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. The working concentration of 4-OHT was 1.5 µM.

Targeting vector construction

The original GnRHR-IRES-Cre (GRIC) targeting vector described in (22) 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. S1A) 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. S1A) 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. S1B 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. S1C). 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/+;*Rosa26*$^{YFP/+}$ pituitary sections were prepared as described in the text Methods section. Sections were incubated in 1:500 dilutions of rabbit anti-GFP and goat anti-LHβ primary antibodies overnight at 4°C and Alexa fluor 594-conjugated donkey anti-goat (1:600) and Alexa fluor 488-conjugated donkey anti-rabbit (1:600) secondary antibodies for 1 h at room temperature. Control and induced F2S4 cKO pituitary sections were prepared as described in the text Methods section. Sections were incubated with rabbit anti-FOXL2, guinea pig anti-FSHβ (1:1000, NIDDK, AFP-1760191) and goat anti-TSH (1:250, Santa Cruz, sc-7815, RRID: AB_2208237) primary antibodies overnight at 4°C and Alexa fluor 647-conjugated donkey anti-rabbit (1:1000, Jackson Lab, 711-605-152, RRID: AB_2492288), Alexa fluor 594-conjugated donkey anti-guinea pig (1:1000, Jackson Lab, 706-605-148, RRID: AB_2340476) and Alexa fluor 488-conjugated donkey anti-goat (1:600, Life Technologies, A-11055, RRID: AB_142672) secondary antibodies for 1 h at room temperature. All sections were washed three times with PBS between primary and secondary antibody incubations. Slides were analyzed on a Zeiss Axio Imager M2 microscope or an LSM800 confocal microscope.

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.

To determine whether reduced FSH levels in the induced F2S4 cKO females impacted *Cyp19a1* expression, ovarian RNA was extracted from the same control and induced F2S4 females used in Fig. 4A. 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 100 µl 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.

Hormone analysis

Serum estradiol (E2) levels were measured by ELISA at the Ligand Assay and Analysis Core of the Center for Research in Reproduction at the University of Virginia. The reportable range was 3-300 pg/ml. Intra-assay CVs were > 20%.

Table S1: 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
Cyp19a1	
Forward	GACAGGCACCTTGTGGAAAT
Reverse	GAGGTTCACGCCACCTACTC

Supplemental Figure Legends

Fig. S1 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 *EcoR*V (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 *EcoR*V 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.

Fig. S2 Co-expression of YFP and LHβ in pituitaries of GRIC/+;*Rosa26*$^{YFP/+}$ mice. Dual-label immunofluorescence for LHβ (red) and YFP (green). The asterisk indicates a rare LHβ+/YFP- cell. Scale bar = 50 μm.

Fig. S3 FOXL2 is deleted in gonadotropes, but not thyrotropes of conditional knockout mice. A) Triple-label confocal immunofluorescence for LHβ (red), FOXL2 (green) and DAPI (blue). Scale bar = 20 μm. B) Triple-label immunofluorescence for FOXL2 (purple), FSHβ (red) and TSHβ (green). Scale bar = 50 μm.

Fig. S4 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.

Fig. S5 Serum estradiol levels and ovarian aromatase (*Cyp19a1*) gene expression in females one month after oil or tamoxifen injections. A) Serum estradiol levels analyzed by ELISA. B) RT-qPCR analysis of relative ovarian *Cyp19a1* gene expression. Data were analyzed by one-way ANOVA followed by Dunn's multiple comparisons test.

Fig. S6 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 Dunn's multiple comparisons test. *$p < 0.05$.

Fig. S7 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 followed by Dunn's multiple comparisons test. Bars with different symbols differ significantly.

Fig. S8 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 (right) 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 S1. Bars represent the means (+ SEM) of three independent experiments. Basal gene expression levels (black bars) were analyzed by one-way ANOVA followed by Dunn's multiple comparisons test. #, significantly different from the other groups.

Fig. S9 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. 6C (panels A-E) and Fig. 6D (panels F-J). Data were analyzed by two-way ANOVA followed by Sidak *post-hoc* tests. Bars with different symbols differ significantly.

Fig. S10 Quantification of LHβ-positive cells co-expressing YFP following the indicated treatments. Animals were treated and pituitaries processed essentially as in Fig. 1A,B, with the indicated differences in the timing of the injections.

Fig. S11 Recombination of the floxed *Foxl2* allele is observed in pituitary, but not testes two months following tamoxifen treatment. Pituitaries and testes were extracted from male mice of the control (GRIC-) and experimental (GRIC+) genotypes after treatment with vehicle (-) or tamoxifen (TAM, +). DNA was extracted and PCR performed using primers that detect the recombined (top) or floxed *Foxl2* allele.

