but not FSH. *, FSH-expressing cells. Scale bar = 50 µm.

Figure 4.3 Inhibin A dose-dependently antagonizes *Fshb* expression in control but *Tgfbr3*-deficient gonadotropes. Pituitary cultures were prepared from mixed genders of *Tgfbr3*$^{fx/fx}$ mice (A, B) or from control (black lines) and T3cKO (red lines) females (C, D). Cells were treated with the indicated concentrations of inhibin A. *Tgfbr3* (A, C) or *Fshb* (B, D) mRNA expression was measured by RT-qPCR. The house keeping gene *Rpl19* was used for normalization. In A and B, cells were transduced with a Cre-expressing (ad-Cre, red lines) or control adenovirus (adGFP, black lines). Data shown in A-D reflect mean ± SEM from three independent experiments. E) Changes in serum FSH levels (ΔFSH) before and 6 h after inhibin A injection in control (black)

and T3cKO (red) males. Serum FSH levels were assessed by multiplex ELISA in this experiment. *p<0.05

Figure 4.4 Increased fertility, ovulation, and implantation in T3cKO females. A) Average litter sizes in 6-month breeding trials. B) Representative ovarian histology (H&E) in 10-week old females. Antral follicles (AF) and corpora lutea (CL) are labeled. Total numbers of C) large AFs and D) CLs were counted and summed from both ovaries. E) Cumulus-oocytes complexes (COCs) were counted on the morning (~7 am) after mating. F) Implantation sites were counted at 5.5 dpc. Data were analyzed by Student's *t*-test in all panels except panel B. Significance was assessed relative to *p<0.05.

Figure 4.5 Intact serum and pituitary FSH levels in control and T3cKO females and males. Serum FSH levels were assessed in 10-week-old females A-B) on diestrus afternoon (~3 pm), proestrus morning (~11 am), or estrus morning (7 am to 12 pm), and in adult males. Serum FSH levels in females on diestrus and proestrus were assessed by Luminex assay. Serum FSH levels in females on estrous morning and in males were assessed by Multiplex ELISA. Pituitary FSH content in 10-week-old D) female and E) male mice, as assessed by RIA. F-G) RT-qPCR analysis of relative pituitary gene expression in 10-week-old females and males. Data were analyzed by Student's *t*-test in each panel. *p<0.05 was considered statistically significant.

Figure 4.6 Serum FSH and pituitary *Fshb* mRNA levels in control and T3cKO males and females before and after gonadectomy. A) Serum FSH levels in 10-week old control and T3cKO females on proestrus morning (~11 am) before ovariectomy (OVX), and 7 h and 1 week after

surgery. B) Serum FSH levels in 14-week old control and T3cKO males one month after sham or castration operation. Serum FSH levels were assessed by Luminex assay (female) or Multiplex ELISA (male). C,D) RT-qPCR analyses of relative *Fshb* mRNA levels in control and T3cKO males and females after gonadectomy (male and female) or sham surgery (male). Data in A, B, and C) were analyzed by two-way ANOVA followed by Tukey *post-hoc* tests. Data in D) were analyzed by Student's *t*-test. *$p<0.05$.

Figure 4.7 T3cKO and control mice respond similarly to exogenous GnRH treatment. Ten-week-old control and T3cKO males were s.c. injected GnRH or vehicle as indicated in the figure. N=5 per group. Blood samples were collected once before injection, as well as 15, 30, and 60 min after GnRH injection. Serum FSH levels were assessed by Luminex assay; serum LH levels were assessed by an in-house ELISA. Data represent mean ± SEM.

Figure 4.8 Control and T3cKO males respond similarly to GnRH antagonist treatment. Serum A) LH and B) FSH levels 24 h after Antide or vehicle injection, as assessed by multiplex ELISA. Pituitary C) *Lhb* and D) *Fshb* mRNA levels in the mice used in A-B. E,F) Serum LH and FSH levels after Cetrorelix injection. In this experiment, serum LH was assessed by an in-house ELISA and serum FSH levels were evaluated by Luminex assay. Data in A-D were analyzed by two-way ANOVA followed by Tukey *post-hoc* tests. Data in E and F were analyzed by one-way ANOVA for each genotype. *$p<0.05$.

Figure 4.9 Control and T3cKO juvenile females respond similarly to exogenous gonadotropin stimulation. COCs were collected from both oviducts of 25 to 28-day-old control

and T3cKO females after exogenous gonadotropin simulation. Data were analyzed by Student's *t*-test.

Supplementary Figure legends

Figure S4.1 Secreted FSH levels in culture medium 24 h after inhibin A treatment. FSH levels in culture medium from the experiments in Fig. 4.3A-D, as assessed by RIA. Data reflect mean ± SEM from three independent experiments.

Figure S4.2 Normal cyclicity and reproductive organ weights in T3cKO animals. A) Mean day of vaginal opening. pnd, post-natal day. B-C) Estrous cyclicity was assessed by daily vaginal smears for at least 21 consecutive days. M/D, metestrus/diestrus; P, proestrus; E, Estrus. D) Ovarian, E) uterine, and F) testicular weights in 10-week-old control and T3cKO females and males. Values in D-F were normalized to body weight. Data were analyzed by Student's *t*-test in each panel.

Figure S4.3 LH levels and pituitary gene expression in control and T3cKO females and males after gonadectomy. A) Serum LH levels in females 2 weeks after OVX and B) in males 1 month after castration. Serum LH levels were assessed by an in-house ELISA (female) or Multiplex ELISA (males). C) Pituitary gene expression 2 weeks after OVX and D-F) 1 month after castration. Pituitary gene expression was analyzed by RT-qPCR and was normalized to the housekeeping gene *Rpl19*. Data in panel A and C were analyzed by Student's *t*-test; data in B and D-F were analyzed by two-way ANOVA followed by Tukey *post-hoc* tests. *$p<0.05$.

Figure S4.4 Pituitary *Gnrhr* expression 24 h after GnRH antagonist (Antide) injection. Relative *Gnrhr* mRNA levels were normalized to the housekeeping gene *Rpl19*. cDNA samples were the same as used in Fig. 4.8A-D. Data were analyzed by two-way ANOVA followed by Tukey *post-hoc* tests. *$p<0.05$.

Figure S4.5 Pulsatile LH release in 8 to 10-week-old control and T3cKO males. Whole blood was collected every 5 mins for a total of 3 h from control A-B) and T3cKO C-D) males. Whole blood LH levels were analyzed by an in-house ELISA.

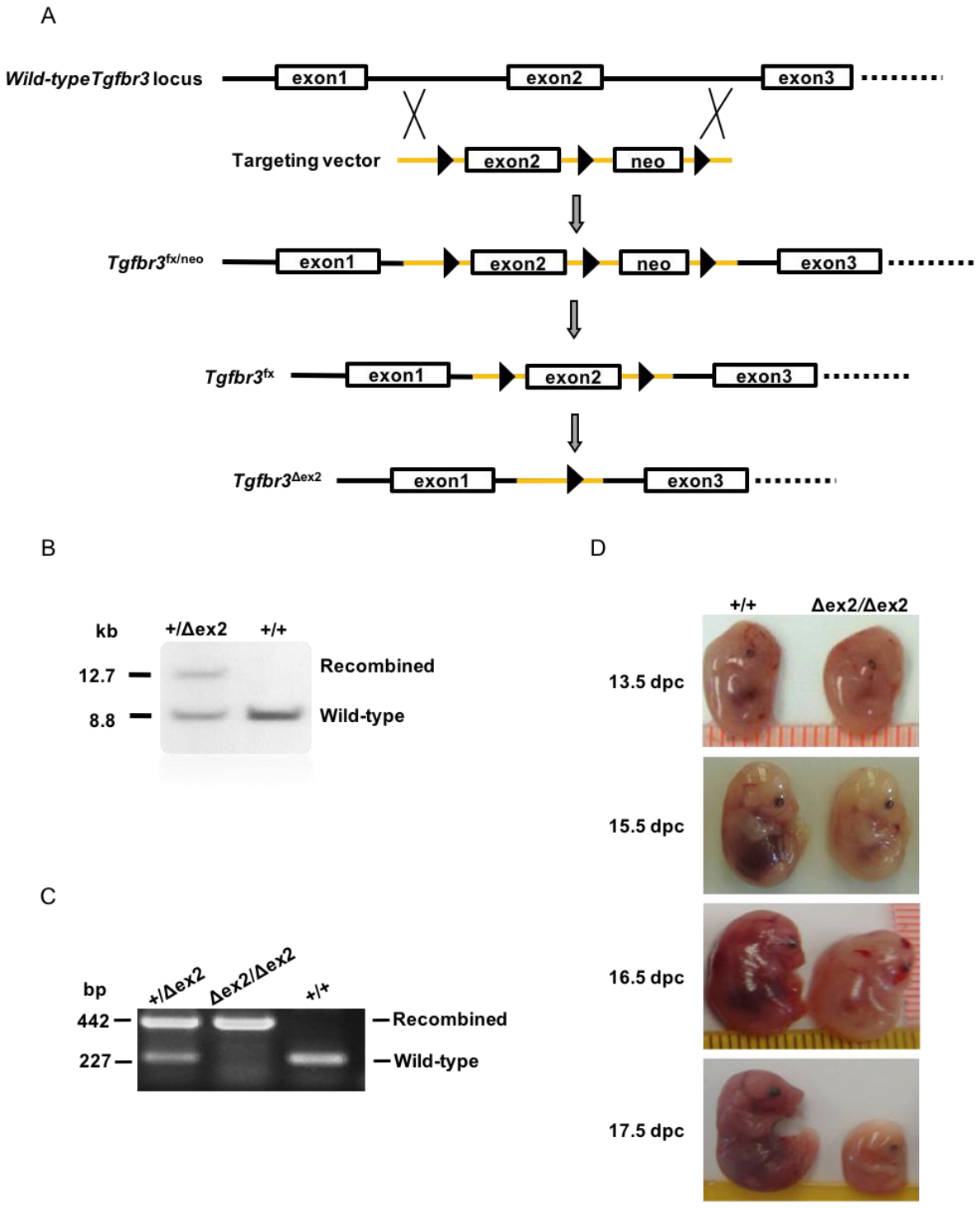
A
Wild-type Tgfbr3 locus
exon1
exon2
exon3
Targeting vector
exon2
neo
Tgfbr3fx/neo
exon1
exon2
neo
exon3
Tgfbr3fx
exon1
exon2
exon3
Tgfbr3Δex2
exon1
exon3
B
kb
+/Δex2
+/+
12.7
8.8
Recombined
Wild-type
C
bp
+/Δex2
Δex2/Δex2
+/+
442
227
Recombined
Wild-type
D
+/+
Δex2/Δex2
13.5 dpc
15.5 dpc
16.5 dpc
17.5 dpc

