Supporting Information

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SI Methods

Animals. All mice were maintained on a C57BL/6;129/SvEv mixed background. *Wt1*$^{+/\mathrm{flox}}$ (1)mice were crossed to mice carrying the *Wt1*-null allele (*Wt1*$^{+/-}$) (2), *Ctnnb1*$^{+/flox(ex3)}$ (3), *Rosa26R*$^{+/flox}$, and *AMH-Cre* transgenic mice (4) to generate *Wt1*$^{-/flox}$*AMH-Cre*, *Wt1*$^{-/flox}$*Rosa26R*$^{+/flox}$*AMH-Cre*, *Wt1*$^{-/flox}$*Ctnnb1*$^{+/flox(ex3)}$ *AMH-Cre*, *Wt1*$^{-/flox}$*Ctnnb1*$^{+/flox(ex3)}$*Rosa26R*$^{+/flox}$*AMH-Cre*, and *Ctnnb1*$^{+/flox(ex3)}$*AMH-Cre* mice. DNA isolated from postnatal tail biopsies was used for genotyping. Genotyping was performed by PCR as described previously (1).

Immunohistochemical Analysis and IF. IHC and IF were performed as described previously (5). Antibodies were diluted according to the manual as follows: WT1 (1:800, Epitomics, 2797–1), 3β-HSD (1:400, Santa Cruz, sc-30820), P450SCC (1:400, Millipore, ABS235), SOX9 (1:1,500, Millipore, AB5535), CTNNB1 (1:400, Abcam, ab6302), and AMH (1:500, Santa Cruz, sc-6886).

Electron Microscopy. Testes were dissected from control and *Wt1* mutant mice and prefixed in 2.5% (vol/vol) glutaraldehyde, 1% paraformaldehyde, 0.1 M sucrose, and 0.1 Mcacodylate for 48 h, then postfixed in 1% OsO_4 for 1 h. After dehydration with an ethanol gradient series, the samples were embedded in Epon/Araldite resin. Next, 100-nm-thick sections were cut and mounted on 200-mesh grids, then stained with uranyl acetate and lead citrate. The images were captured using a Hitachi S-3000N electron microscope.

Primary Leydig Cell Isolation and Culture. Testes from 21-d-old mice were collected and washed with PBS three times. After removal of the testes' tunica albuginea, seminiferous tubules were enzymatically digested in DMEM/F12 with 1 mg/mL collagenase IV (Sigma) and 1 mg/mL hyaluronidase type III (Sigma) and incubated in a shaking water bath at 100 oscillations (osc)/min for 15 min at 37 °C (6). Separated interstitial cells from tubules were filtered through 200 t~m sieves. Tubules left on the sieve were washed with DMEM/F12 and centrifuged for 3 min at 300 × *g*. Cell precipitates were then resuspended with DMEM/F12 containing 10% FCS, placed into culture dishes, and incubated at 37 °C with 5% CO_2.

Cell Isolation for Quantitative RT-PCR. Sertoli and Leydig cells from control adult testes were isolated as previously reported (6, 7). Leydig cells were collected as described above. The remaining tubules were digested with 2 mg/mL collagenase I, 0.5 mg/mL DNase I, and 1 mg/mL hyaluronidase type III for 20–30 min at 37 °C. Tubules were precipitated, washed twice, and incubated with 2 mg/mL collagenase I, 0.5 mg/mL DNase I, 2 mg/mL hyaluronidase, and 1 mg/mL trypsin for 40–60 min at 37 °C. The dispersed cells, containing primarily Sertoli cells and type A spermatogonia, were then washed twice with DMEM/F12 and placed into culture dishes in DMEM/F12 with 10% FCS and incubated at 37 °C and 5% CO_2. After culture overnight, cells were exposed to 20 mM Tris (pH 7.4) to remove spermatogonia by hypotonic treatment. The testicular tumors from 5-mo-old double KO mice were dissected out with forceps under the stereoscope.