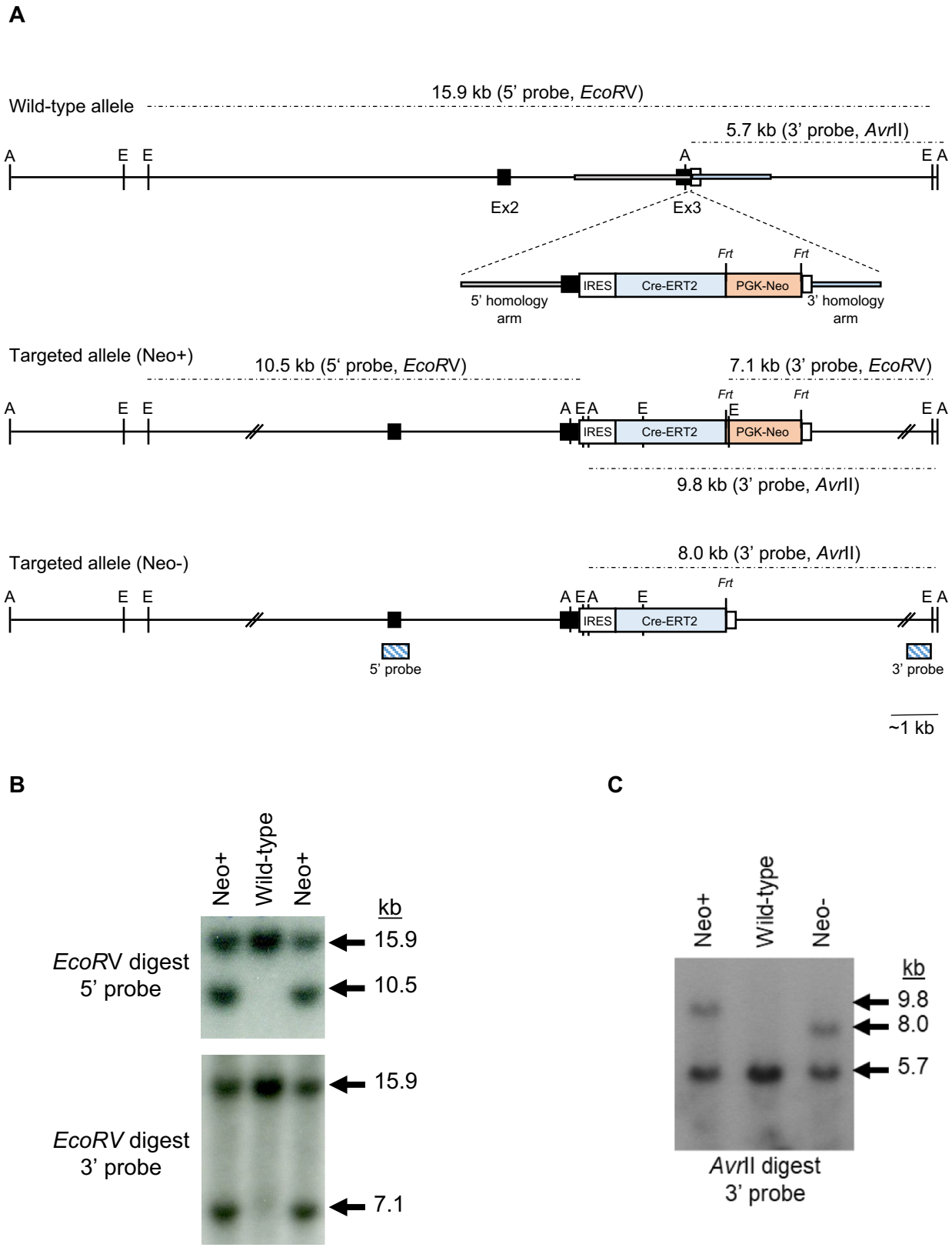

A
Wild-type allele
15.9 kb (5' probe, EcoRV)
5.7 kb (3' probe, AvrII)
A E E
A
E A
Ex2
Ex3
Frt
Frt
IRES
Cre-ERT2
PGK-Neo
5' homology arm
3' homology arm
Targeted allele (Neo+)
10.5 kb (5' probe, EcoRV)
7.1 kb (3' probe, EcoRV)
A E E
A E A
E
E
E A
9.8 kb (3' probe, AvrII)
Targeted allele (Neo-)
8.0 kb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