Figure 4.1

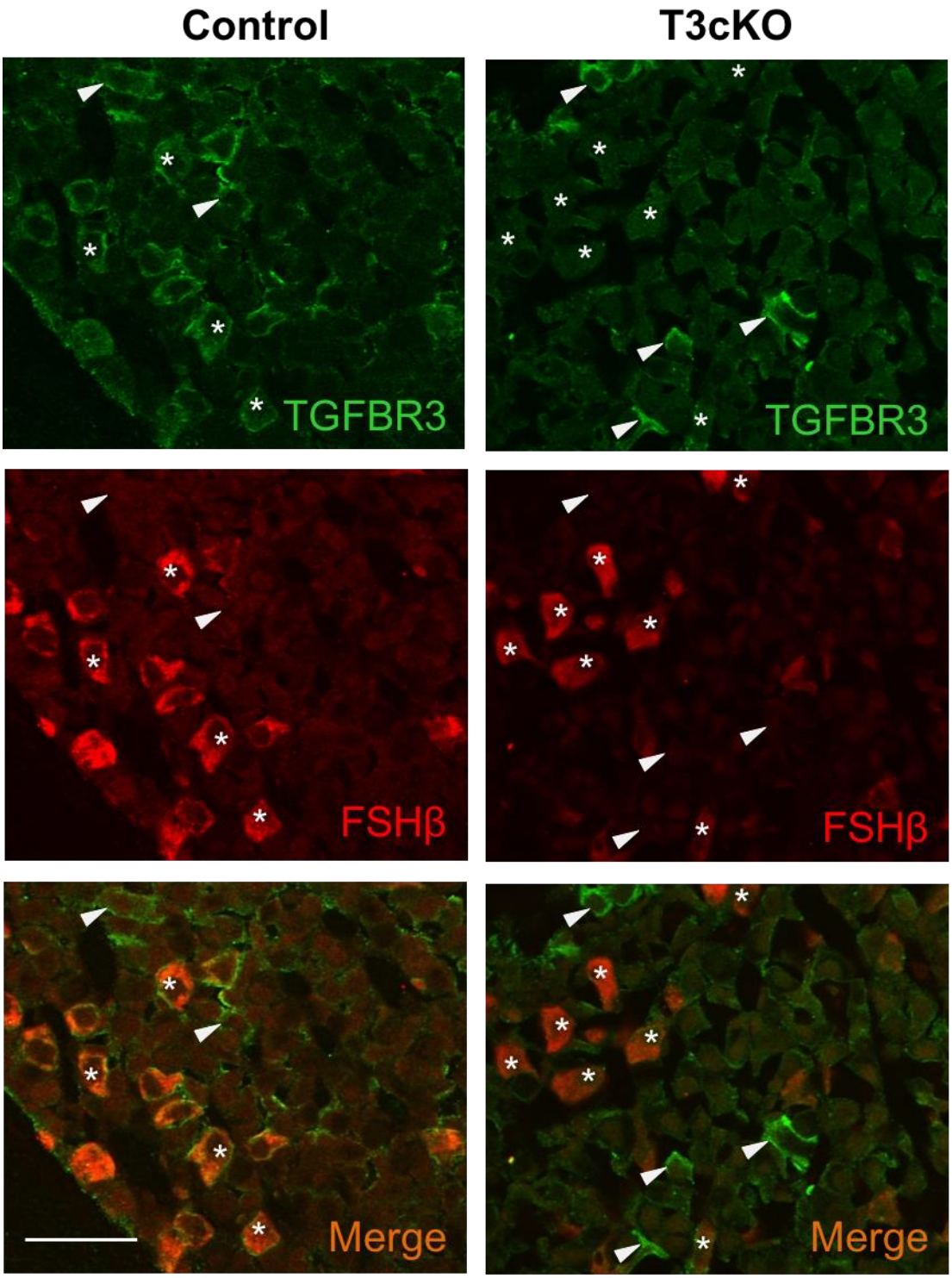

Figure 4.2

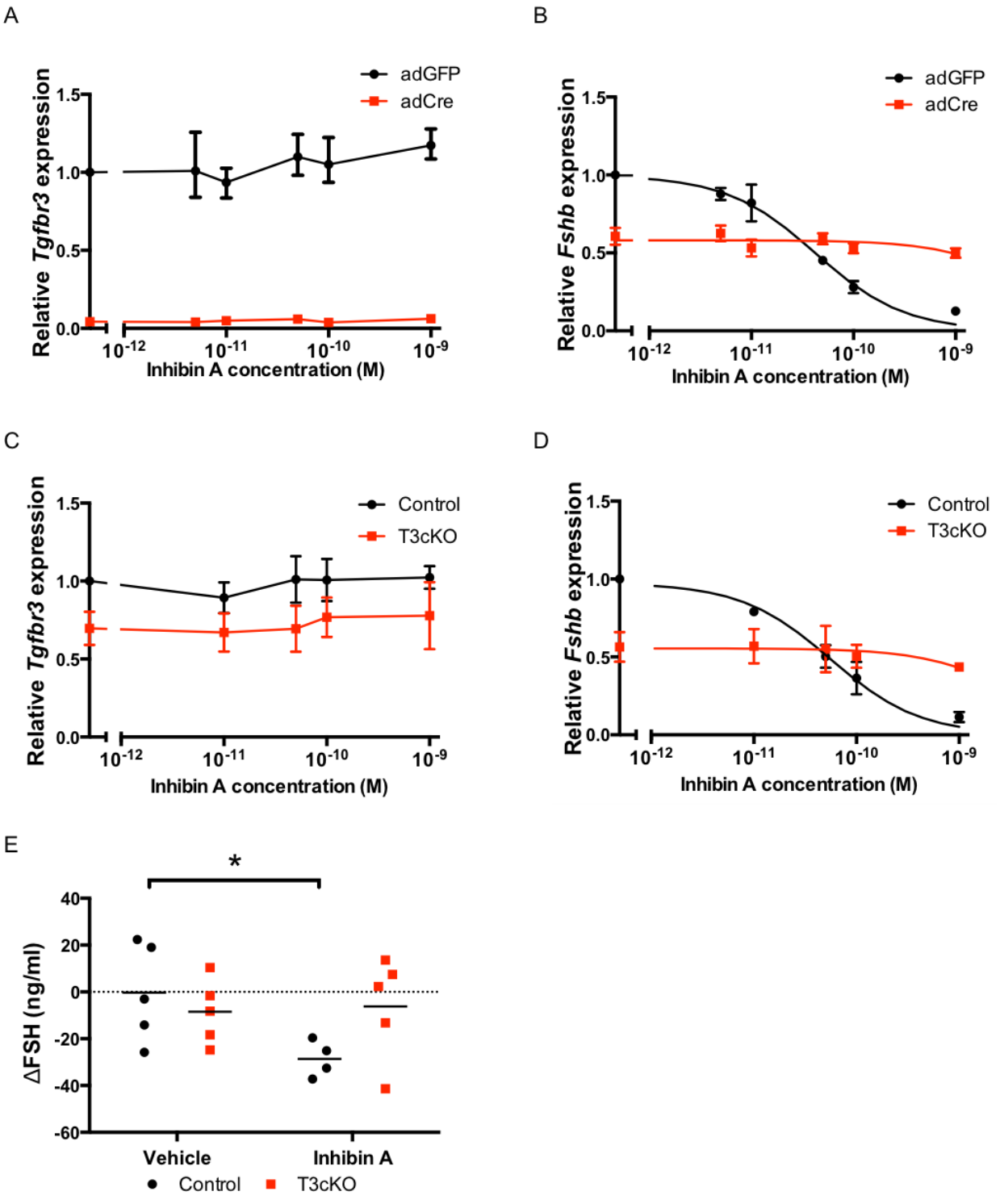
A
B
C
D
E
adGFP
adCre
Control
T3cKO
Relative Tgfbr3 expression
Relative Fshb expression
Inhibin A concentration (M)
ΔFSH (ng/ml)
Vehicle
Inhibin A
*

Figure 4.3

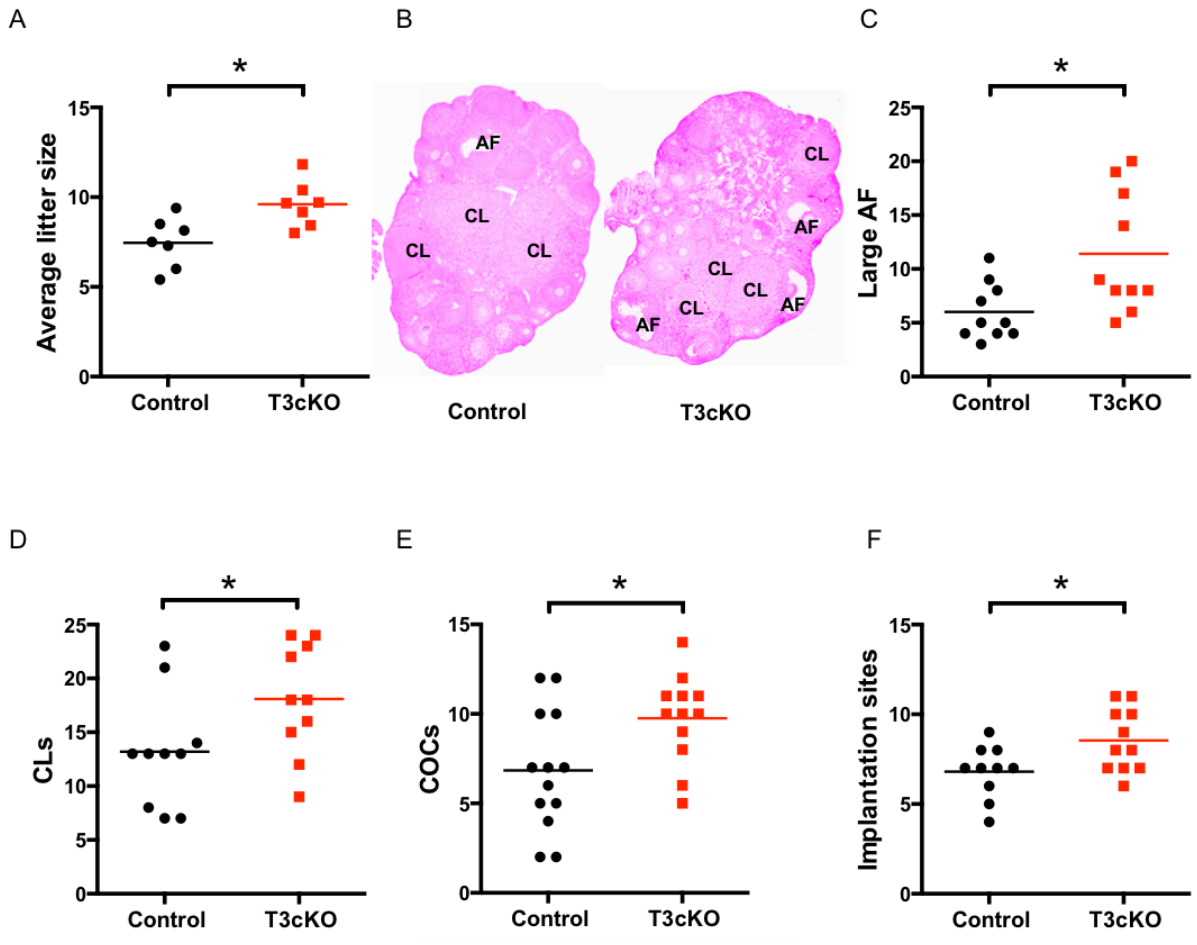
A
B
C
*
Average litter size
15
10
5
0
Control
T3cKO
AF
CL
CL
CL
CL
AF
CL
CL
AF
AF
Control
T3cKO
Large AF
25
20
15
10
5
0
Control
T3cKO
D
E
F
CLs
COCs
Implantation sites
Control
T3cKO