Sertoli and Leydig cells from control and *Wt1*$^{-/flox}$*AMH-Cre* P1 testes were sorted by flow cytometry according to the instructions for the BD Pharmingen Transcription Factor Buffer Set. After digestion, the single-cell suspension was incubated with Fix/Perm Buffer at 2–8 °C for 40–50 min. After washing with Perm/Wash Buffer, the single-cell suspension was incubated with primary antibodies (anti-WT1 and anti–3β-HSD) in 80–100 μL of Perm/Wash Buffer for 40–50 min at 2–8 °C. The cell suspensions were then incubated with a fluorescent-conjugated secondary antibody for 40–50 min. After washing with Perm/Wash Buffer three times, the cell pellet was resuspended in 350 μL of flow cytometry staining buffer and analyzed with a BD FACSAria. Cell suspensions processed in the same way but without primary antibody were defined as the negative control. In control testes, WT1-positive and 3β-HSD–positive cells were sorted as normal Sertoli cells and Leydig cells, respectively. In *Wt1*$^{-/flox}$*AMH-Cre* testes, because the truncated WT1 protein was recognized by the anti-WT1 antibody, WT1-positive cells were sorted as *Wt1*-deficient Sertoli cells.

Construction of Adenovirus Vectors. The adenoviruses containing *Wt1* cDNA were generated using the Gateway Expression System (Invitrogen). Candidate genes were amplified by PCR and inserted into the pENTR3C vector (Invitrogen). The desired plasmids were then generated by homologous L/R recombination. Viral constructs were transduced into the 293A cell line, and a high viral particle titer (108 IU/mL) was obtained with four rounds of amplification. The viral titer was determined as previously described (8).

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2. Kreidberg JA, et al. (1993) WT-1 is required for early kidney development. *Cell* 74(4): 679–691.
3. Harada N, et al. (1999) Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. *EMBO J* 18(21):5931–5942.
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5. Gao F, et al. (2004) The Wt1+/R394W mouse displays glomerulosclerosis and early-onset renal failure characteristic of human Denys-Drash syndrome. *Mol Cell Biol* 24(22): 9899–9910.
6. Chang YF, Lee-Chang JS, Panneerdoss S, MacLean JA, 2nd, Rao MK (2011) Isolation of Sertoli, Leydig, and spermatogenic cells from the mouse testis. *Biotechniques* 51(5): 341–342, 344.
7. Wang XN, et al. (2013) The Wilms tumor gene, Wt1, is critical for mouse spermatogenesis via regulation of sertoli cell polarity and is associated with non-obstructive azoospermia in humans. *PLoS Genet* 9(8):e1003645.
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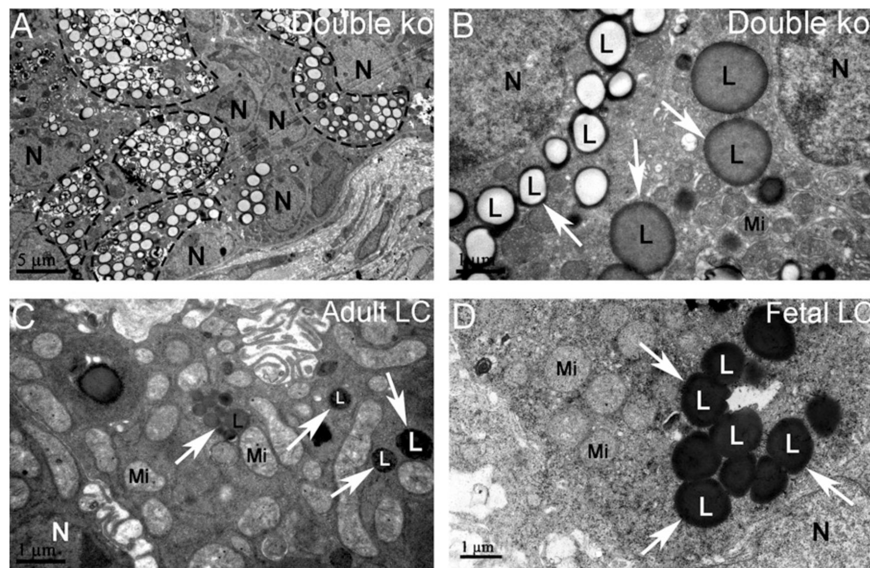

