Knockdown of *DDX4* decreases the number of germ cells in male and female chicken embryonic gonads

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Short title: Effects of *DDX4* KD on chicken embryonic gonads

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Abstract

DEAD-box helicase 4 (*DDX4*, also known as *vasa*) is essential for the proper formation and maintenance of germ cells. Although *DDX4* is conserved in a variety of vertebrates and invertebrates, its roles differ between species. This study investigated the function of *DDX4* in chicken embryos by knocking down its expression using retroviral vectors that encoded *DDX4*-targeting microRNAs. *DDX4* was effectively depleted *in vitro* and *in vivo* via this approach. Male and female gonads of *DDX4*-knockdown embryos contained a decreased number of primordial germ cells, indicating that *DDX4* is essential to maintain a normal level of these cells in chicken embryos of both sexes. *DMRT1* and *SOX9*, which are involved in testis determination and differentiation, were expressed as normal in male gonads of *DDX4*-knockdown embryos. By contrast, expression of *CYP19A1*, which encodes aromatase and is essential for ovary development, was significantly decreased in female gonads of *DDX4*-knockdown embryos. Expression of *FOXL2*, which plays an important role in ovary differentiation, was also slightly reduced, but this was not statistically significant. FOXL2 was previously hypothesized to regulate aromatase expression based on several pieces of evidence. These results indicate that aromatase expression is also regulated by several additional pathways.

Introduction

DEAD-box helicase 4 (DDX4, also called vasa) is a member of the DEAD-box protein family, which is important for the proper formation of germ cells. The roles of DDX4 are reported to differ between species. Expression of DDX4 has been depleted in *Drosophila melanogaster* (Styhler et al, 1998), *Caenorhabditis elegans* (Roussell and Bennett, 1993; Gruidl et al, 1996; Kawasaki ei al, 1998; Kuznicki et al, 2000; Spike et al, 2008), mice (Tanaka et al, 2000), and zebrafish (Braat et al, 2001) via gene knockout (KO), RNA interference (RNAi), and morpholino treatment. DDX4 is essential for oogenesis in most species and is necessary for male germ cells to progress through spermatogenesis in several species, including mice (Tanaka et al, 2000; Kuramochi-Miyagawa et al, 2010; Ewen-Campen et al, 2013). These diverse functions of DDX4 evolved for reproductive adaptation.

The chicken *DDX4* homolog (also called chicken vasa homologue, *CVH*) was first cloned by Tsunekawa et al. (2000). This gene encodes 663 amino acids and is located on the Z chromosome (male: ZZ, female: ZW). DDX4 protein localizes to the cytoplasm of germ cells, including presumptive primordial germ cells (PGCs) in uterine-stage embryos and spermatids and oocytes in adult gonads. These findings indicate that the chicken germ lineage is maternally predetermined (Tsunekawa et al 2000). Taylor et al. (2017) efficiently knocked out the *DDX4*

54 locus via homologous recombination mediated by transcription activator-like effector nucleases.
55 Targeted male PGCs were injected into surrogate host chicken embryos. Seventeen targeted G1
56 offspring were generated following mating of a host male cockerel with wild-type (WT) hens.
57 G1 females (hemizygous KO chickens) were sterile and did not exhibit detectable follicles
58 post-hatching. The germ cell lineage was initially formed in early embryos, but PGCs were
59 subsequently lost during meiosis. These findings indicate that DDX4 plays an important role in
60 the female reproductive system. By contrast, homozygous (null) male KO chickens could not be
61 generated because it was impossible to cross G1 female and male KO chickens (Taylor et al,
62 2017). Therefore, the function of DDX4 in male PGCs remains unclear in chickens.
63 Herein, we show that *DDX4* is essential for PGCs in chicken embryos of both sexes. We
64 knocked down *DDX4* using retroviral vectors that encoded *DDX4*-targeting microRNAs
65 (miRNAs). There was a decreased number of PGCs in male and female gonads of
66 *DDX4*-knockdown (KD) embryos. *DMRT1* and *SOX9*, which are involved in testis
67 determination and differentiation, were expressed as normal in male gonads of *DDX4*-KD
68 embryos. By contrast, expression of *CYP19A1*, which encodes aromatase, was significantly
69 decreased in female gonads of *DDX4*-KD embryos.
70

71 **Materials and Methods**

72 ***Animals and ethics statement***

73 Fertilized chicken eggs (*Gallus gallus domesticus*) were purchased from Takeuchi Hatchery
74 (Nara, Japan). The Hy-Line Maria chicken strain was used in this study. Fertilized eggs were
75 incubated at 37.8°C. The sex of each embryo was determined by PCR genotyping using
76 genomic DNA as the template (Fridolfsson & Ellegren 1999).

77 All animal experiments described in this study were approved by the Institutional Animal
78 Care and Use Committee of National University Corporation Hokkaido University and were
79 performed in accordance with the Guidelines for the Care and Use of Laboratory Animals
80 issued by Hokkaido University. This study did not involve any human participants or
81 specimens.

82

83 ***Construction of RNAi vectors***

84 Two miRNAs targeting different regions of chicken *DDX4* mRNA and two non-targeting
85 (scrambled) controls were designed using BLOCK-iT RNAi Designer (Thermo Fisher Scientific,
86 MA) and siRNA Wizard v3.1 (Thermo Fisher Scientific) (Table 1). The miRNA constructs were
87 cloned as described previously (Tanaka et al., 2017). pRFPRNAi.C and RCASRNAi were

88 provided by ARK-Genomics, The Roslin Institute (Das et al, 2006). The vectors encoding
89 *DDX4*-targeting and scrambled miRNAs were named RCASA.miRNA.DDX4.eGFP and
90 RCASA.miRNA.Sc.eGFP, respectively.