Figure 4.4

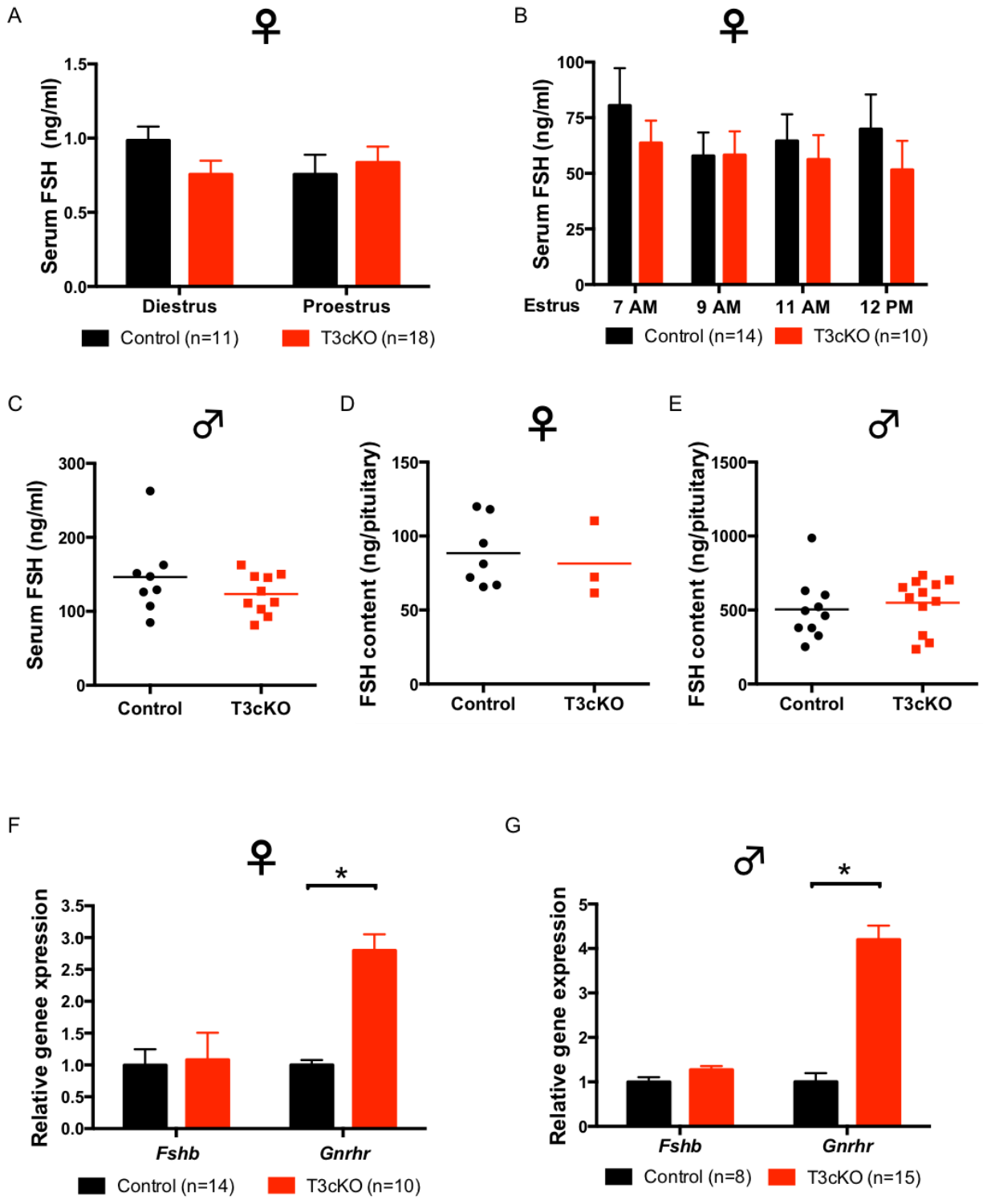
A
♀
Serum FSH (ng/ml)
1.5
1.0
0.5
0.0
Diestrus
Proestrus
Control (n=11)
T3cKO (n=18)
B
♀
Serum FSH (ng/ml)
100
75
50
25
0
Estrus
7 AM
9 AM
11 AM
12 PM
Control (n=14)
T3cKO (n=10)
C
♂
Serum FSH (ng/ml)
300
200
100
0
Control
T3cKO
D
♀
FSH content (ng/pituitary)
150
100
50
0
Control
T3cKO
E
♂
FSH content (ng/pituitary)
1500
1000
500
0
Control
T3cKO
F
♀
*
Relative genee xpression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Fshb
Gnrhr
Control (n=14)
T3cKO (n=10)
G
♂
*
Relative gene expression
5
4
3
2
1
0
Fshb
Gnrhr
Control (n=8)
T3cKO (n=15)

Figure 4.5

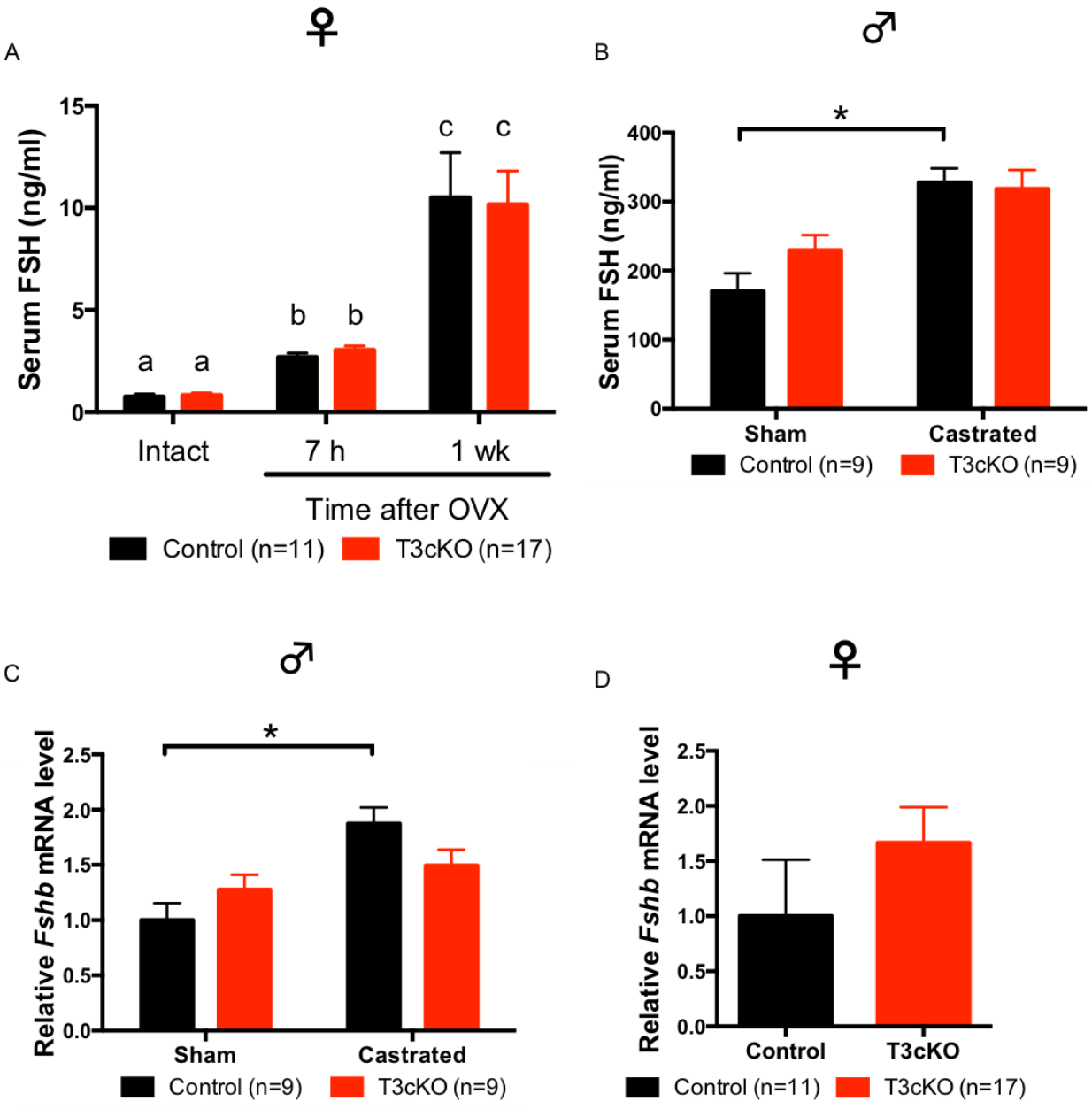
A
♀
Serum FSH (ng/ml)
15
10
5
0
a a
b b
c c
Intact
7 h
1 wk
Time after OVX
Control (n=11)
T3cKO (n=17)
B
♂
Serum FSH (ng/ml)
400
300
200
100
0
*
Sham
Castrated
Control (n=9)
T3cKO (n=9)
C
♂
Relative Fshb mRNA level
2.5
2.0
1.5
1.0
0.5
0.0
*
Sham
Castrated
Control (n=9)
T3cKO (n=9)
D
♀
Relative Fshb mRNA level
2.5
2.0
1.5
1.0
0.5
0.0
Control
T3cKO
Control (n=11)
T3cKO (n=17)