Fig. S1

GRIC/+;*Rosa26*$^{YFP/+}$

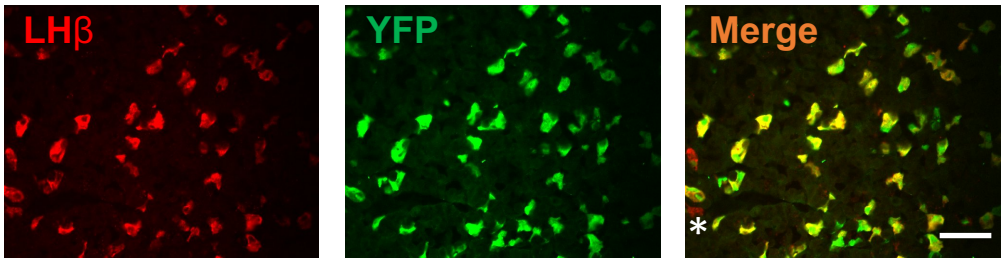


Fig. S2

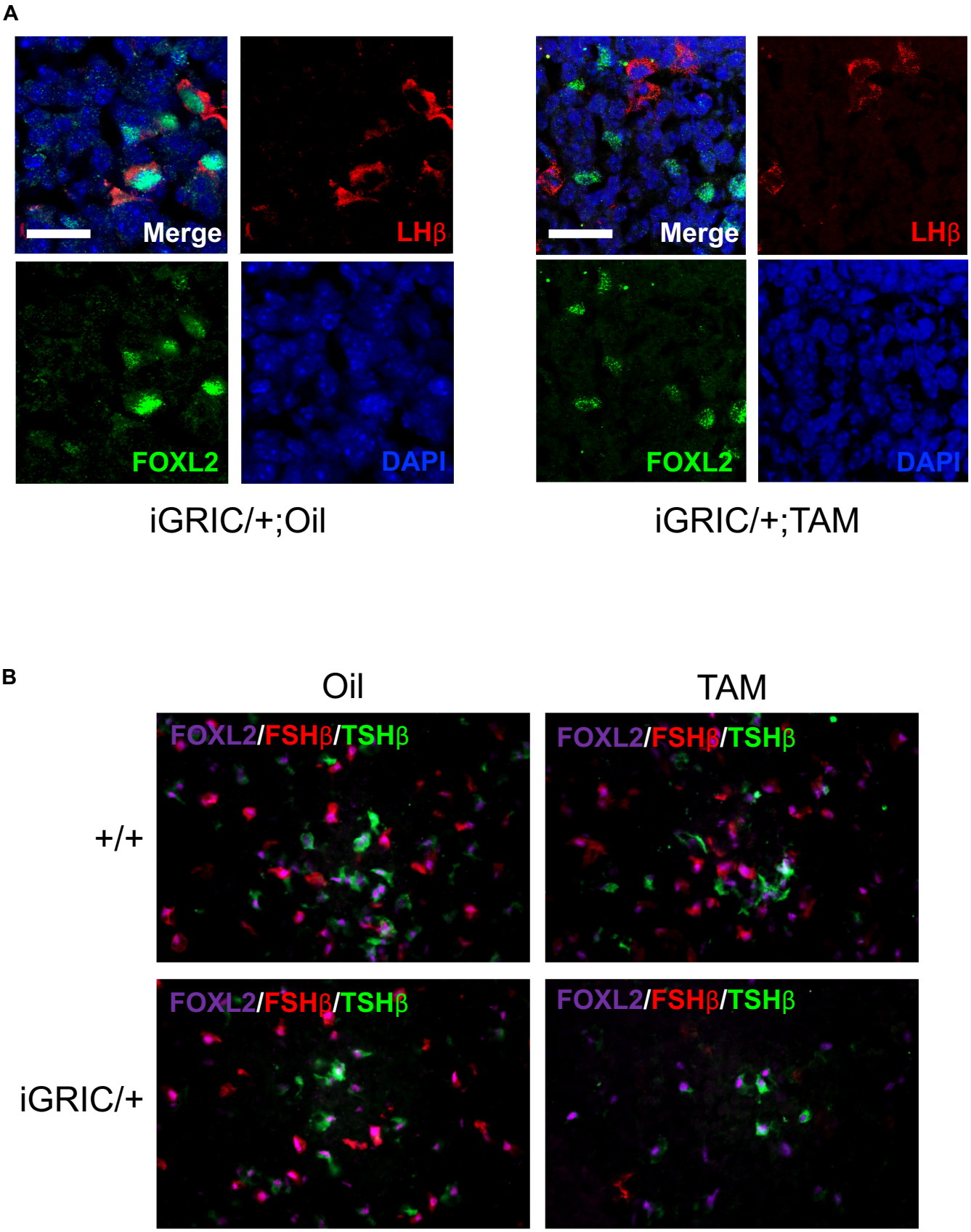

A
Merge
LHβ
FOXL2
DAPI
iGRIC/+;Oil
Merge
LHβ
FOXL2
DAPI
iGRIC/+;TAM
B
Oil
TAM
+/+
iGRIC/+
FOXL2/FSHβ/TSHβ
FOXL2/FSHβ/TSHβ
FOXL2/FSHβ/TSHβ
FOXL2/FSHβ/TSHβ