91 To test the KD efficiency using DF-1 cells, RCASA.miRNA.DDX4 and
92 RCASA.miRNA.Sc, which lacked the eGFP sequence, were constructed. A vector expressing
93 DDX4 fused to GFP was also generated for *in vitro* experiments. A forward primer containing a
94 *Cla*I site and a reverse primer containing a *Spe*I site were designed to amplify the coding DNA
95 sequence of *DDX4* (Table 1). Following digestion with *Cla*I and *Spe*I, the PCR product was
96 cloned into the eGFP-harboring RCAS.B vector that had been digested with the same restriction
97 enzymes. This construct was named RCASB.DDX4_eGFP. RCAS.B proviral DNA, RCAS.A,
98 and RCAS.B were kindly provided by Dr. Hughes, National Cancer Institute (Hughes et al,
99 1987).

100

101 ***Preparation and injection of viruses***

102 Endotoxin-free proviral DNA was prepared using a PureYield™ Plasmid Miniprep Kit
103 (Promega, WI). DF-1 cells were transfected with this DNA using Lipofectamine 2000 (Thermo
104 Fisher Scientific) according to the manufacturer's protocol, transferred to 10-mm dishes

105 (surface area of 78.5 cm^2), and cultured to sub-confluency in Dulbecco's Modified Eagle's
106 Medium supplemented with 10% (v/v) fetal bovine serum. Active viruses in the pooled medium
107 were concentrated. Briefly, 100 ml of medium was centrifuged overnight at 6,000 × *g*, and then
108 the supernatant was carefully decanted to leave a small pellet in 200 µl of medium. The
109 resuspended viral solution was aliquoted and stored at -80°C until use. The viral titer was
110 determined as described previously (Nakata et al, 2013).
111 Embryos were injected as described previously (Nakata et al, 2013) with minor
112 modifications. Approximately 3 µl of concentrated viruses containing 0.025% Fast Green
113 tracking dye was injected into the subgerminal cavity of blastderms at day 0 (Hamburger and
114 Hamilton stage X; Hamburger & Hamilton 1951). Viruses carrying RCASA.miRNA.DDX4
115 were injected into 189 eggs to KD *DDX4*. The eggs were sealed and incubated until embryonic
116 day (E)8.5. The number of embryos developed at ~E8.5 are 18 (ZW) and 15 (ZZ).
117
118 ***Reverse-transcription polymerase chain reaction (RT-PCR) and quantitative real-time***
119 ***polymerase chain reaction (qRT-PCR)***
120 For RT-PCR, total RNA was extracted from DF-1 cells infected with RCASB.DDX4_eGFP or
121 with RCASA.miRNA.DDX4 followed by RCASB.DDX4_eGFP. For qRT-PCR, total RNA was

122 extracted from gonads of male and female *DDX4*-KD embryos infected with
123 RCASA.miRNA.DDX4.eGFP and from gonads of male and female WT embryos at E8.5. Total
124 RNA was extracted using an RNeasy Kit (Invitrogen) according to the manufacturer's
125 instructions. RNA was treated with DNase I and reverse-transcribed using SuperScript III
126 reverse transcriptase (Invitrogen) and an oligo(dT) primer. qRT-PCR was performed using
127 Power SYBR® Green PCR Master Mix (Thermo Fisher Scientific) and an ABI 7300 Fast
128 Real-Time PCR System (Thermo Fisher Scientific). To ensure the amplification efficiency was
129 maximal in all reactions, the amplicon size was restricted to 90–101 bp. Reactions were
130 performed in triplicate using 96-well plates in a reaction volume of 10 μl. Data were analyzed
131 using the $^{\Delta\Delta}$Ct method, and the mRNA level of each target gene was normalized against that of
132 beta-actin (*ACTB*). The primers used to quantify *DDX4* expression are listed in Table 1, while
133 those used to measure the expression levels of other genes were previously described (Nakata et
134 al, 2013).
135
136 ***Immunohistochemistry***
137 Urogenital tissues of chicken embryos were fixed in formalin for 1 h at room temperature.
138 Paraffin sections (10 μm thick) were cut along the dorsal-ventral axis and thaw-mounted onto

MAS-coated glass slides. The experimental conditions of immunohistochemistry were described previously (Nakata et al, 2013). The anti-DDX4 antibody was kindly provided by Dr. Hattori, Kyushu University (Aramaki et al, 2009).

Alkaline phosphatase (AP) staining

Urogenital tissues of chicken embryos were fixed in 4% paraformaldehyde prepared in phosphate-buffered saline (PBS). Serial paraffin sections (10 μm thick) were cut along the anterior-posterior axis and mounted onto MAS-coated glass slides. De-waxed slides were treated with PBS containing 0.5% Triton X-100 for 20 min and incubated with the NBT/BCIP substrate (Promega).

In situ hybridization

A fragment of DND microRNA-mediated repression inhibitor 1 (*DND1*, also known as *CDH*) was amplified by RT-PCR using cDNA obtained from gonads at E8.5 as the template. The primers are listed in Table 1. RNA extraction and cDNA synthesis were performed as described earlier. The PCR product of 568 bp was subcloned using a pGEM T-Easy vector system (Promega). cDNA clones were labeled using Digoxigenin RNA Labeling Mix (Roche, Basel,

156 Switzerland) and T7 or SP6 RNA polymerase (MAXIscript™; Thermo Fisher Scientific).
157 Hybridization to serial frozen sections was performed as described previously (Nakata et al,
158 2013). The images were captured using a cooled CCD camera (DS-Ri1, Nikon corporation,
159 Tokyo, Japan) mounted on a Nikon ECLIPSE E800 microscope, and were analyzed with the
160 NIS ELEMENTS application program of Nikon corporation.
161
162 ***Statistical analysis***
163 The data are presented as mean ± standard deviation. Statistical comparison was made by
164 Student *t* test. $P < 0.005$ was considered statistically significant.
165
166 **Results**
167 ***DDX4-targeting miRNAs efficiently deplete DDX4***
168 We first examined the efficiency of *DDX4* KD using *DDX4*-targeting miRNAs in DF-1 cells.
169 These cells were infected with RCASB.DDX4_eGFP (Fig. 1a, b), an RCAS vector expressing
170 DDX4 fused to eGFP, or with RCASA.miRNA.Sc followed by RCASB.DDX4_eGFP (Fig. 1c,
171 d) as controls. eGFP was observed in both cases. However, eGFP was not detected in DF-1 cells
172 infected with RCASA.miRNA.DDX4, which encoded two *DDX4*-targeting miRNAs, prior to

RCASB.DDX4_eGFP (Fig. 1e, f). In addition, *DDX4* was detected by RT-PCR in DF-1 cells infected with RCASB.DDX4_eGFP, but not in those infected with RCASA.miRNA.DDX4 prior to RCASB.DDX4_eGFP (Fig. 1g).