Figure 4.6

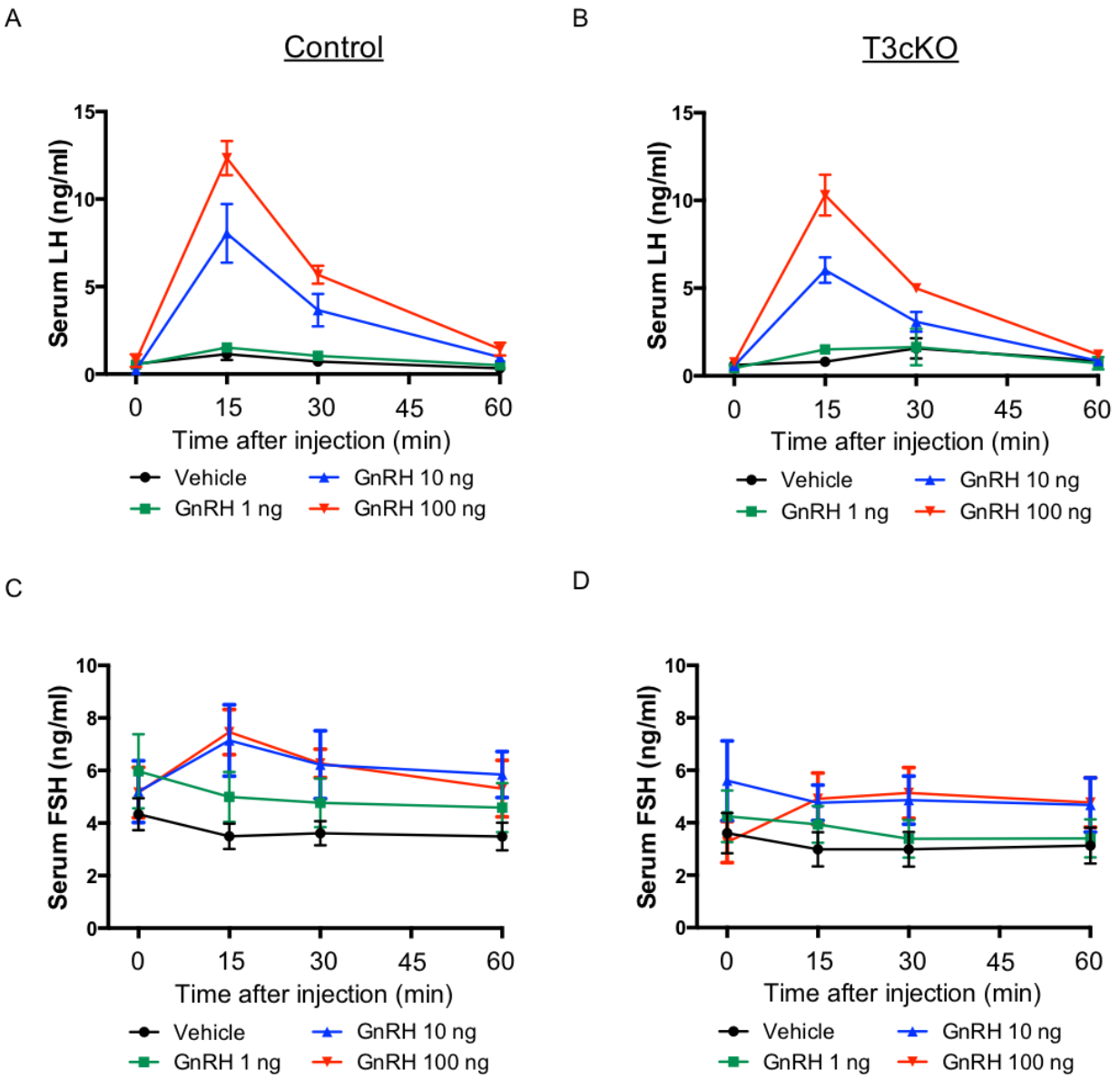
A
Control
Serum LH (ng/ml)
15
10
5
0
0
15
30
45
60
Time after injection (min)
Vehicle
GnRH 10 ng
GnRH 1 ng
GnRH 100 ng
B
T3cKO
Serum LH (ng/ml)
15
10
5
0
0
15
30
45
60
Time after injection (min)
Vehicle
GnRH 10 ng
GnRH 1 ng
GnRH 100 ng
C
Serum FSH (ng/ml)
10
8
6
4
2
0
0
15
30
45
60
Time after injection (min)
Vehicle
GnRH 10 ng
GnRH 1 ng
GnRH 100 ng
D
Serum FSH (ng/ml)
10
8
6
4
2
0
0
15
30
45
60
Time after injection (min)
Vehicle
GnRH 10 ng
GnRH 1 ng
GnRH 100 ng

Figure 4.7

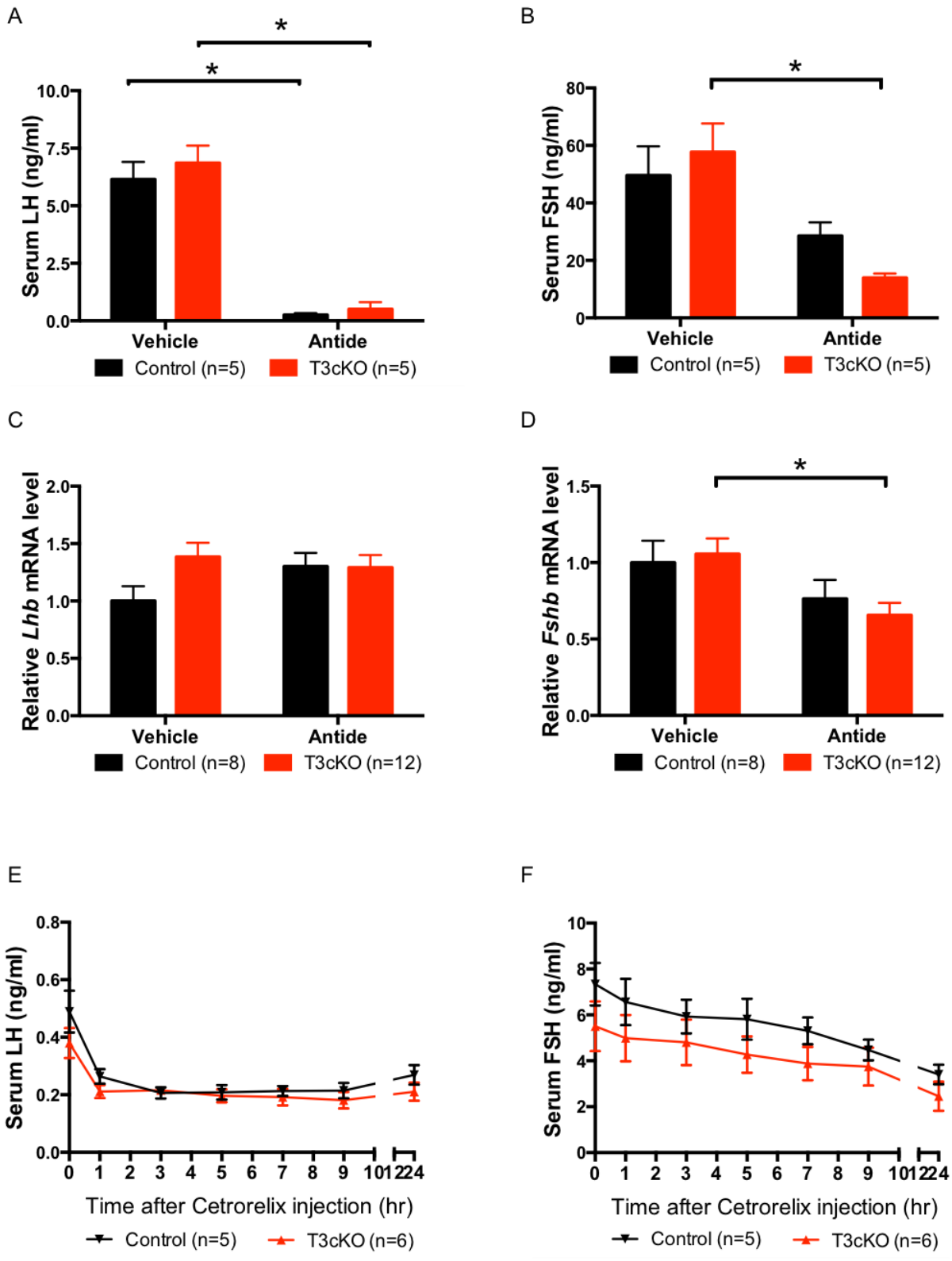
A
*
*
Serum LH (ng/ml)
10.0
7.5
5.0
2.5
0.0
Vehicle
Antide
Control (n=5)
T3cKO (n=5)
B
*
Serum FSH (ng/ml)
80
60
40
20
0
Vehicle
Antide
Control (n=5)
T3cKO (n=5)
C
Relative Lhb mRNA level
2.0
1.5
1.0
0.5
0.0
Vehicle
Antide
Control (n=8)
T3cKO (n=12)
D
*
Relative Fshb mRNA level
1.5
1.0
0.5
0.0
Vehicle
Antide
Control (n=8)
T3cKO (n=12)
E
Serum LH (ng/ml)
0.8
0.6
0.4
0.2
0.0
0 1 2 3 4 5 6 7 8 9 10 12 24
Time after Cetrorelix injection (hr)
Control (n=5)
T3cKO (n=6)
F
Serum FSH (ng/ml)
10
8
6
4
2
0
0 1 2 3 4 5 6 7 8 9 10 12 24
Time after Cetrorelix injection (hr)
Control (n=5)
T3cKO (n=6)

Figure 4.8

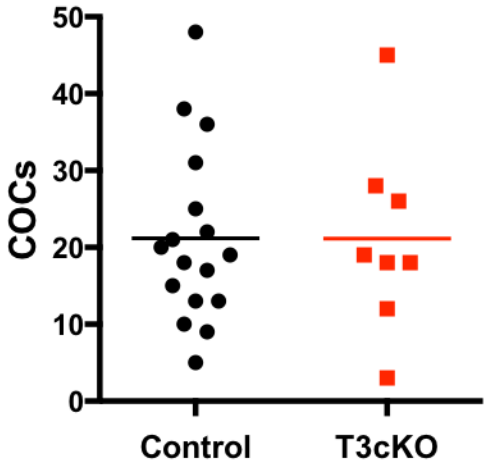

Figure 4.9

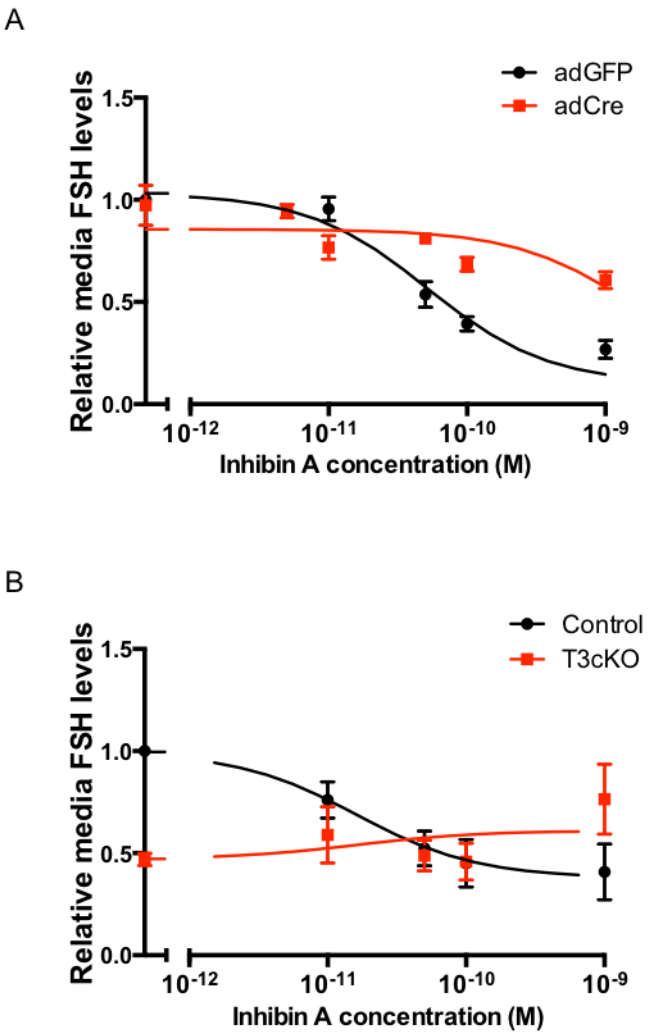
A
adGFP
adCre
Relative media FSH levels
1.5
1.0
0.5
0.0
10-12
10-11
10-10
10-9
Inhibin A concentration (M)
B
Control
T3cKO
Relative media FSH levels
1.5
1.0
0.5
0.0
10-12
10-11
10-10
10-9
Inhibin A concentration (M)