Fig. S1. Ultrastructure of Leydig-like tumor cells in double KO mice. To identify the tumor cells in double KO mice, TEM was performed. (*A*) Numerous large lipid droplet clusters were noted in Leydig-like tumor cells from double KO mice (circled with black dotted line). (*B*) Higher magnification image showing the large lipid droplets in double KO testes (white arrows). (*C*) Scattered small lipid droplets were observed in adult Leydig cells of control testes (white arrows). (*D*) Large lipid droplet clusters were observed in control fetal Leydig cells (white arrows). L, lipid droplets; Mi, Mitochondria; N, nuclei.

Fig. S2. Differential expression of Leydig cell- and Sertoli cell-specific genes in tumor cells from double KO mice. (*A*) Leydig cell-specific genes were abundantly expressed in tumor cells from double KO mice, comparable to levels in control Leydig cells. (*B*) Sertoli cell-specific genes were dramatically reduced in tumor cells from double KO mice. Data are presented as the mean ± SEM. $*P < 0.05$; $**P < 0.01$.

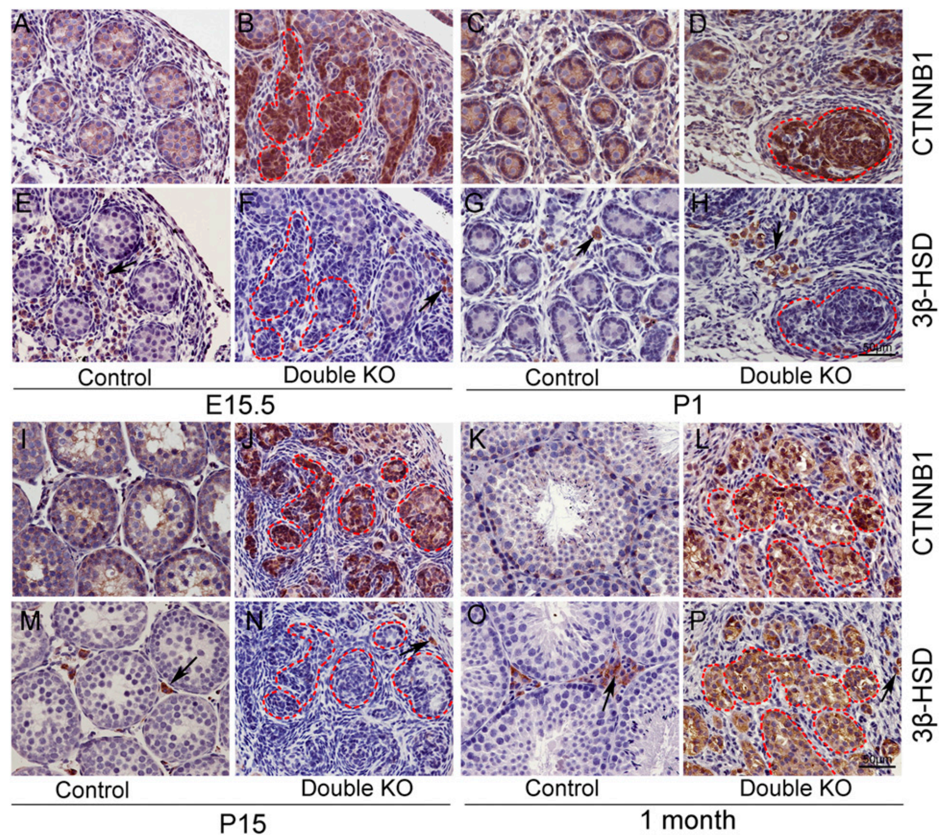

Fig. S3. Immunostaining of CTNNB1 and 3β-HSD. To examine the cell fate of *Wt1* and *Ctnnb1* (exon3) double KO Sertoli cells, the expression of CTNNB1 and 3β-HSD was analyzed by IHC using adjacent serial sections. In control testes, CTNNB1 protein was detected at Sertoli and germ cell junctions (*A*, *C*, *I*, and *K*), and 3β-HSD protein was expressed in Leydig cells (*E*, *G*, *M*, and *O*, black arrows). In double KO testes, CTNNB1-positive tumor-like cell clusters (circled with red dotted lines) were noted at E15.5 (*B*), P1 (*D*), P15 (*J*), and 1 mo (*L*). 3β-HSD protein was detected in the CTNNB1-positive tumor-like cell clusters (circled with red dotted lines) at 1 mo of age (*P*), but not at E15.5 (*F*), P1 (*H*), orP15 (*N*).