We performed immunohistochemical staining of DDX4 in gonads of *DDX4*-KD embryos at E8.5. DDX4 staining was reduced in female (Fig. 2a–d) and male (Fig. 2e–h) gonads of *DDX4*-KD embryos. *DDX4* expression in gonads of WT and *DDX4*-KD embryos at E8.5 was quantified by qRT-PCR. This demonstrated that *DDX4* expression was significantly reduced in *DDX4*-KD embryos of both sexes (Fig. 2i). These results demonstrate that *DDX4*-targeting miRNAs efficiently deplete *DDX4*.

The number of PGCs is decreased in male and female gonads of DDX4-KD embryos

We performed AP staining and *DND1 in situ* hybridization to label PGCs in gonads of *DDX4*-KD embryos. PGCs exhibit AP staining in many species. DND1 co-localizes with DDX4 in germ cell lines generated from chicken embryonic gonads (Kito et al 2010) and is thus a marker of PGCs.

PGCs were detected by AP staining in female and male gonads of WT embryos at E8.5 (Fig. 3a, b). However, AP staining was not clearly observed in *DDX4*-KD embryos of either sex

(Fig. 3c, d). The *DND1 in situ* hybridization experiments yielded similar results (Fig. 3e–h).

The total number of PGCs was counted in serial sections subjected to AP staining (Fig. 3i) and *DND1 in situ* hybridization (Fig. 3j). Based on AP staining and *DND1 in situ* hybridization, the number of PGCs was decreased by 74% and 79% in *DDX4*-KD female embryos, respectively, and by 61.4% and 62.2% in *DDX4*-KD male embryos, respectively. The detection sensitivity of each method is different: the sensitivity of AP staining is relatively higher than that of *DND1 in situ* hybridization. Although total number of PGCs detected by AP-staining is larger than *DND1* in situ hybridization, the reduction rate of number of PGCs is almost same between both methods.

KD of DDX4 decreases CYP19A1 expression

We quantified expression of *DMRT1*, *SOX9*, *FOXL2*, and *CYP19A1*, which are involved in gonadal differentiation, in gonads of WT and *DDX4*-KD embryos at E8.5 (Fig. 4). In both WT and *DDX4*-KD embryos, expression of *DMRT1* and *SOX9* was high in male gonads, but low in female gonads (Fig. 4), consistent with previous reports (Smith et al, 1999a; 1999b). Expression of *DMRT1* and *SOX9* did not significantly differ between WT and *DDX4*-KD embryos.

Expression of *FOXL2* and *CYP19A1* was reported to be high in female embryonic gonads,

207 but extremely low in male embryonic gonads (Govoroun et al, 2004). We confirmed this finding
208 in WT embryos. However, *CYP19A1* expression in female gonads was significantly lower in
209 *DDX4*-KD embryos than in WT embryos (Fig. 4). Expression of *FOXL2* was slightly lower in
210 female gonads of *DDX4*-KD embryos than in those of WT embryos, but this difference was not
211 statistically significant.
212
213 **Discussion**
214 The *DDX4* gene is conserved in a variety of vertebrates and invertebrates. Although *DDX4* is
215 essential for the development and maintenance of germ cells in a wide range of species, its roles
216 differ between species. A null mutation of *DDX4* in *D. melanogaster*, in which this gene was
217 initially identified, causes female sterility due to severe defects in oogenesis, while males
218 remain fertile (Styhler et al, 1998). However, male *DDX4*-KO mice exhibit a reproductive
219 deficiency; male homozygotes do not produce sperm in the testes, and the proliferation of PGCs
220 is hampered (Tanaka et al, 2000). By contrast, disruption of *DDX4* does not affect the
221 development of female germ cells in mice, despite the fact that this gene is expressed in both
222 male and female germ cells of mice (Tanaka et al, 2000). The current study demonstrated that
223 *DDX4*-targeting miRNAs effectively suppressed *DDX4* expression *in vitro* and *in vivo* (Fig. 1,

224 2). The number of proliferative cells detected by EdU is significantly reduced in female gonads
225 of *DDX4*-KO chicken embryos at E10.5, indicating that germ cells form at an early
226 developmental stage and are subsequently lost (Taylor et al, 2017). Our results confirmed this
227 previous finding and demonstrated that the number of PGCs was also reduced in male gonads of
228 *DDX4*-KD embryos (Fig. 3). Therefore, *DDX4* is also involved in the proliferation of PGCs in
229 males, suggesting it is important for both male and female fertility in chickens.

230 We investigated the expression levels of genes involved in gonadal differentiation and
231 development in *DDX4*-KD embryos (Fig. 4). Expression of *DMRT1* and *SOX9*, which are
232 markers of testis determination and differentiation, were quantified. *DMRT1* is located on the Z
233 chromosome in birds, is a major avian male-determining factor, and begins to be expressed at
234 ~E3.5. Expression of *DMRT1* is detected in the medulla of gonads and is higher in males than in
235 females. Upon high *DMRT1* expression, *SOX9* is continuously expressed for testis development
236 in male chicken embryos after E6.5. Expression of *DMRT1* and *SOX9* was higher in male
237 gonads than in female gonads of both *DDX4*-KD and WT embryos (Fig. 4). This suggests that
238 testis differentiation and development occurred as normal in male gonads with a decreased
239 number of PGCs.