Figure S4.1

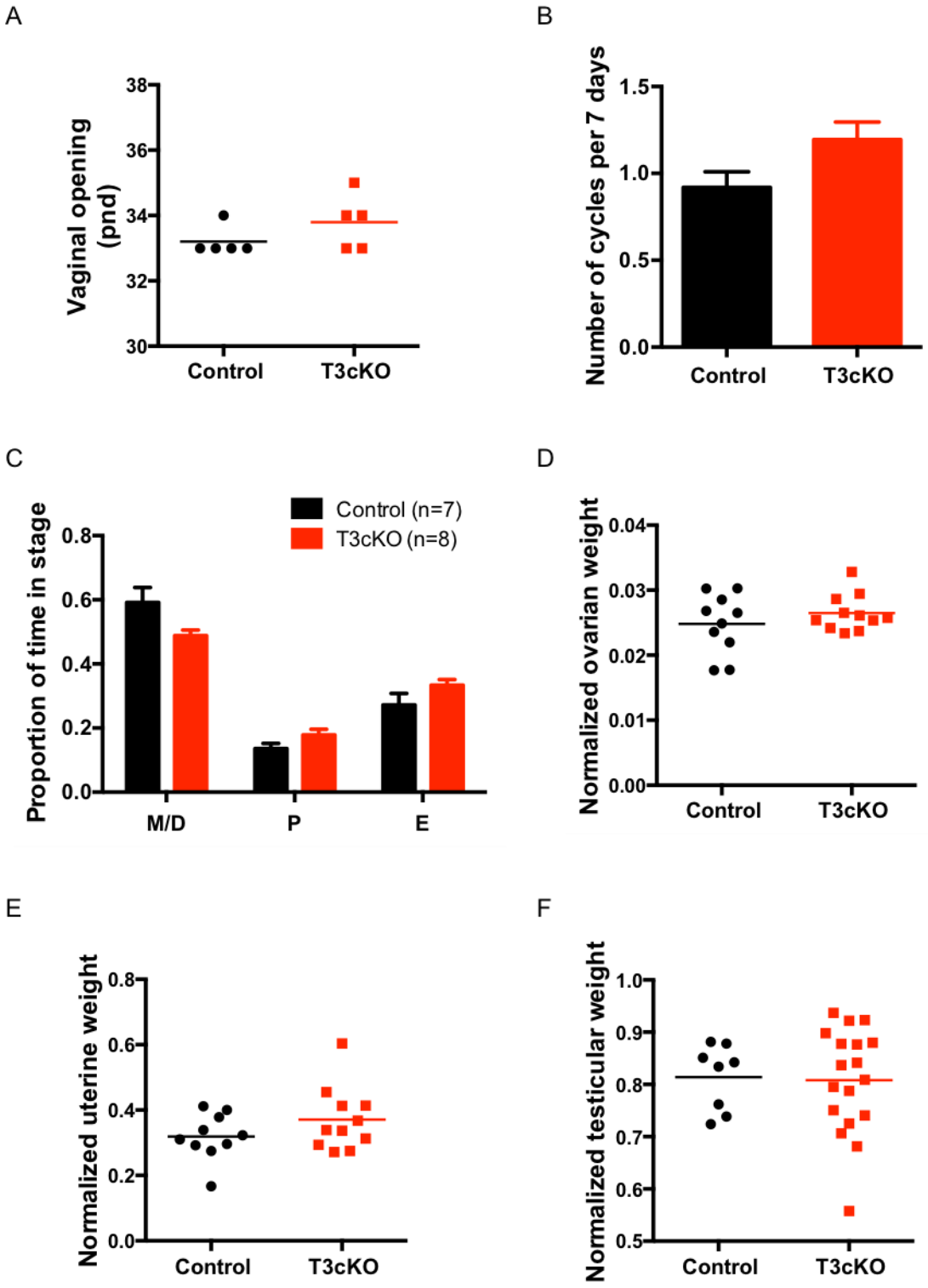
A
Vaginal opening (pnd)
38
36
34
32
30
Control
T3cKO
B
Number of cycles per 7 days
1.5
1.0
0.5
0.0
Control
T3cKO
C
Control (n=7)
T3cKO (n=8)
Proportion of time in stage
0.8
0.6
0.4
0.2
0.0
M/D
P
E
D
Normalized ovarian weight
0.04
0.03
0.02
0.01
0.00
Control
T3cKO
E
Normalized uterine weight
0.8
0.6
0.4
0.2
0.0
Control
T3cKO
F
Normalized testicular weight
1.0
0.9
0.8
0.7
0.6
0.5
Control
T3cKO

Figure S4.2

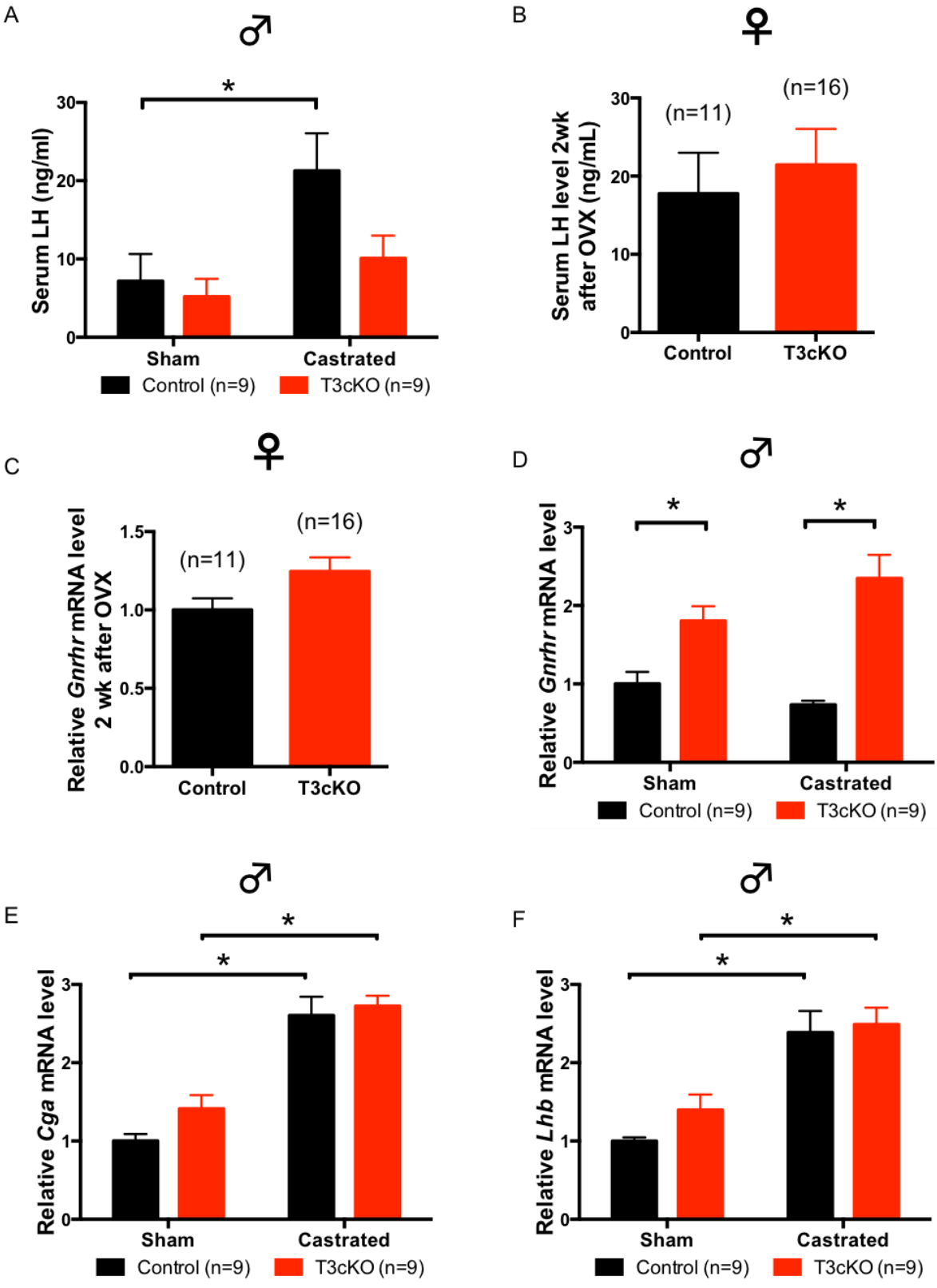
A
♂
*
Serum LH (ng/ml)
30
20
10
0
Sham
Castrated
Control (n=9)
T3cKO (n=9)
B
♀
(n=11)
(n=16)
Serum LH level 2wk after OVX (ng/mL)
30
20
10
0
Control
T3cKO
C
♀
(n=11)
(n=16)
Relative Gnrhr mRNA level 2 wk after OVX
1.5
1.0
0.5
0.0
Control
T3cKO
D
♂
*
*
Relative Gnrhr mRNA level
3
2
1
0
Sham
Castrated
Control (n=9)
T3cKO (n=9)
E
♂
*
*
Relative Cga mRNA level
3
2
1
0
Sham
Castrated
Control (n=9)
T3cKO (n=9)
F
♂
*
*
Relative Lhb mRNA level
3
2
1
0
Sham
Castrated
Control (n=9)
T3cKO (n=9)

Figure S4.3

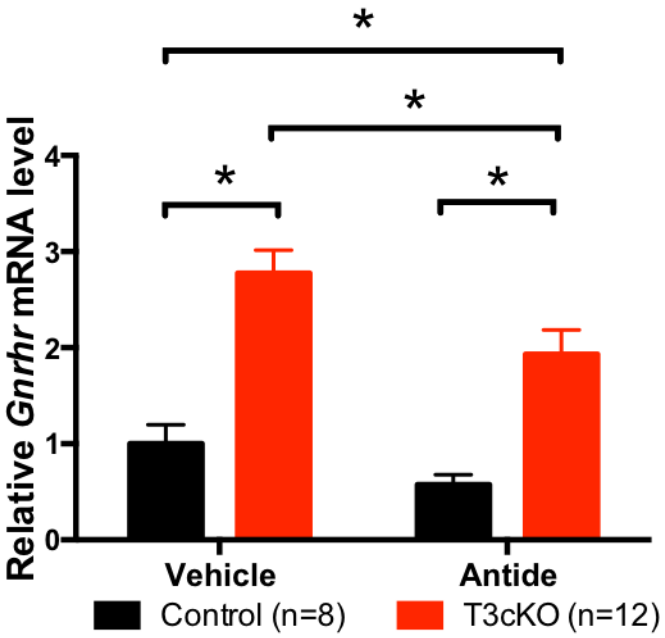
*
*
*
*
Relative Gnrhr mRNA level
4
3
2
1
0
Vehicle
Antide
Control (n=8)
T3cKO (n=12)