Fig. S3

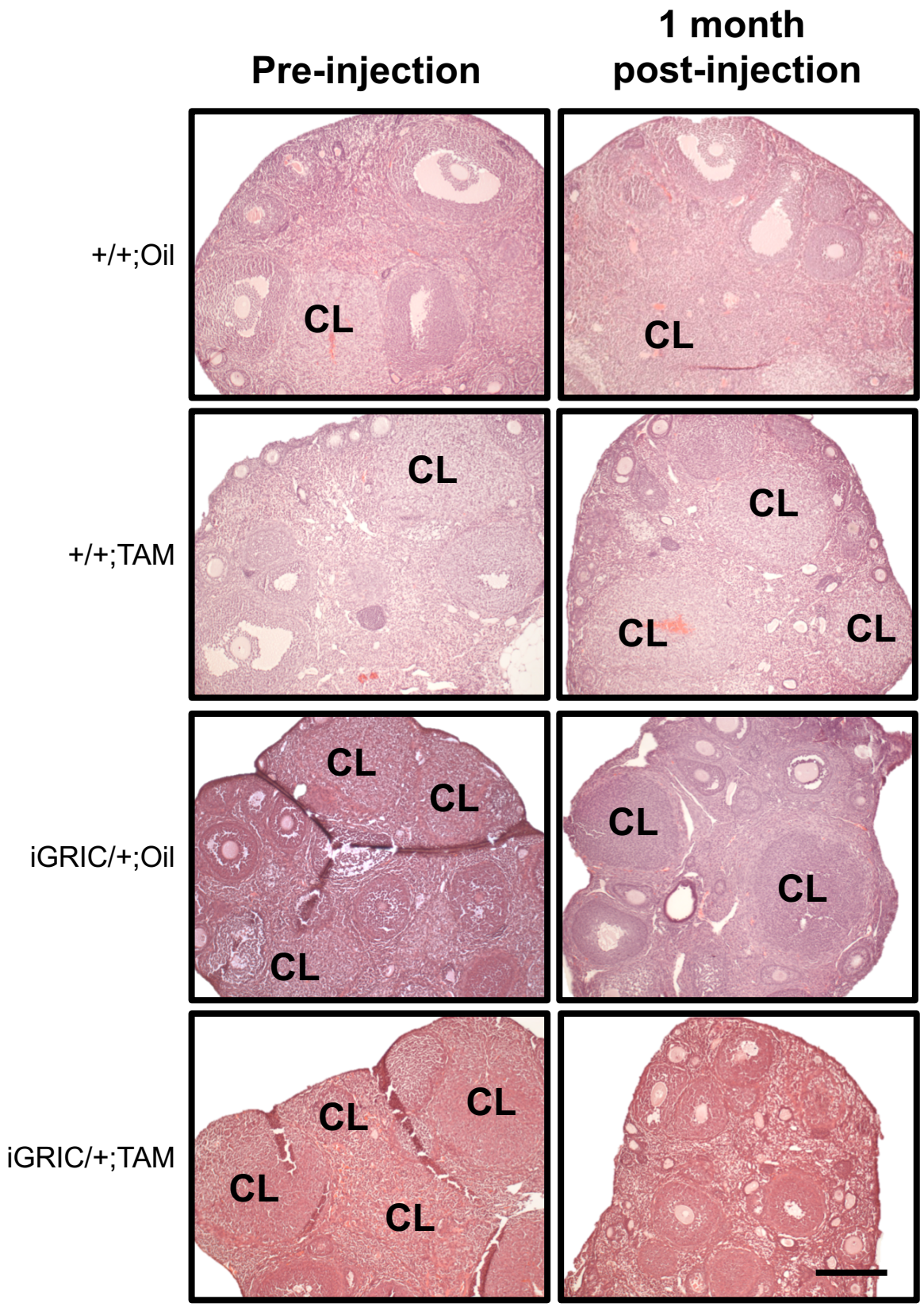

Pre-injection
1 month post-injection
+/+;Oil
+/+;TAM
iGRIC/+;Oil
iGRIC/+;TAM
CL

Fig. S4

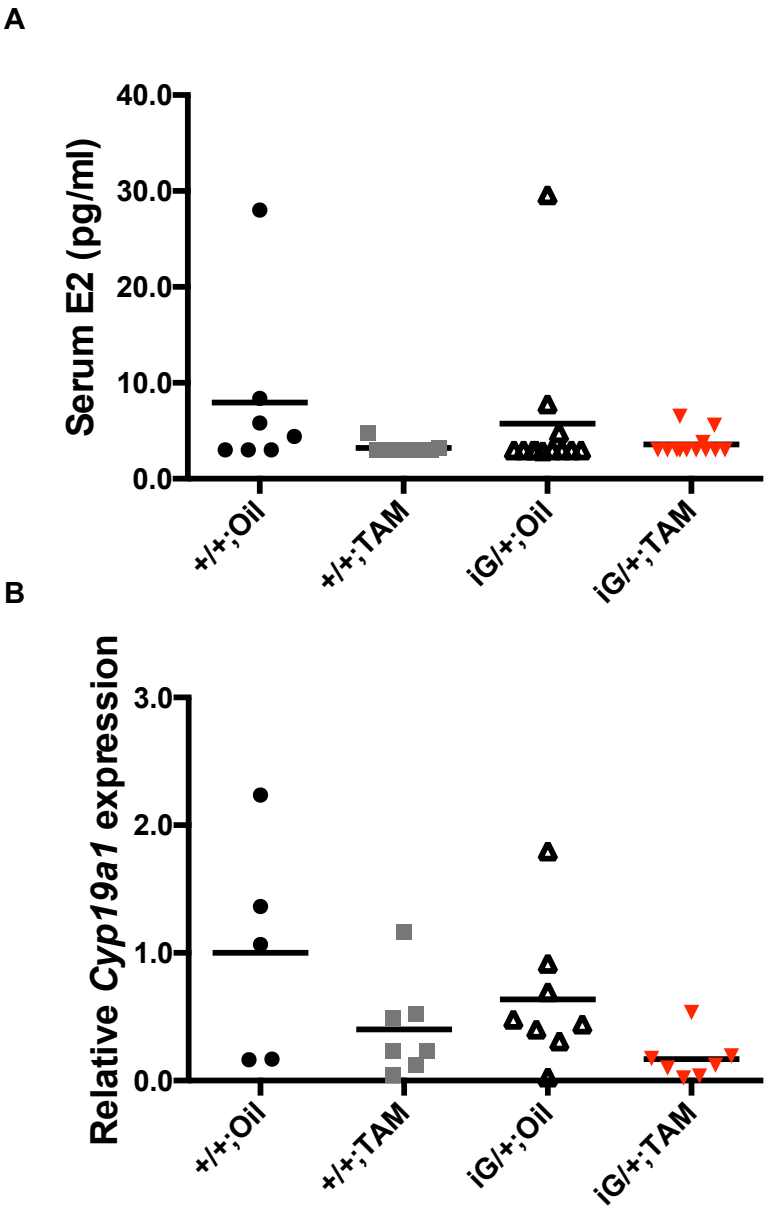

A
Serum E2 (pg/ml)
40.0
30.0
20.0
10.0
0.0
+/+;Oil
+/+;TAM
iG/+;Oil
iG/+;TAM
B
Relative Cyp19a1 expression
3.0
2.0
1.0
0.0
+/+;Oil
+/+;TAM
iG/+;Oil
iG/+;TAM

Fig. S5

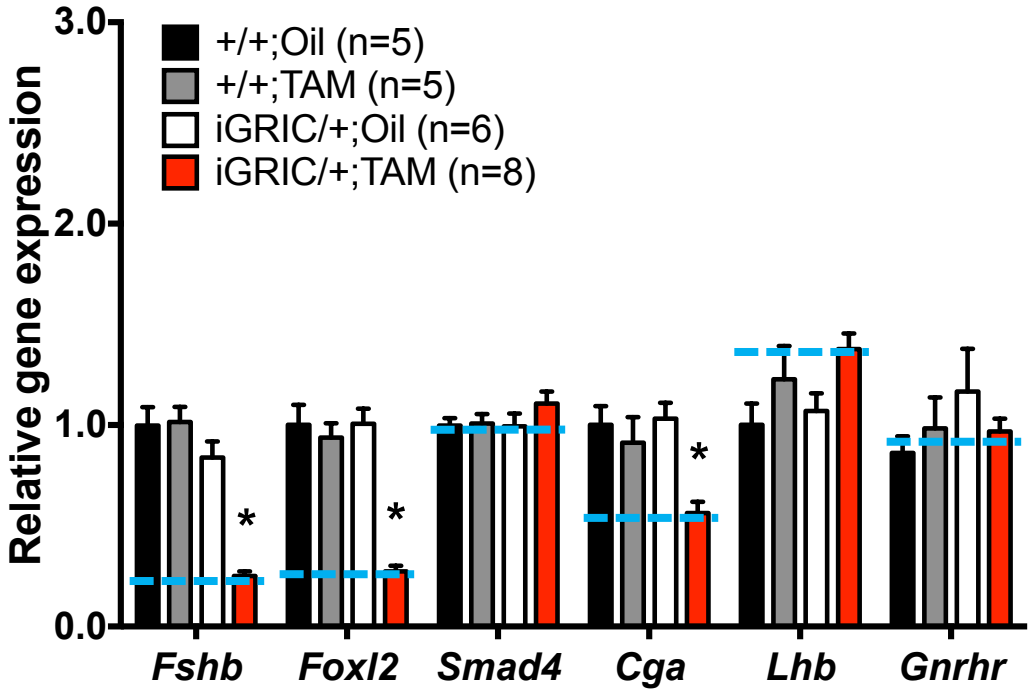

3.0
2.0
1.0
0.0
Relative gene expression
+/+;Oil (n=5)
+/+;TAM (n=5)
iGRIC/+;Oil (n=6)
iGRIC/+;TAM (n=8)
*
*
*
Fshb
Foxl2
Smad4
Cga
Lhb
Gnrhr