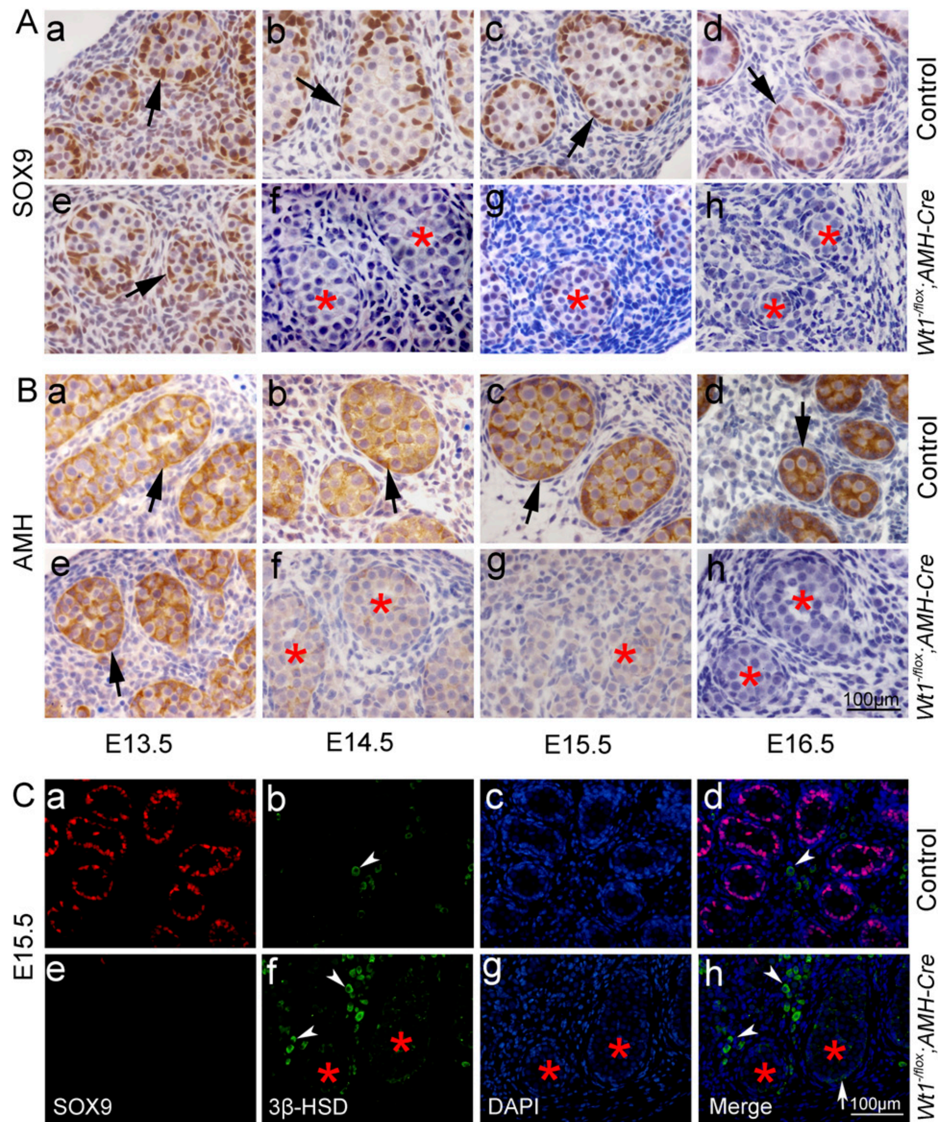

Fig. S4. SOX9 and AMH expression was absent in *Wt1*-deficient Sertoli cells. In control testes, SOX9 (*A*, *a*–*d*) and AMH (*B*, *a*–*d*) proteins were detected in Sertoli cells (black arrows) from E13.5 to E16.5. SOX9 (*A*, *e*, black arrows) and AMH (*B*, *e*, black arrows) proteins were also detected in Sertoli cells of *Wt1*$^{-/flox}$ *AMH-Cre* mice at E13.5, whereas these proteins were absent at E14.5 (*A*, *f*, and *B*, *f*), E15.5 (*A*, *g*, and *B*, *g*), and E16.5 (*A*, *h*, and *B*, *h*). IF results further showed that SOX9 protein (red) was expressed in control Sertoli cells (*C*, *a* and *d*), and 3β-HSD protein (green, white arrowheads) was expressed in Leydig cells (*C*, *b* and *d*) at E15.5. No SOX9 protein was detected in Sertoli cells (*C*, *e* and *h*) of *Wt1*$^{-/flox}$*AMH-Cre* mice at E15.5, whereas 3β-HSD protein (green, white arrowheads) was detected in the remnant testicular cords (*C*, *f* and *h*, red asterisks) at this stage.

Fig. S5. 3β-HSD protein was detected in most of the remnant testicular cords in *Wt1*$^{-/flox}$*AMH-Cre* testes. The expression of 3β-HSD protein in control and Wt1 mutant testes was examined by immunohistochemistry. In control testes, 3β-HSD protein was expressed in only Leydig cells, and no 3β-HSD signal was detected inside testicular cords at E15.5 (*A*), E16.5 (*B*), or E18.5 (*C*). By contrast, 3β-HSD protein was observed in not only normal Leydig cells of *Wt1*$^{-/flox}$ *AMH-Cre* testes but also most of the remnant testicular cords (asterisks) at E16.5 (*E*, black arrows) and E18.5 (*F*, black arrows).