240 By contrast, expression of *CYP19A1* was significantly lower in female gonads of

241 *DDX4*-KD embryos than in those of WT embryos (Fig. 4). Gonadal sex differentiation in female
242 birds is sensitive to the sex steroid hormone estradiol. This hormone is only detected in female
243 embryonic gonads and is required and sufficient for ovarian development (Elbrecht & Smith
244 1992). Aromatase converts androgens into estradiol. Consequently, *CYP19A1* is essential for the
245 female reproductive system. Indeed, treatment with aromatase inhibitors, such as fadrozole, can
246 effectively inhibit to feminization in ZW embryos (Elbrecht & Smith 1992). Aromatase
247 localizes to the cytoplasm of somatic cells in the medulla of female embryonic gonads from
248 E6.5 onwards and its expression increases during ovarian development (Govoroun et al, 2004;
249 Smith et al. 2005). DDX4 is thought to increase the translation of several proteins required for
250 meiotic progression and the assembly of cytoplasmic granules in germ cells. The
251 ATP-dependent catalytic activity of RNA helicases regulates the translation of multiple mRNAs
252 (Carrera et al, 2000). Considering its expression pattern and function, DDX4 is not thought to
253 directly regulate expression of *CYP19A1*. Repression of the *DDX4* expression and/or a
254 decreased number of PGCs might indirectly affect aromatase expression.

255 Expression of *FOXL2*, another marker of ovary differentiation, was slightly lower in
256 female gonads of *DDX4*-KD embryos than in those of WT embryos; however, this difference
257 was not statistically significant (Fig. 4). *FOXL2* is an essential factor that is widely conserved in

vertebrates, including chickens (Loffler et al, 2003; Wang et al, 2007; Pisarska et al, 2011). The expression patterns of *FOXL2* and *CYP19A1* highly correlate with each other in the developing ovary after 4.7–12.7 days of incubation (Govoroun et al. 2004). The proteins encoded by these genes co-localize at the nuclei of medullar cord cells in female embryonic gonads. *FOXL2* is expressed just prior to *CYP19A1*, suggesting it directly or indirectly regulates transcription of *CYP19A1*, at least in embryos. This hypothesis is supported by the finding that FOXL2 expression is induced in aromatase-overexpressing male embryos (Lambeth et al, 2013). FOXL2-binding sites (5'-CACAACA-3') are located in the promoter region of *CYP19A1* (-668 and -3453 bp in the antisense strand and -3642 bp in the sense strand; Govoroun et al, 2004). However, FOXL2 fails to upregulate *CYP19A1* expression in luciferase assays (Wang and Gong, 2017). The expression patterns of FOXL2 and aromatase differ in the ovaries of adult chickens, with the former protein localizing to the granulosa cell layer and the latter protein localizing to the theca cell layers, indicating that the regulatory mechanism of aromatase differs between embryos and adults (Wang and Gong, 2017). Our data suggest that several pathways regulate expression of *CYP19A1*, in addition to that involving *FOXL2*. Furthermore, treatment with an aromatase inhibitor reduces *FOXL2* expression *in vivo*, suggesting that a regulatory feedback loop exists between *FOXL2* and *CYP19A1* (Hudson et al, 2005). Therefore, the slight reduction

in *FOXL2* expression observed in female gonads of *DDX4*-KD embryos might be due to the substantial decrease in *CYP19A1* expression.

In summary, this study demonstrates that DDX4 is important for the female and male reproductive systems of chickens. Furthermore, our data indicate repression of the *DDX4* expression and/or a decreased number of PGCs affect the regulation of *CYP19A1* expression.

Declaration of interest

The authors declare that no competing interests exist.

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Authors' contributions

NA performed all the experiments and analyzed all the data. HI constructed the retroviral vectors. AK conceived and designed the study and SM and AK wrote the manuscript. All authors read and approved the final manuscript.

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394 **Figure legends**

395 **Figure 1 *DDX4* expression is efficiently suppressed *in vitro***

396 DF-1 cells were infected with (a, b) RCASB.DDX4_eGFP alone, (c, d) RCASA.miRNA.Sc and
397 RCASB.DDX4_eGFP, or (e, f) RCASA.miRNA.DDX4 and RCASB.DDX4_eGFP. Although
398 eGFP was observed in both cases (a-d), eGFP was not detected in DF-1 cells infected with
399 RCASA.miRNA.DDX4, which encoded two *DDX4*-targeting miRNAs, prior to
400 RCASB.DDX4_eGFP (e, f). Scale bar, 100 μm. (g) RT-PCR analysis of *DDX4* in DF-1 cells
401 infected with RCASB.DDX4_eGFP alone or together with RCASA.miRNA.DDX4. The DDX4
402 expression is not detected in DF-1 cells infected with RCASA.miRNA.DDX4 prior to
403 RCASB.DDX4_eGFP (g). M: molecular marker.

404

405 **Figure 2 *DDX4* expression is efficiently suppressed *in vivo***

406 (a–h) Immunohistochemical staining of DDX4 in the left gonads of (a–d) female and (e–h) male
407 embryos at E8.5 was performed to examine the KD efficiency of *DDX4*-targeting miRNAs. (a,
408 b) Female and (e, f) male gonads of WT embryos at E8.5 exhibited normal expression of DDX4,
409 indicating that PGCs localized to the cortical and interior of the embryonic gonad, respectively.
410 DDX4 expression was suppressed in (c, d) female and (g, h) male embryos infected with

411 RCASA.miRNA.DDX4. FITC fluorescence is shown in a, c, e, g. Hoechst staining is shown in

412 B, D, F, H. Scale bar, 100 μm. (i) qRT-PCR analysis of *DDX4* in gonads at E8.5. Delivery of

413 *DDX4*-targeting miRNAs effectively suppressed *DDX4* expression. Black, dark gray, white, and

414 light gray bars correspond to WT female, *DDX4*-KD female, WT male, and *DDX4*-KD male

415 embryos, respectively. The data are presented as mean ± standard deviation. *$P < 0.005$; $n \geq 3$.

416

417 **Figure 3 The number of PGCs is decreased in gonads of *DDX4*-KD embryos**

418 (a–d) AP staining in gonads of WT and KD embryos at E8.5. Gonad sections (dorsal-ventral

419 axis) of (a) WT female, (b) WT male, (c) *DDX4*-KD female, and (d) *DDX4*-KD male embryos.