Fig. S6

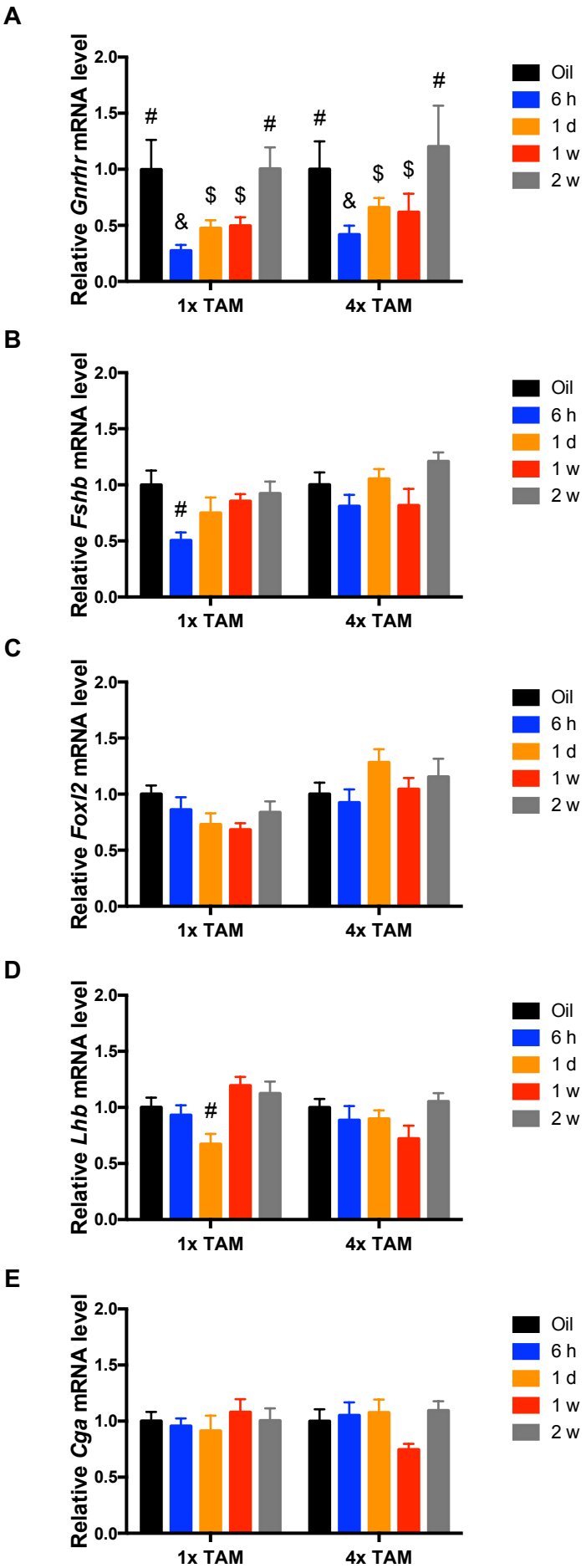

A
Relative Gnrhr mRNA level
2.0
1.5
1.0
0.5
0.0
#
&
$
$
#
#
&
$
$
#
1x TAM
4x TAM
Oil
6 h
1 d
1 w
2 w
B
Relative Fshb mRNA level
#
C
Relative Foxl2 mRNA level
D
Relative Lhb mRNA level
#
E
Relative Cga mRNA level