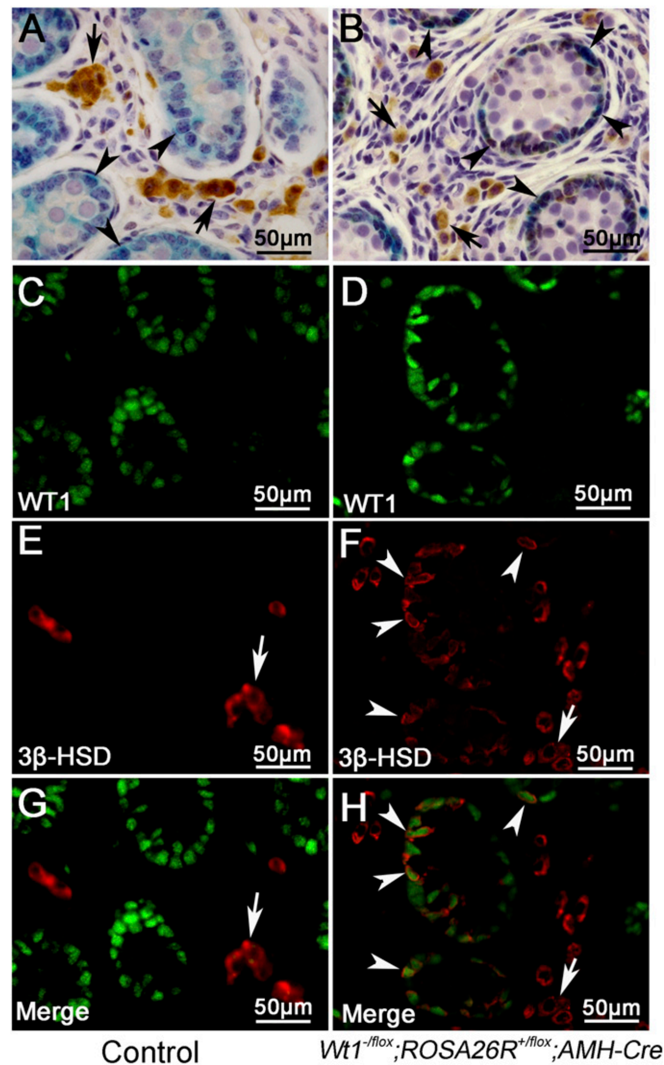

Fig S6. 3β-HSD was expressed in *Wt1*-deficient Sertoli cells. 3β-HSD and X-Gal double-positive Sertoli cells were noted in $Wt1^{-/flox}$ $Rosa26R^{+/flox}$ *AMH-Cre* testes. In control testes (*A*), the Sertoli cells were labeled with X-Gal (blue, black arrowheads) and Leydig cells were labeled with anti–3β-HSD antibody (black arrows). In $Wt1^{-/flox}$ $Rosa26R^{+/flox}$ *AMH-Cre* testes (*B*), X-Gal was detected in Sertoli cells (blue, black arrowheads) of remnant seminiferous tubules (asterisks), and 3β-HSD was detected in both Leydig cells and Sertoli cells (black arrowheads) of remnant seminiferous tubules (asterisks). The expression of Wt1 and 3β-HSD in testes of control and $Wt1^{-/flox}$ *AMH-Cre* mice at P1 was examined by IF. In control testes, Wt1 was expressed in Sertoli cells (*C* and *G*, green), and 3β-HSD was only detected in Leydig cells (*E* and *G*, red, white arrows). In $Wt1^{-/flox}$ *AMH-Cre* testes, Wt1 protein was detected in Sertoli cells (*D* and *H*, green), and 3β-HSD was detected in both Leydig cells (*F* and *H*, white arrows) and Wt1-positive Sertoli cells (*H*, white arrowheads).

Fig. S7. Sertoli cell-derived 3β-HSD–positive cell clusters were observed in *Wt1* mutant testes at P15 and 1 mo. To trace the cell fate of Sertoli cell-derived 3β-HSD–positive cells, the expression of WT1 and 3β-HSD in $Wt1^{-/flox}$*AMH-Cre* testes was examined by IF. In control testes, 3β-HSD protein (green, white arrowheads) was observed in Leydig cells, and WT1 protein (red) was expressed in Sertoli cells at P15 (*A*–*C*) and 1 mo (*G*–*I*). In $Wt1^{-/flox}$*AMH-Cre* testes, several Wt1 and 3β-HSD double-positive cell clusters (white arrows) were observed at P15 (*D*–*F*) and 1 mo of age (*J*–*L*).

Fig. S8. X-Gal and ORO double-positive cell clusters were observed in adult $Wt1^{-/flox}$*AMH-Cre* testes. Serial testis sections from 4-mo-old $Wt1^{-/flox}$*AMH-Cre* mice were stained with X-Gal (*A* and *B*) and ORO (*C* and *D*). Several cell clusters were labeled with X-Gal (*B*, black arrows); these cell clusters were also stained with ORO (*D*, black arrows).