420 Marked staining was observed in female and male gonads of WT embryos (a, b), but not in

421 those of *DDX4*-KD embryos (c, d). Arrows indicate AP staining. Scale bar, 100 μm. (e–g)

422 *DND1 in situ* hybridization in frozen gonad sections of WT and KD embryo at E8.5. Gonad

423 sections of (e) WT female, (f) WT male, (g) *DDX4*-KD female, and (h) *DDX4*-KD male

424 embryos. Marked staining was observed in female and male gonads of WT embryos (E, F), but

425 not in those of *DDX4*-KD embryos (g, h). Arrows indicate *DND1* staining. Scale bar, 100 μm.

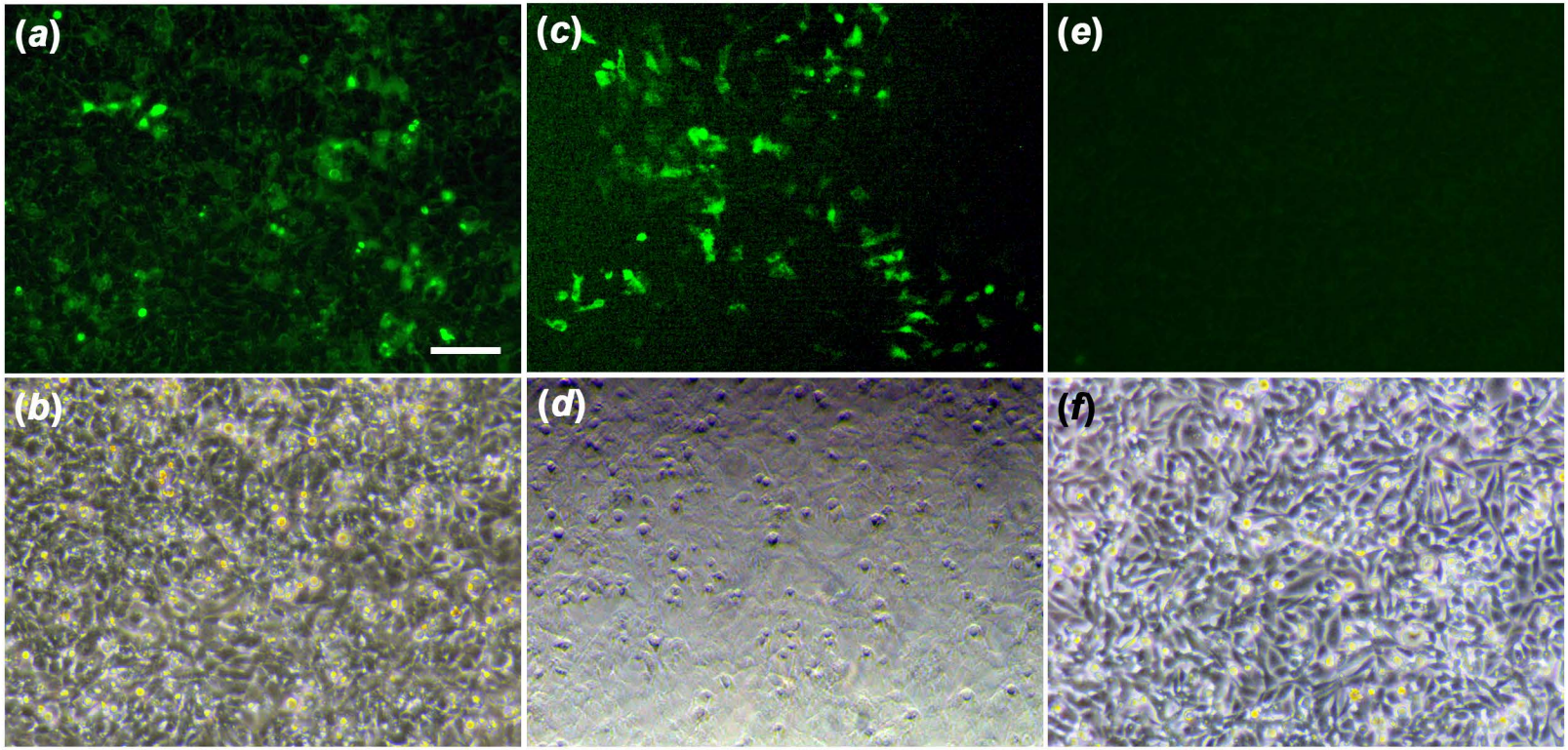
426 (i) AP staining and (j) *DND1 in situ* hybridization. Black, dark gray, white, and light gray bars

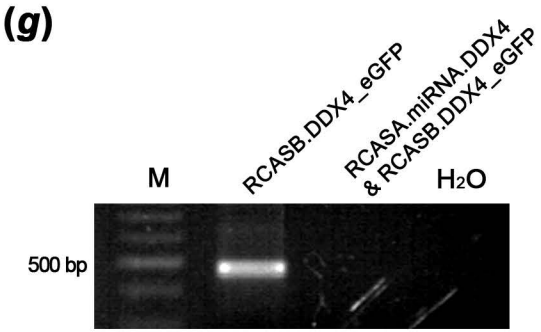
427 show the numbers of positively stained cells in gonads of WT female, *DDX4*-KD female, WT