Fig. S7

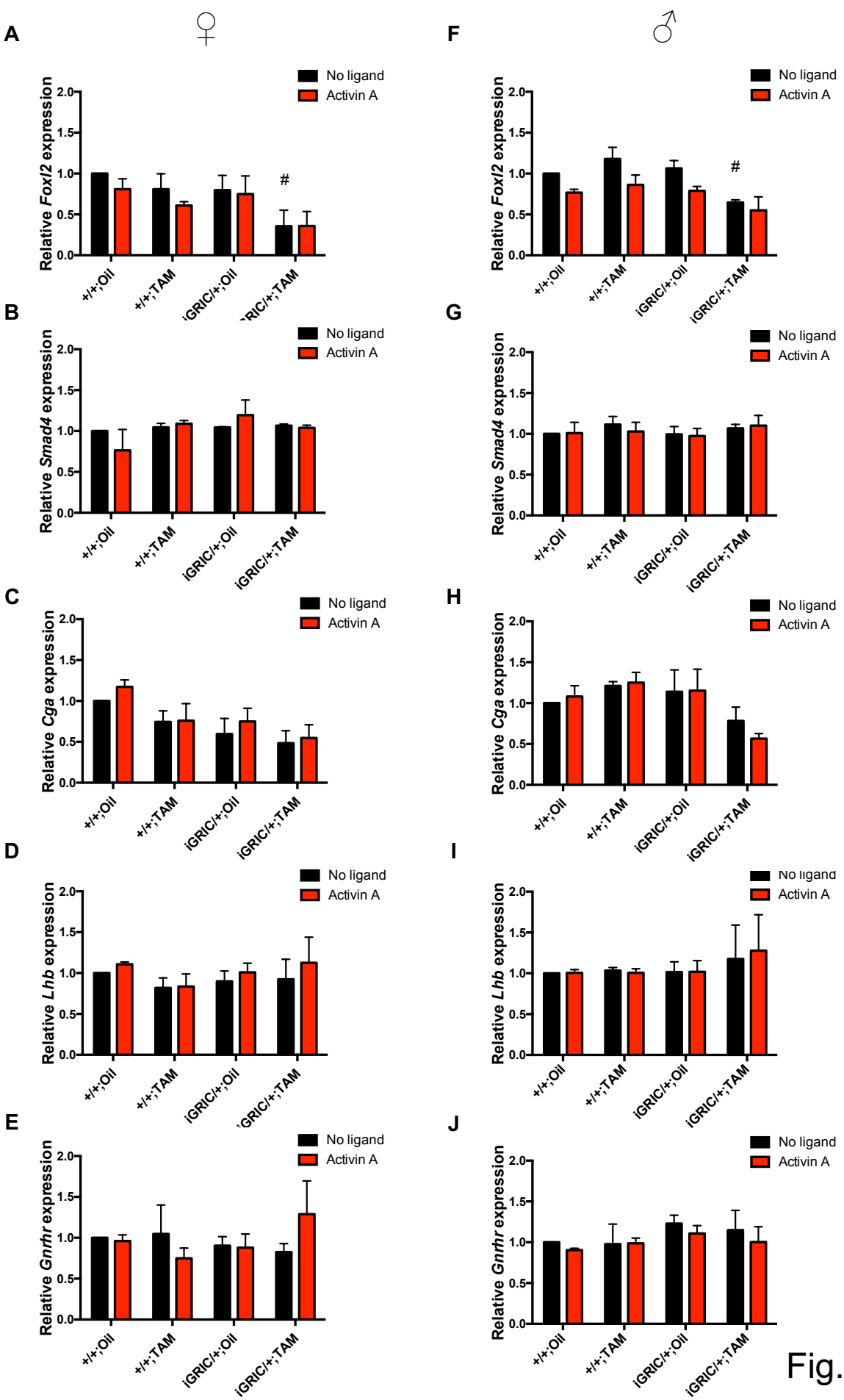
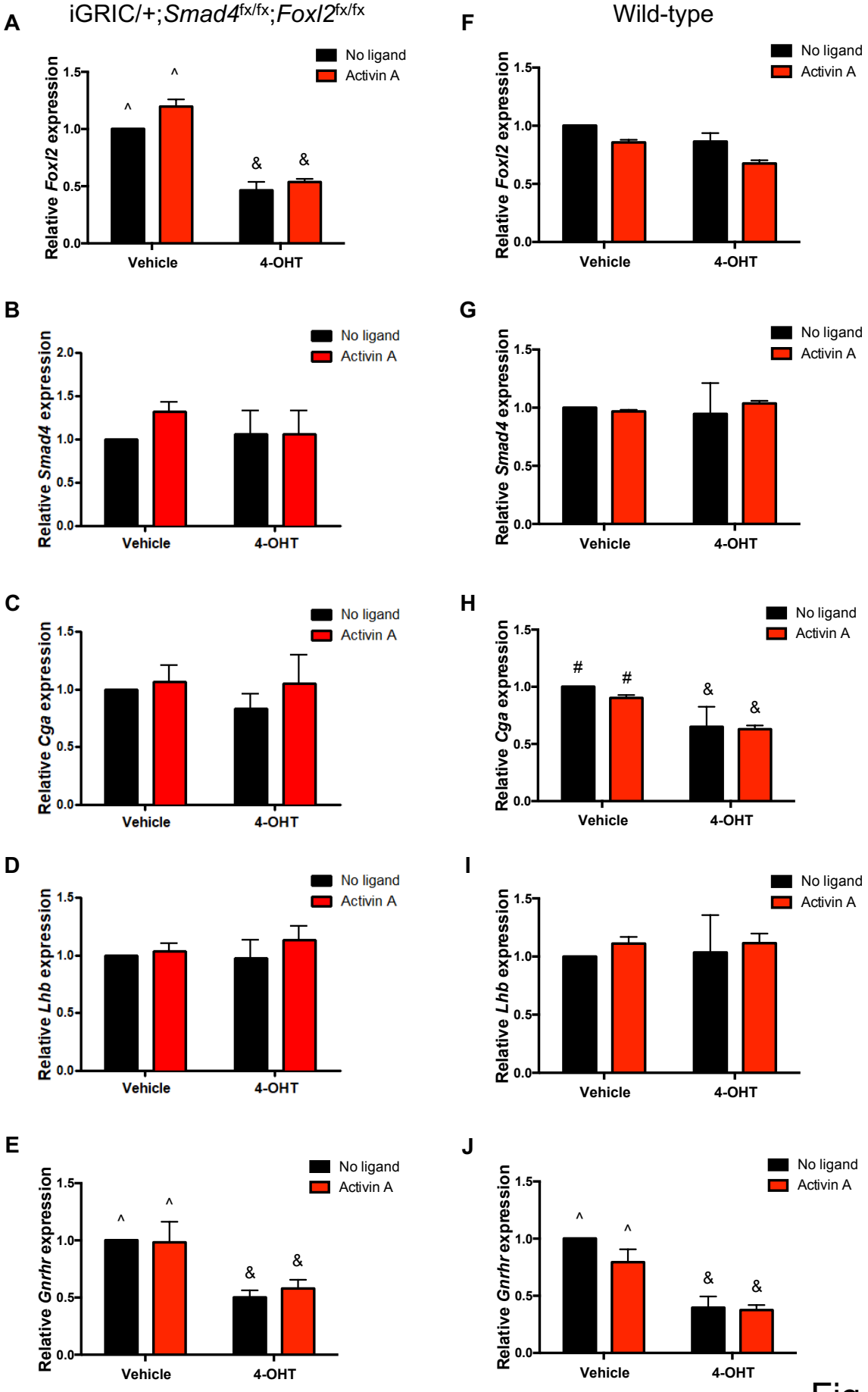