Figure S4.4

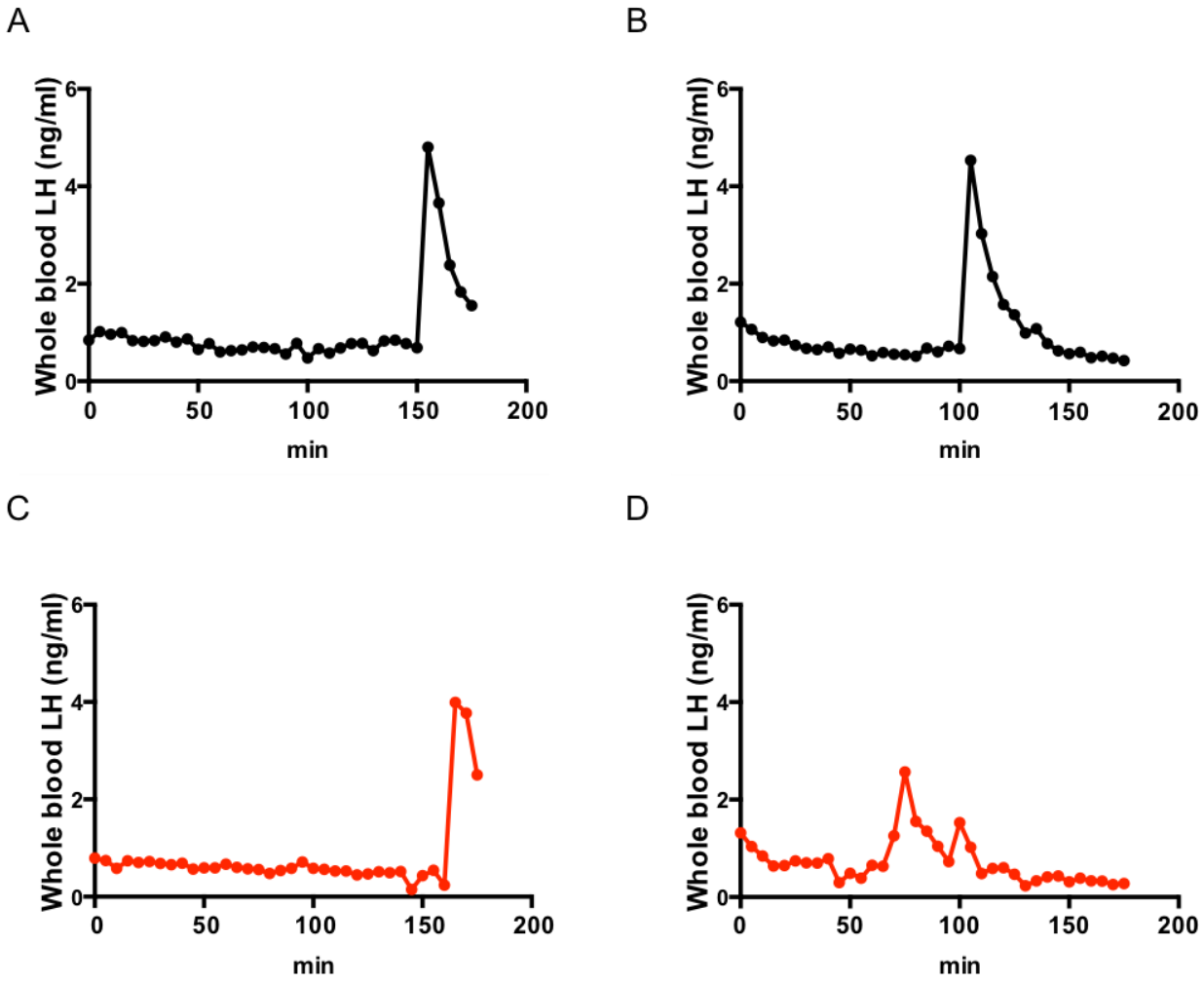

Figure S4.5

Chapter 5 General discussion

The data presented in my thesis provide new insights into the molecular basis of the regulation of FSH production by TGFβ superfamily ligands *in vivo*. The results shown in **Chapters 2 and 3** provide further evidence that activin-like signaling is the dominant regulator of FSH production in mice. Ablation of critical activin signaling molecules (*Smad4* with either *Smad3* or *Foxl2* in combination) during development (**Chapter 2**) or adulthood (**Chapter 3**) causes profound FSH deficiencies and female infertility in mice. Furthermore, the data presented in **Chapter 3** indicate that FSH reduction during development, but not in adults, impairs spermatogenesis, challenging the idea that FSH could be a target for male contraception. The results in **Chapter 4** provide, for the first time, *in vivo* evidence that inhibin A antagonism of FSH synthesis requires TGFBR3 expression in murine gonadotropes. Although female conditional knockouts in this study ovulated more eggs during each cycle and exhibited enhanced fertility, we were unable to detect changes in FSH synthesis or secretion. Why FSH is not elevated and how fertility is enhanced are important, but presently unsolved mysteries. In this final chapter, I will discuss these and other questions arising from our investigation of activin/inhibin signaling in gonadotropes. Additionally, I will discuss the potential implications of my results for future studies.

5.1 Regulation of murine GnRH receptor (*Gnrhr*) expression in gonadotropes *in vivo*

Responsiveness of gonadotropes to GnRH stimulation is at least partially dependent on the cell surface density of GnRHR [757-759]. In rodents, the main regulators of *Gnrhr* expression include GnRH [749-751, 760], steroids [294-297, 749, 752], and perhaps activins [761, 762]. The effects of activins on *Gnrhr* expression appear may be species- or context-dependent manner. For example, activins stimulate *Gnrhr* expression in primary cultured rat pituitary cells whereas in

primary cultured ovine cells, they suppress *Gnrhr* expression [763, 764]. Furthermore, activins induce murine *Gnrhr* promoter activity in two immortalized murine gonadotrope cell lines, LβT2 and αT3-1 [764, 765]. However, previous [409] and current *in vivo* results (**Chapters 2 and 3**) demonstrate that *Gnrhr* expression is either elevated or unchanged in gonadotropes in the absence of activin signaling molecules. Specifically, gonadotropes express increased levels of *Gnrhr* when lacking *Smad4* alone [409] or *Smad4* together with *Smad3* (**Chapter 2**), though ablating *Smad3* alone has no impact on *Gnrhr* expression [408]. As a result, we argue that loss of *Smad4* is likely responsible for the increase of *Gnrhr* expression *in vivo*. Opposing to our idea, however, *Gnrhr* mRNA levels in gonadotropes are unchanged when *Smad4* and *Foxl2* are deleted in combination whether during development [409] or in adulthood (**Chapter 3**). Notably, knocking out *Foxl2* alone in gonadotropes has no impact on *Gnrhr* expression, ruling out a simple negative regulatory role of FOXL2 [417]. Furthermore, *Gnrhr* expression is dramatically increased in murine gonadotropes in the absence of the inhibin co-receptor TGFBR3 (**Chapter 4**). Recall that activin signaling in these mice is at least intact, if not enhanced. However, *Gnrhr* mRNA levels are nevertheless comparable between the controls and the conditional knockouts upon dispersing the pituitaries in culture, suggesting that the regulator of *Gnrhr* expression is located outside of pituitary gland, e.g. GnRH from the brain or estrogen from the gonads. Consistent with this idea, the difference in *Gnrhr* expression between the control and the T3cKO is diminished 1 week following ovariectomy, suggesting that ovarian factors (perhaps estrogens) play critical roles in murine GnRH receptor expression in gonaodotropes. In contrast, *Gnrhr* expression remains elevated in T3cKO male mice relative to controls one month following castration. Although the basis for sex differences in control and T3cKO mice is unclear, it may due to the fact that the dominant sex steroid hormones (androgens vs estrogens) is different in males and females.

However, assessing steroid levels using ELISA or RIA in murine serum appears unreliable. For example, females of *Smad4* and *Foxl2* conditional knockout (S4F2 cKO) show thread-like uteri, nevertheless, estradiol levels were comparable between control and S4F2 cKO, leading to the doubt of the assay accuracy [409]. Therefore, a more reliable assay for the assessment of estradiol in murine sera is desired to evaluate the role of steroid hormone in the regulation of *Gnrhr* expression in murine gonadotropes *in vivo*.

Moreover, there is no available evidence showing whether estrogen directly regulates *Gnrhr* expression or acts via modulating GnRH frequency or amplitude released from the brain. An *in vitro* study shows that pulsatile, but not continuous, administration of GnRH caused a remarkable increase in *Gnrhr* mRNA levels in primary cultured rat pituitary cells [749]. If this observation also reflects the regulatory mechanism in murine gonadotropes *in vivo*, the altered *Gnrhr* expression we observe in conditional knockout models may be attributed, at least in part, to the changes in GnRH secretion from the hypothalamus. Interestingly, although T3cKO pituitaries express significantly higher levels of *Gnrhr* compared to the control littermates, the T3cKO pituitaries responded similarly to the controls when challenged with exogenous GnRH at different concentrations (Fig. 4.7). Notably, GnRH was injected once only in these animals. Therefore, one may argue that this method may not reflect the true phenotype when GnRH is released in a pulsatile manner endogenously. Therefore, we attempted to assess LH pulses in control and T3cKO mice. However, LH pulsatile release does not appear significantly different between the two genotypes in our pilot experiment, at least in males (Fig. S4.5).

Additionally, T3cKO pituitaries express higher levels of *Gnrhr* compare to the controls, nevertheless, the equivalent responsiveness to GnRH agonist/antagonist treatment between the control group and the T3cKOs may reflect the fact that *Gnrhr* transcripts are not fully translated

to GnRHR protein or the fact that they are not expressed on the cell membrane in the T3cKO pituitaries [749, 766, 767]. Therefore, whether changes in *Gnrhr* expression translates into alterations in GnRH signaling in mice lacking TGFBR3 *in vivo* remains to be determined.

On the whole, both previous and current data suggest that *Gnrhr* expression in gonadotropes is either directly or indirectly regulated by gonadal estrogen production. In the latter case, estrogen may function by altering GnRH's pulsatile frequency or amplitude from the hypothalamus, thereby influencing its action on the pituitary.

5.2 The relative role of GnRH and activins in the regulation of FSH production *in vivo*

Although both GnRH and activins have been shown to be essential for FSH synthesis *in vivo*, the relative contribution of their actions on FSH synthesis is unclear [161, 340, 417, 712, 768-770]. This gap is in part due to the lack of knowledge of the mechanisms by which GnRH stimulates FSH synthesis [161]. The critical role of GnRH in the synthesis of both gonadotropins is best demonstrated in *hpg* mice, which lack the GnRH decapeptide [768]. Additionally, GnRH antagonist (e.g. antide) administration in the adults inhibits the synthesis and secretion of both gonadotropins productions, suggesting that GnRH is required in maintaining gonaodotropin levels in normal conditions [771-774]. Whereas, the data presented in Chapters 2 and 3 in this thesis clearly indicate that activin-like signaling is also required for FSH production during both development (**Chapter 2**) and adulthood (**Chapter 3**). In **Chapter 2**, we first show that mice lacking SMAD3/4 in their gonadotropes produce very little, if any, FSH. We argued that the early (E12.75) deletion of *Smad3* and *Smad4* during development could impair gonadotrope development, which means that the cells are therefore unable to respond to GnRH stimulation for FSH production later in life. However, we went on to show that inducible disruption of activin-

like signaling (via FOXL2 and SMAD4) during adulthood also resulted in profoundly reduced FSH production, though to a lesser extent than the reduction caused by gene deletion during development [409]. Although it is not clear whether the residual FSH is generated by GnRH or by the incomplete recombination of *Foxl2* and *Smad4* in the inducible knockouts, the amount of FSH produced in the inducible knockout females is not sufficient (in most cases) to stimulate follicle development and maintain fertility (**Chapter 3**). These data demonstrate that GnRH action alone is not sufficient for maintaining FSH production. Nevertheless, GnRH is necessary for FSH [507, 775], though, GnRH may function by stimulating the synthesis of molecules that are required for activin signaling (e.g. ligand, receptor, dimeric assembly or transcription factors). Further studies are required to clarify the role of GnRH function in the regulation of adult FSH *in vivo* and to demonstrate how and where GnRH and activin signaling cascades interact.