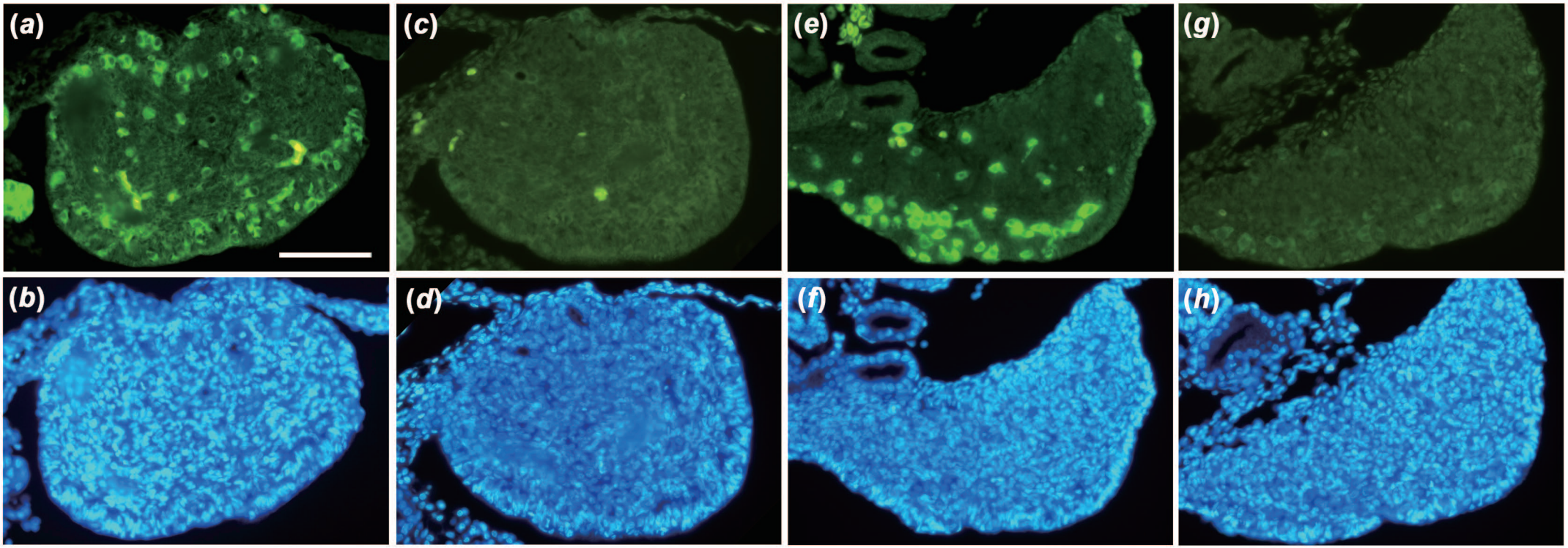
male, and *DDX4*-KD male embryos at E8.5, respectively.

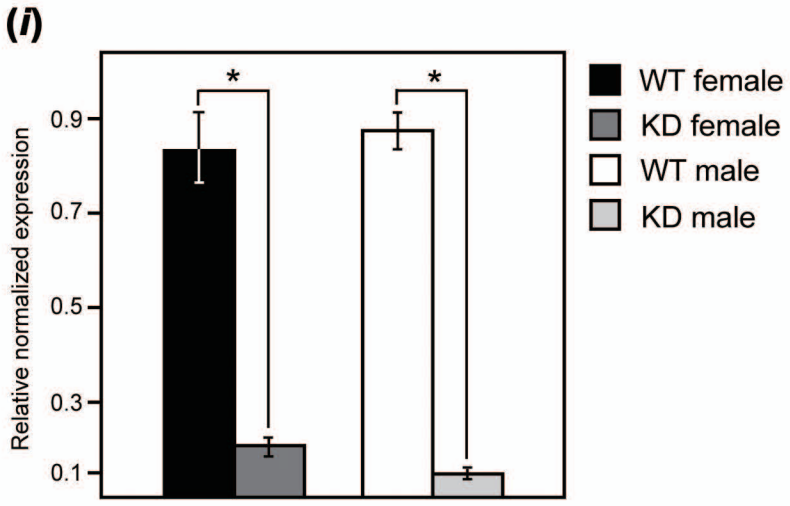
Figure 4 KD of *DDX4* decreases *CYP19A1* expression in female embryonic gonads

qRT-PCR analysis of *CYP19A1*, *FOXL2*, *DMRT1*, and *SOX9* expression in gonads of WT female (black bars), *DDX4*-KD female (dark gray bars), WT male (white bars), and *DDX4*-KD male (light gray bars) embryos at E8.5. Expression of *DMRT1* and *SOX9* did not significantly differ between WT and *DDX4*-KD embryos. *CYP19A1* expression in female gonads was significantly lower in *DDX4*-KD embryos than in WT embryos. Expression of *FOXL2* was slightly lower in female gonads of *DDX4*-KD embryos than in those of WT embryos, but this difference was not statistically significant. The data are presented as mean ± standard deviation. *$P < 0.005$; $n \geq 3$.