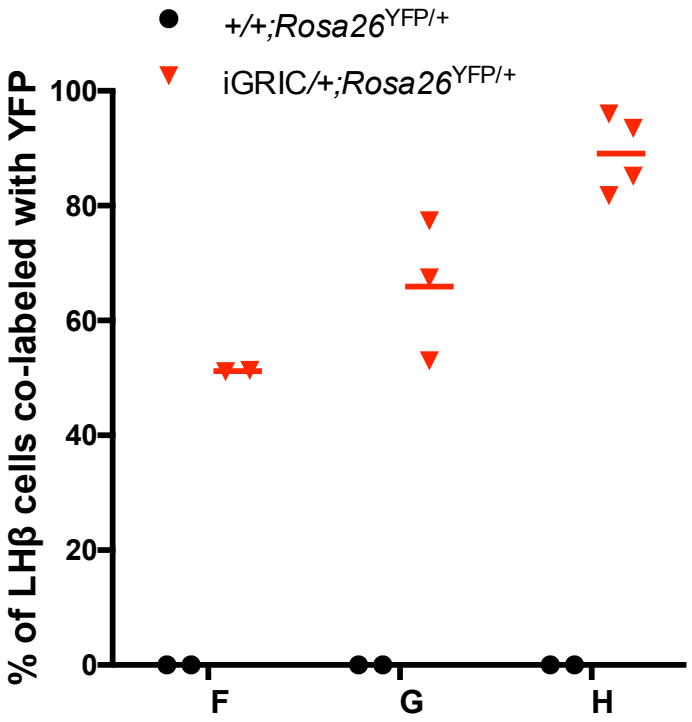
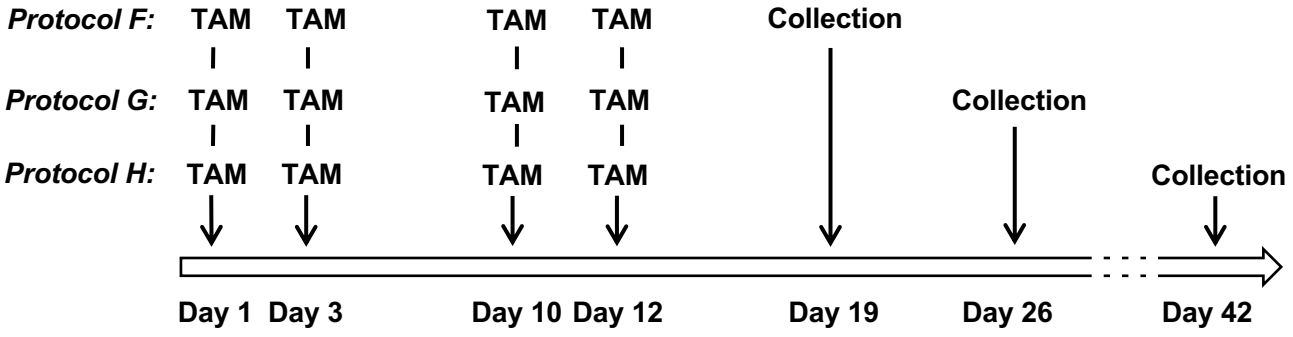