5.3 FSH bioactivity *in vivo*

Although both gonadotropins are synthesized in gonadotropes, the release of FSH and LH is differentially regulated. Specifically, LH is first stored in dense-core granules and then released in response to GnRH stimulation whereas FSH is mainly secreted constitutively upon its synthesis (see section 1.1.3.2) [776-778].

A precious study has demonstrated that redirecting FSH to the regulated secretory pathway dramatically influences ovarian responsiveness to FSH stimulation [212]. However, it is not clear whether sorting to the regulated pathway leads to a pulsatile release of FSH or result in producing a differentially glycosylated form of FSH that is different than FSH in the constitutive pathway [212]. In **Chapter 4**, we demonstrated enhanced fertility but intact FSH levels in T3cKO females when compared to their control littermates. However, whether the enhanced fertility in T3cKO

females is due to a re-programmed endogenous FSH secretion pattern is unknown. To address this question, we are currently trying to establish a sensitive in-house FSH ELISA assay to measure FSH levels every 10 min over 6 hours. This will enable us to assess whether T3cKO animals display a more pulsatile-like pattern of FSH release.

In addition to the releasing manner, the levels of glycosylation also determine glycoprotein bioactivity (see section 1.1.3.2). Although the post-translational modification of both FSH and LH are currently considered "naturally occurring" [197]. However, *in vitro* studies demonstrate that activin A treatment in primary cultured rat pituitary cells results in a more acidic (less active) form of FSH [779], while GnRH stimulates a less acidic (more active) form of FSH in human and in dispersed rat pituitaries [780, 781]. If this is also true in murine FSH regulation, then GnRH and activin might stimulate different isoforms of FSH, thereby differentially regulating glycoprotein potency, a fact that could explain the phenotype we observed in T3cKO females. That is, the T3cKO females exhibit enhanced fertility and increased ovulation, however, these animals produce comparable FSH levels when compare to the littermate controls (**Chapter 4**).

Additionally, it is not clear whether the enhanced fertility in T3cKO females is associated with a shift from activin to GnRH dependence for FSH production. If this is true, the increased level of *Gnrhr* expression in the T3cKO pituitaries might be consistent with such an idea (**Chapter 4**). However, gonadotrope responsiveness to exogenous GnRH stimulation to release FSH is comparable between control and T3cKO male, indicating that FSH is not more dependent on GnRH in the conditional knockouts (**Chapter 4**). Therefore, it remains unclear what drives the enhanced fertility in the mice lacking *Tgfbr3* in the gonadotropes.

Learning about the actions of FSH isoforms can also help in the reproductive clinic. Human gonadotropin preparations have been used in IVF and assisted reproduction for more than five

decades [782]. Since the 1960s, gonadotropins have been extracted from menopausal human (hMG) urine and applied for ovulation induction [783], and in the early 1990s, human recombinant FSH (hrFSH) replaced hMG and since then has achieved great success in fertility clinics [784]. However, large issues with using recombinant FSH still remain today: first, not all women respond to exogenous gonadotropin stimulation; second, even when they do, in many cases gonadotropin supra-stimulation results in multi-fetal pregnancies and increases the chance of ovarian cyst formation [785]. The ideal treatment for anovulatory women is allowing their bodies to mimic healthy natural ovulation (mono-ovulation). However, the ratio of active to less active isoforms of FSH in hMG and hrFSH are mostly at a fixed ratio of 2:8, which is different from the physiological conditions in women of reproductive age [191, 192]. Additionally, hrFSH also has a different carbohydrate structure compared to endogenous active forms of FSH [786]. Therefore, it is still on demand for other gonadotropin preparations, which can reach the mono-ovulation goal for clinical purposes. Furthermore, there is still ongoing seeking for new drug targets for infertility treatment. Although we are currently uncertain whether blocking TGFBR3 can increase FSH bioactivity and hence fertility in women, it is still an important new direction for future drug development.

5.4 Implication of the tamoxifen-inducible system in the study of gene function in murine gonadotropes

The robust phenotypes we observe in conditional *Foxl2* and *Smad4* knockouts (F2S4 cKO) [409] as well as in *Smad3* and *Smad4* conditional knockout animals presented in **Chapter 2** raise the question whether early deletion of genes disrupts aspects of gonadotrope development that impair FSH production later in life. Thus, in **Chapter 3**, we employed the tamoxifen-inducible system, which enables the temporal control of gene deletion only after the animals reach adulthood.

Indeed, most of the phenotypes we observed in *Foxl2*/*Smad4* inducible knockouts (S4F2 icKO) are the same as those in the F2S4 cKO that occur during development [409]. Particularly, both animals are profoundly FSH deficient (Fig. 3.4A-B) [409]. Whereas, the F2S4 cKO females are completely sterile; in contrast, some of the F2S4 icKO females reproduce a few pups after tamoxifen-induced recombination (Fig. 3.2A). Additionally, both F2S4 cKO females and males are hypogonadal, and the females exhibit hypoplastic (thread-like) uteri, indicating hypoestrogenemia [409]. In contrast, reproductive organ weights in F2S4 icKO females were comparable to those of control females (Fig. 3.2C-D). Nevertheless, follicle development was clearly impaired in female of both models (Fig. 3.2 B) [409]. The lack of differences in uterine weights between control and the F2S4 icKO females could have reflected the timing of sampling during the cycle and/or the fact that the F2S4 icKO females are not as hypoestrogenemic as F2S4 cKO females. We cannot assess this in the current case because we did not measure E2 levels in our model, because of lack of confidence in available E2 assays. Most remarkably, spermatogenesis is impaired in F2S4 cKO males but not in F2S4 icKO males, suggesting a variable requirement for FSH at different life stages. These results suggest that, to study gene function in adult animals and avoid of developmental defects, a strategy that enables temporal control of gene recombination is required.

The data presented in **Chapter 3** are the first investigation of gene function in the adult gonadotropes using the tamoxifen-inducible recombination system (TAM-icKO). TAM-inducible Cre-ERT/Cre-ERT2 recombination systems have been established and widely applied in multiple research fields; however, they have never before been employed in studying pituitary gland [624-626]. This is in part due to the fact that the tamoxifen metabolite 4-OHT binds to endogenous estrogen receptors (see section 1.3.2 and 1.3.3) [623]. Consistent with previous observations, our

data presented in **Chapter 3** demonstrate that short-term TAM exposure has no toxic effect in mice, since all animals survived and appeared indistinguishable from those injected with oil vehicle [637]. Although TAM exposure transiently suppressed *Gnrhr* expression in gonadotropes, this inhibitory effect lasted no longer than two weeks after the last injection (Fig. S3.6). Furthermore, four weeks after the last injection, control animals injected with TAM exhibited phenotypes (including pituitary gene expression, serum gonadotropin levels and fertility) no different from control animals injected with oil vehicle. Moreover, since the Cre recombinase is introduced in *Gnrhr* locus, which is also expressed in the male germline cells (spermatid) [649, 725]. Therefore, one might worry that the induced recombination will also occur in the testes, thereby influencing testicular function. However, short-term TAM effects in the testes, if any, are transient because Cre activity is only active in the round spermatid stage [649]. To confirm the temporal action of Cre recombinase in the male germline cells, we assessed gene recombination in pituitary and testis using PCR analysis. Our data demonstrate that one month after the last injection, *Foxl2* gene recombination could be detected in the pituitary gland, but not in the testis and epididymis (data not shown). Therefore, we are confident in using this model for future studies investigating gene function specifically in adult gonadotropes.

To optimize TAM-induced gene recombination efficiency in adult gonadotropes, we used mice carrying a reporter allele *Rosa26*$^{\text{LSL-eYFP}}$. Our data suggest that the recombination efficiency is dose- and time-sensitive (Fig. 3.1B and S3.9). First, one or two injections of 2 mg of TAM failed to induce eYFP expression up to 1 month after the last injection (Fig. 3.1B). Whereas, when mice were injected with 2 mg of TAM every other day, four times in total, we observed that ~55% of LH+ gonadotropes expressed eYFP after one week, and ~75% after four weeks (Fig. 3.1A-B), suggesting that the recombination efficiency is time- -sensitive. Using the 4 injection protocol we

successfully recombined almost all floxed *Foxl2* alleles in gonadotropes in the iGRIC animals (Fig. 3.1C), suggesting that the recombination efficiency is also allele-dependent. Next, we asked whether the recombination efficiency could be further improved. Cre activity is dependent on the expression of *Gnrhr* in iGRIC mice, but *Gnrhr* expression is reduced for at least one week after exposure to TAM; therefore, Cre expression might similarly be impaired by TAM in iGRIC mice. Therefore, we optimized the protocol by leaving one week between the first two and the last two injections. Under these conditions, we observed on average ~87% with up to 99% gonadotropes expressed eYFP. All in all, our experience suggests that recombination efficiency varies significantly in a time-, dose-, and allele-dependent manner. Therefore, some modifications of the TAM injection protocol may prove necessary when employing this strategy in the other studies.

5.5 The complex roles of TGFBR3 in gonadotropes *in vivo*

As presented in **Chapter 4**, in the absence of *Tgfbr3*, gonadotrope responsiveness to inhibin A for FSH production is significantly impaired both *in vivo* and *ex vivo*. As predicted, T3cKO females are supra-fertile, ovulate more eggs per cycle, and produce approximately two more pups per litter than the control littermates. Surprisingly, T3cKO mice have comparable serum FSH levels to control animals. Additionally, although expressing significantly higher levels of the *Gnrhr* gene in T3cKO pituitaries, the mice respond equivalently to GnRH stimulation when compared to the controls. These results indicate that TGFBR3's role in pituitary gonadotropes may be more complex than originally conceived.

5.5.1 TGFBR3 is shared by TGFβ ligands in gonadotropes

In primary cultured murine pituitary cells, basal *Fshb* expression is significantly lower (40%) in the cells lacking *Tgfbr3* than in controls (Fig. 4.3B,D and S3.1). Additionally, T3cKO animals produce equivalent quantities of FSH production *in vivo* (Fig. 4.5A-E). These observations suggest that TGFBR3 may be required by another ligand, which positively regulates FSH synthesis in the pituitary.

Inhibins bind to TGFBR3 through their α subunit; activins, in contrast, lack the α subunit, aand do not bind to TGFBR3 [449]. Therefore, it is possible that another TGFβ ligand, other than activins, binds to TGFBR3 and stimulates *Fshb* transcription in cultured pituitary cells. The identity of this ligand is presently unclear. However, here we speculate that the ligand must meet the following criteria: it must belong to the TGFβ family; it must function through binding to ACVR2A, ALK4, 5 or 7 receptors; and it must signal through activation of SMAD3, SMAD4 and FOXL2 transcription molecules. Other than activins, activin type II receptors interact with other TGFβ family proteins, including TGFβ2 [440, 443, 444, 465], nodal [787], BMP7 [788], GDF8 (also known as myostatin) [789, 790], and GDF11 (also known as BMP11) [790]. Among these ligands, TGFβs, BMP7, and GDF11 have been demonstrated binding to TGFBR3 in non-gonadotrope cells [445, 791]. Intriguingly, both GDF8 and GDF11 stimulate FSH release in LβT2 cells, with significantly higher induction under GDF11 stimulation [790]. Moreover, both GDF8 and GDF11 can bind and transduce signals through activin type II receptors (ACVR2B) and activin type I receptors, including ALK4 and ALK5 [789, 792, 793]. In addition, GDF11 and GDF8 can also signal through ALK7 [792]. Furthermore, binding of GDF11, and to a lesser extent binding of GDF8, to activin receptors activates SMAD3 and SMAD4 signaling molecules [790]. Additionally, like activin, GDF11 and GDF8 activities are bio-neutralized by follistatins [794,

795]. However, the role of GDF11 or GDF8 in adult animals remains elusive due to the embryonic lethality in the global knockouts [796]. Hence, a conditional knockout of *Gdf11* and/or *Gdf8* in gonadotropes is necessary to determine the function of GDF11 in the regulation of FSH *in vivo*, and to determine whether it acts via TGFBR3 to stimulate FSH production in gonadotropes.