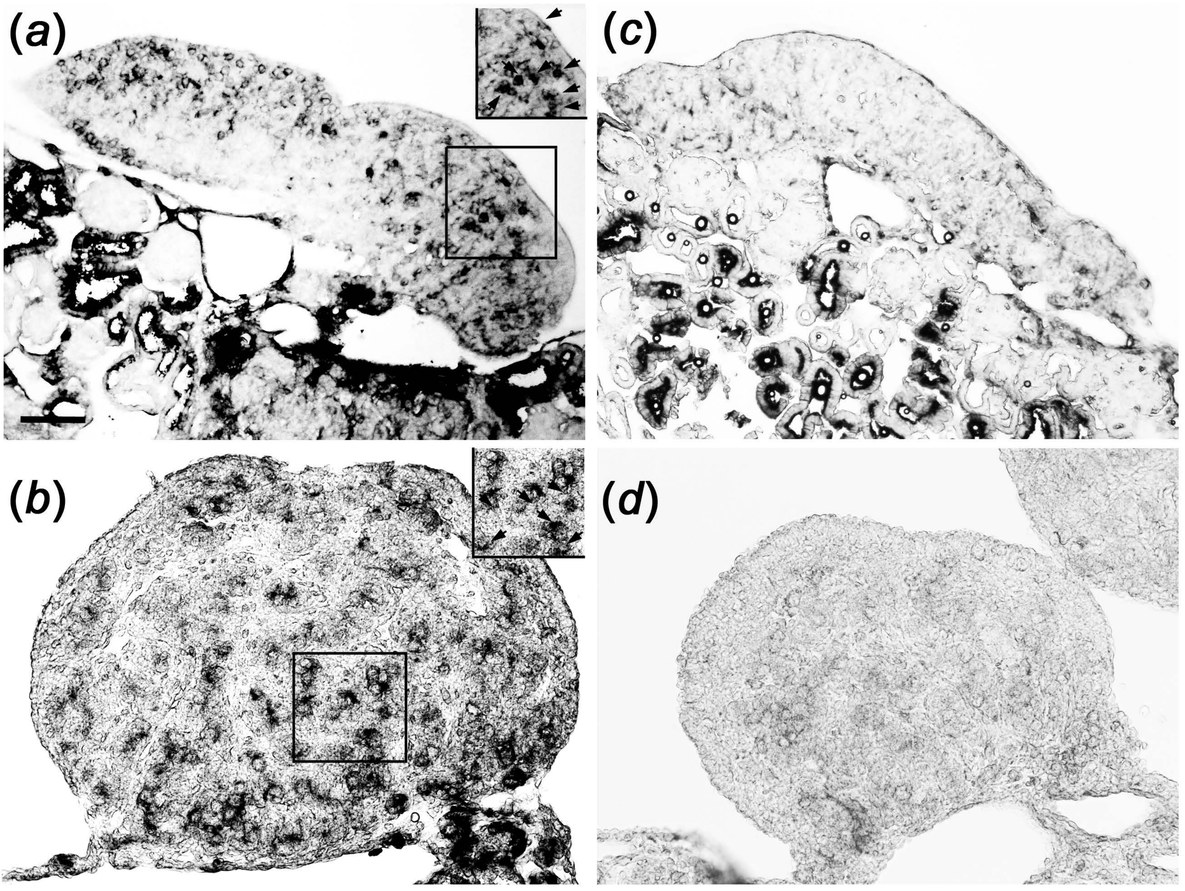

(a)
(c)
(e)
(b)
(d)
(f)


(g)
M
RCASB.DDX4_eGFP
RCASA.miRNA.DDX4
& RCASB.DDX4_eGFP
H2O
500 bp

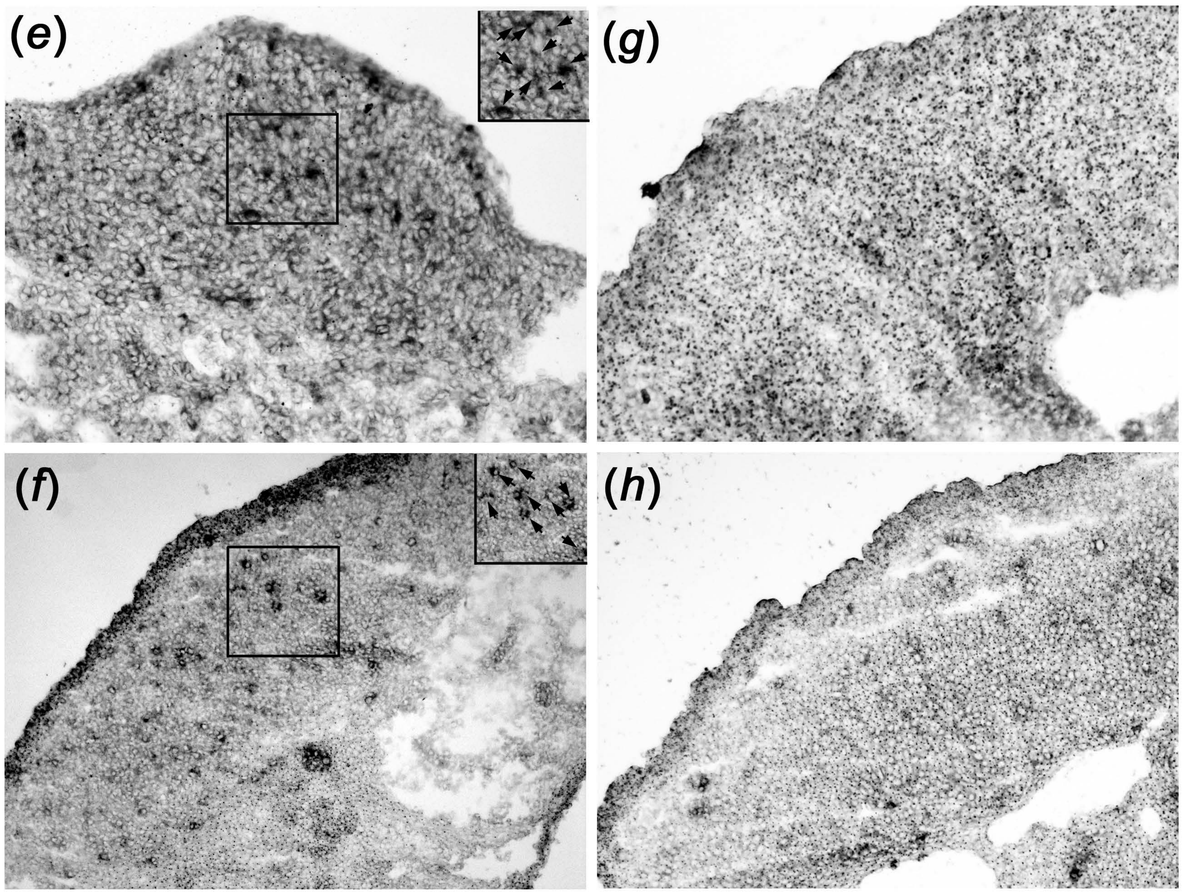

(a)
(c)
(e)
(g)
(b)
(d)
(f)
(h)

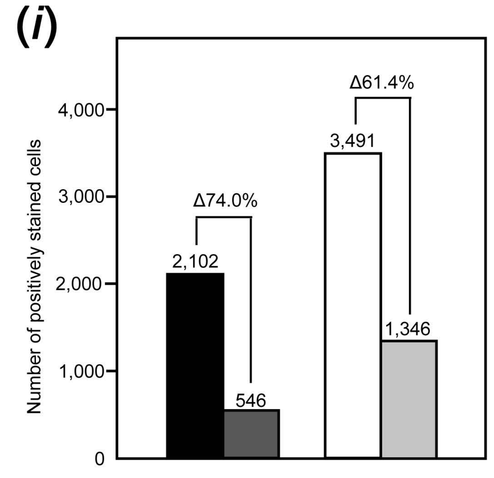

(i)
Relative normalized expression
0.9
0.7
0.5
0.3
0.1
*
*
WT female
KD female
WT male
KD male