A
♀
F
♂
No ligand
Activin A
Relative Foxl2 expression
Relative Smad4 expression
Relative Cga expression
Relative Lhb expression
Relative Gnrhr expression
+/+;Oil
+/+;TAM
iGRIC/+;Oil
iGRIC/+;TAM
#
B
G
C
H
D
I
E
J

Fig. S8


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
Fig. S9


+/+;Rosa26YFP/+
iGRIC/+;Rosa26YFP/+
% of LHβ cells co-labeled with YFP
100
80
60
40
20
0
F
G
H


Protocol F:
Protocol G:
Protocol H:
TAM
TAM
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Collection
Collection
Collection
Day 1
Day 3
Day 10
Day 12
Day 19
Day 26
Day 42

Fig. S10

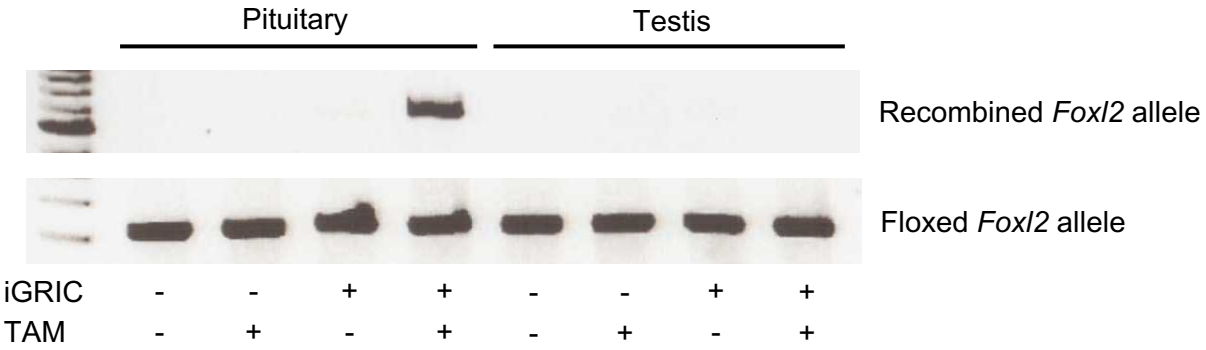
Pituitary
Testis
Recombined *Foxl2* allele
Floxed *Foxl2* allele
iGRIC - - + + - - + +
TAM - + - + - + - +

Fig. S11