Furthermore, it is necessary to identify which domain (membrane distal or proximal) of TGFBR3 that GDF11 may bind. Inhibins specifically bind to the membrane-proximal domain of TGFBR3, therefore if GDF11 binds to the same compartment, inhibin antagonism may also be conducted through competitively binding to TGFBR3 through its α subunit. This also suggests that the assessment of the potential stimulatory effects of GDF11 on FSH synthesis should be assessed in both the presence and absence of inhibins.

5.5.2 TGFBR3's role in mediating inhibin antagonism

In addition to functioning as a co-receptor for TGFβ superfamily ligands, TGFBR3 also directly interacts with and influences the fate of the type II and I TGFβ receptors. At the cell surface, type II and I receptors can either be internalized by clathrin-mediated endocytosis and activate SMAD signaling molecules, or they can undergo clathrin-independent membrane pathway, which leads to the receptors' degradation [797, 798]. Additionally, several studies suggest that TGFBR3 facilities TGFβ and BMP signaling by stabilizing the type II and I receptors' complex presence on the cell membrane and preventing cytoplasmic receptor clearance [797, 799]. Like the TGFβ type I and II receptors, TGFBR3 also undergoes internalization. Depending on the ligand to which it binds, TGFBR3 either undergoes degradation or receptor recycling [800]. In Y1 mouse adrenocortical cells, TGFβ2 treatment efficiently reduces >50% of TGFBR3 at the cell surface in 4 hours and further eliminates 90% of TGFBR3 within 24 hours. Inhibin A can similarly decrease

surface TGFBR3 by 50% in 4 hours; however, the receptor internalization reaches a plateau during the following 21 hours [798, 800]. A further study demonstrates that TGFβ2 directs the internalized TGFBR3 to lysosomes for degradation while total TGFBR3 protein levels remain intact when treated with inhibin A, suggesting that inhibin leads to TGFBR3 receptor recycling rather than degradation [800]. The fate of TGFBR3 endocytosis upon binding to inhibins and activin type II receptors in gonadotropes remains unclear. Therefore, we cannot exclude the fact that the inhibins negatively regulate FSH production via TGFBR3 by mediating TGFBR3 intracellular trafficking, thereby blocking its role as co-receptors for the other agonists (e.g. GDF11 and GDF8). Additionally, although inhibin A and inhibin B can both bind to TGFBR3, their binding affinities to TGFBR3 are quite different (see section 5.4.3) [429]. Thus, it is possible that inhibin A and B act differently to suppress FSH due to distinct interactions with TGFBR3. For example, one may competitively bind to and sequester activin type II receptors with the association of TGFBR3 at the ectodomain while the other may bind to TGFBR3 and then internalize the receptor, thereby reducing the chances of activin-like ligands binding to the receptor. To this end, we have only demonstrated the antagonism by inhibin A in the presence and absence of TGFBR3 *ex vivo* and *in vivo* (Fig. 4.3B, D-E and S4.1). The action of inhibin B through TGFBR3, however, remains known.

5.5.3 Inhibin antagonism to FSH production may be more complicated than currently thought

Although TGFBR3 is the only confirmed inhibin co-receptor, it is not clear whether additional proteins are involved in inhibin B antagonism of FSH. Previous research using mouse

adrenocortical cell lines has revealed an additional undefined protein (145-148kDa) involved in the inhibin-TGFBR3-activin type II receptor [801].

As described previously, inhibin B more potently antagonizes FSH production than inhibin A both *in vivo* (1.45-fold) and *in vitro* (4.2-fold) [737]. In contrast, inhibin A has higher (5 to 6.2 fold) affinity for TGFBR3/activin type II receptor complexes than inhibin B [745]. These data suggest that the increased inhibin B antagonism potency may be mediated by co-receptors in addition to or in place of TGFBR3 [737]. If this proves true, the presence of the unidentified protein may compensate for the loss of TGFBR3 in the T3cKO mice for maintaining FSH levels. Further study is necessary to clarify whether such a component exists at all and whether it participates in inhibin B's actions in gonadotropes. In fact, inhibin B is the predominant form of inhibin in females and the only one in males, has not been assessed [286, 802]. In female rodent, inhibin B level is elevated and FSH level is low in metestrus and diestrus (see section 1.1.3.3.2 and Fig 1.4B). Inhibin B level begins to decline in the following afternoon (proestrus), prior to the pre-ovulatory FSH and LH surge [286]. Therefore, a following experiment should be conducted for assessing inhibin B actions at the stages of proestrus afternoon into early morning of estrus in control and T3cKO females.

5.6 Inhibins' actions outside of the pituitary gland

In recent years, inhibins' role in bone metabolism, adrenal function, and gonadal tumors has been more broadly revealed [801, 803-805]. Today, serum inhibin levels are assessed in the clinic as biomarkers for diagnosis of pregnancy and monitoring for pregnancy loss and for diagnosis of prenatal Down syndrome, pre-eclampsia, ovarian cancer and ovarian reserve [806, 807]. Therefore, it is important to illustrate the mechanism of inhibins' actions in specific cell

types. As described in section 1.1.3.3, inhibins are expressed in Sertoli cells in the testes and in granulosa (and luteal) cells in the ovaries [808, 809]. Inhibin levels are undetectable in gonadectomized rats, confirming that the gonads are the main source of circulating inhibins [257, 810]. In fact, inhibins also perform important functions in an autocrine or paracrine manner within the gonads, particularly in the ovaries [811] where they influence folliculogenesis by regulating granulosa cell proliferation and oocyte growth, as well as stimulating steroid hormone production [812-815]. Furthermore, inhibin homeostasis is critical for ovarian function. Male and female mice that lack the inhibin α subunit (*Inha-/-*), which only makes activins, develop mixed or incompletely differentiated gonadal stromal tumors as early as 4 weeks of age [804]. The development of gonadal tumors in inhibin-deficient mice demonstrates that inhibins act as tumor-suppressors in the gonads. In the absence of the inhibin α subunit, the free β subunits form excess activin production in the gonads. Therefore, it is possible that the enhanced activin signaling in the gonads is responsible for the development of gonadal tumors [816]. Consist with this finding, mice lacking both inhibin α and the ACVR2A showed slower tumor progression [816]. However, due to the loss of inhibin negative feedback, *Inha-/-* pituitaries produce more FSH, and hence tumor growth may be promoted by both elevated FSH and activin. It therefore follows that deletion of the *Fshb* gene in *Inha-/-* mice significantly slows tumor growth [816]. These data suggest that abnormally elevated FSH due to the loss of inhibin negative feedback can also contribute to gonadal tumorigenesis.

Additionally, *Inha-/-* mice also display a precocious follicular development phenotype as early as the first week after birth and this eventually leads to infertility in the adults [817]. During early follicular development, oocyte growth and gransulosa cell proliferation occur in a synchronous manner and are mediated by locally produced growth factors including GDF9, BMPs,

TGFβ, Anti-Müllerian hormone (AMH), and activins/inhibins [812]. In juvenile *Inha* knockout ovaries, abnormally small oocytes were enclosed by large secondary follicles, indicating that inhibin is required for the interaction and synchronous growth between germ cells and somatic cells [817].

The mechanism of inhibin-mediated granulosa cell/oocyte interaction and whether it acts through TGFBR3 has yet to be clarified. Previously, Sarraj *et al.* reported that during follicular development, TGFBR3 was robustly expressed in all oocytes starting from the day of birth and in granulosa cells starting from the primary follicle stages, whereas little expression was detected in thecal cells [818]. Thus, we attempted to investigate whether inhibins interact with oocytes through TGFBR3 in female mice using our floxed *Tgfbr3* animals and GDF9-iCre [819] mice to drive recombination in oocytes. However, our data suggest that TGFBR3's role in the oocyte is dispensable for follicular development and female fertility (data not shown). In fact, in contrast to the immunostaining results demonstrated by Sarraj *et al.* [818], our RT-qPCR analysis showed little to nearly no expression of *Tgfbr3* genes in purified oocytes from control mice (data not shown). Whether TGFBR3 is required for inhibin action in granulosa cells, however, remains unknown, but can be addressed with our *Tgfbr3* floxed mice and granulosa cell Cre-drivers.

Taken together, the presence of TGFBR3 on cell membranes determines the potency of inhibin A actions both *in vivo* and *in vitro*. However, TGFBR3 modulates functions of many other TGFβ superfamily ligands, such as the TGFβ1-3 and some BMPs. These ligands may simultaneously require binding to TGFBR3, which means that one may competitively antagonize the other. In light of this relationship, it is important to determine the function of one ligand associated with TGFBR3 without the disruption from other competitors. Furthermore, inhibin A and B may antagonize the actions of activins through different mechanisms. Therefore, future

studies should assess the role of inhibin A and B independently. Moreover, given the context-dependent nature of TGFBR3, research on TGFBR3's roles must be conducted in cell-type-specific manner. Taking advantage of our newly established *Tgfbr3*$^{fx/fx}$ mice, we and others now can explore the function of TGFBR3 in most, if not all, cell types.

Conclusion

The results presented in this thesis contribute to our understanding of the mechanisms of activin and inhibin regulation of FSH production and female fertility *in vivo*. Although GnRH has generally been considered the most important mediator of gonadotropin synthesis and secretion *in vivo*, my data demonstrate the essential role of activin-like signaling in FSH production. Future studies should seek to uncover the interplay between GnRH and activin-like signaling in FSH synthesis *in vivo*. Do the pathways act in parallel or are they interdependent?

In contrast to activins, inhibins suppress FSH synthesis. Though the current model suggests that inhibins function as competitive receptor antagonists in the presence of TGFBR3 (betaglycan), my data challenge this idea. Whereas inhibin A actions on FSH do appear to be TGFBR3-dependent, mice lacking the co-receptor in gonadotropes produce FSH seemingly normally. These observations suggest that inhibin B may produce its actions in whole or in part via a distinct co-receptor. Future investigations must determine the actions of inhibin B on FSH synthesis and secretion in the *Tgfbr3* conditional knockout model described here. If inhibin B actions remain largely intact, the hunt for novel inhibin B binding proteins should start in earnest.

Finally, the inducible Cre-driver line described here (iGRIC) provides a valuable new tool for our lab and others to investigate gene function in gonadotropes at essentially all life stages. It will be exciting to see how these mice accelerate our understanding of GnRH, TGFβ, and steroid signaling, and more, in the years to come.

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