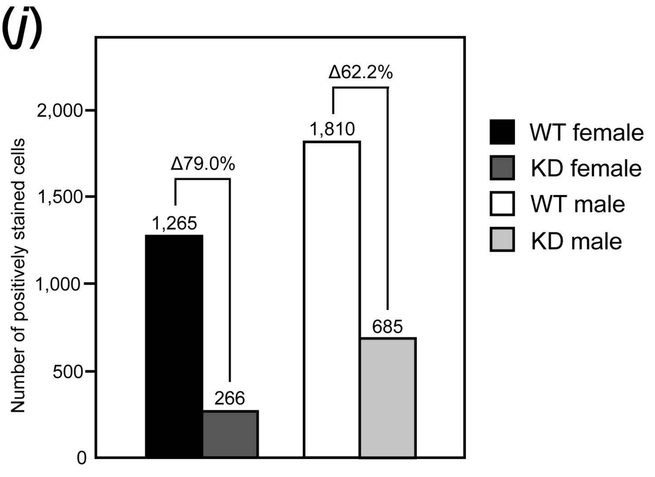
AP staining



DND1 in situ hybridization







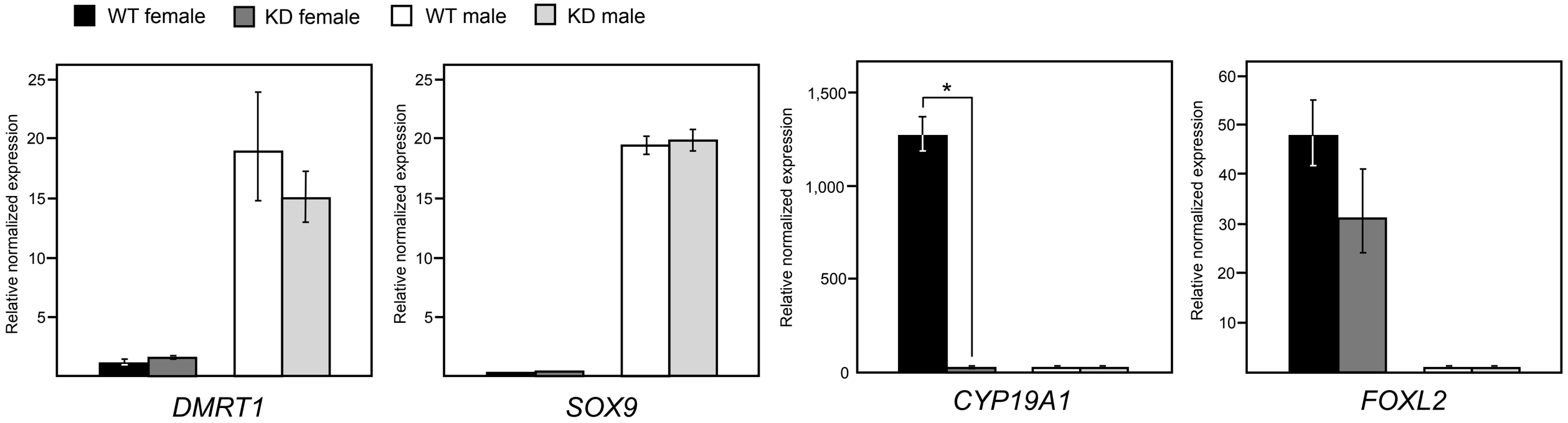

WT female
KD female
WT male
KD male
Relative normalized expression
25
20
15
10
5
DMRT1
Relative normalized expression
25
20
15
10
5
SOX9
Relative normalized expression
1,500
1,000
500
0
*
CYP19A1
Relative normalized expression
60
50
40
30
20
10
FOXL2

Table 1 Primer sequences

Experiment	Direction of primer	Sequence
Construction of miRNA vectors		
DDX4-targeting miRNA #1	F	5'- GAG AGG TGC TGC TGA GCG AGA TCC TGG TAT GCA AGA TCA TAG TGA AGC CAC AGA TGT A -3'
	R	5'- ATT CAC CAC CAC TAG GCA TGA TCC TGG TAT GCA AGA TCA TAC ATC TGT GGC TTC ACT -3'
DDX4-targeting miRNA #2	F	5'- CTG GTT CCT CCG TGA GCG AGG TGC TAA TGA AGG ACT TAA TAG TGA AGC CAC AGA TGT A -3'
	R	5'- CCT GAA GAC CAG TAG GCA TGG TGC TAA TGA AGG ACT TAA TAC ATC TGT GGC TTC ACT -3'
Scrambled miRNA #1	F	5'- GAG AGG TGC TGC TGA GCG AAC CTT CTC AAT TCT CAT CAT TAG TGA AGC CAC AGA TGT A -3'
	R	5'- ATT CAC CAC CAC TAG GCA GAC CTT CTC AAT TCT CAT CAT TAC ATC TGT GGC TTC ACT -3'
Scrambled miRNA #2	F	5'- CTG GTT CCT CCG TGA GCG ACA TGT TCC CTC GTC ACT TTA TAG TGA AGC CAC AGA TGT A -3'
	R	5'- CCT GAA GAC CAG TAG GCA GCA TGT TCC CTC GTC ACT TTA TAC ATC TGT GGC TTC ACT -3'
Construction of the DDX4_GFP vector	F	5'- TAA TCG ATG GAG GAG GAC TGG G -3'
	R	5'- GCA CTA GTC TCC CAT GAC TTA AAT GTT G -3'
DND1 in situ hybridization probe	F	5'- CAA CCG GAC CAA TAA GAT GG -3'
	R	5'- ATT CCC TTC CAC CAG AGC TT -3'
qRT-PCR analysis of DDX4	F	5'- GTA GCA TCA AGA GGC CTG GA -3'
	R	5'- ACG ACC AGT TCG TCC AAT TC -3'