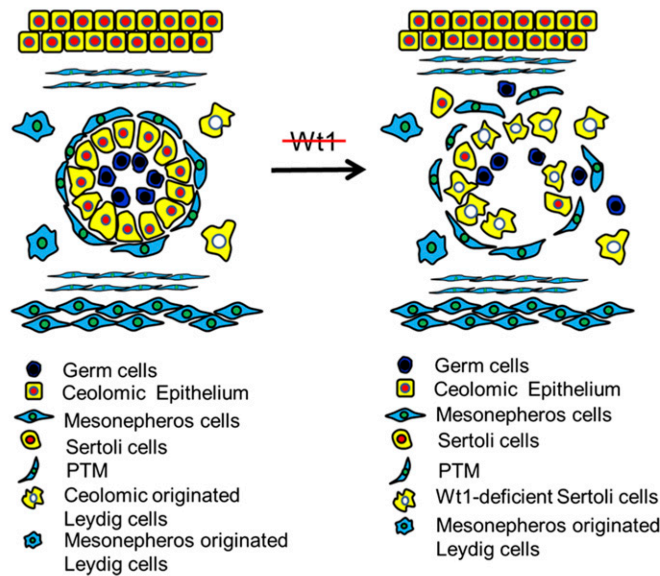

Fig. S9. Model for *Wt1* function in testis development. *Wt1* is required for Sertoli cell lineage maintenance. Inactivation of *Wt1* results in Sertoli cell-to-Leydig cell transdifferentiation, which in turn leads to the loss of Sertoli cell-specific gene expression and testicular cord disruption.

Table S1. Primers used for real-time PCR

Gene symbol	Forward primer	Reverse primer
Wt1	5′ CCAGTGTAAAACTTGTCAGCGAAA 3′	5′ ATGAGTCCTGGTGTGGGTCTTC 3′
AMH	5′ TGGTGCTAACCGTGGACTTC 3′	5′ AGCCAAATAGAAAGGCTTGCA 3′
Dhh	5′ GGACCTCGTACCCAACTACAA 3′	5′ CGATGGCTAGAGCGTTCACC 3′
Sox9	5′ GCATCTGCACAACGCGG 3′	5′ AGCCTCCAGAGCTTGCCC 3′
Erbb4	5′ CAGCGCTTCTCAGTCAGTGT 3′	5′ CTGCTGTTCCAGGTCAGAGA 3′
Shbg	5′ CCAAAATCAGCAAACCCCATT 3′	5′ CTGGATCCCAGGTTCGAAAC 3′
Ptgds	5′ GGCTCCTGGACACTACACCT 3′	5′ CATAGTTGGCCTCCACCACT 3′
Gdnf	5′ GGGTGCGTTTTAACTGCCATA 3′	5′ GCCCAAACCCAAGTCAGTGA 3′
Dmrt1	5′ GGAGTCTCCCAGCACCTTACG 3′	5′ TCTGCCACTGGTTTCCAGTCT 3′
Clu	5′ TGAAGGGCCAGTGTGAAAAGT 3′	5′ TGAACAGTCCACAGACAAGATCTC 3′
3β-HSDI	5′ CTCAGTTCTTAGGCTTCAGCAATTAC 3′	5′ CCAAAGGCAAGATATGATTTAGGA 3′
P450scc	5′ CCAGTGTCCCCATGCTCAAC 3′	5′ TGCATGGTCCTTCCAGGTCT 3′
LHR	5′ AATGAGTCCATCACGCTGAAAC 3′	5′ CCTGCAATTTGGTGGAAGAGA 3′
StAR	5′ CCGGAGCAGAGTGGTGTCA 3′	5′ CAGTGGATGAAGCACCATGC 3′
Cyp17a1	5′ GATCTAAGAAGCGCTCAGGCA 3′	5′ GGGCACTGCATCACGATAAA 3′
17β-HSDIII	5′ ATGGCATCGGGAAAGCCTAT 3′	5′ CTCTTCTGCAATGGTCTGTAGC 3′
SUR2	5′ GGTTGGTGCTGCGGTCA 3′	5′ TCCGCCAGCTTCGTGG 3′
EH	5′ GGACATCCACTTCATCCACGT 3′	5′ AGCAAGGGCTTTGGAGTGC 3′
GAPDH	5′ TTGTCTCCTGCGACTTCAACA 3′	5′ ACCAGGAAATGAGCTTGACAAAG 3′