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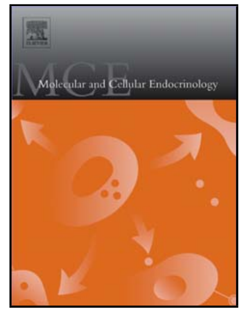

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WNT4 is Expressed in Human Fetal and Adult Ovaries, and Its Signaling Contributes to Ovarian Cell Survival

Minna Jääskeläinen[a,b], Renata Prunskaite-Hyyryläinen[c,d], Florence Naillat[c,d], Helka Parviainen[f,g,h], Mikko Anttonen[g,h], Markku Heikinheimo[f,j], Annikki Liakka[e], Roxana Ola[i], Seppo Vainio[c,d], Tommi E. Vaskivuo[b], Juha S. Tapanainen[a,b,*]

[a]Department of Obstetrics and Gynecology, Oulu University Hospital, Finland;
[b]Clinical Research Center, Oulu University Hospital, Finland
[c]Oulu Center of Cell-Matrix Research, Biocenter Oulu, Laboratory of Developmental Biology,
[d]Institute of Biomedicine, Department of Medical Biochemistry and Molecular Biology and
[e]Department of Pathology, University of Oulu, Finland;
[f]Children's Hospital, University of Helsinki, Finland;
[g]Program for Women´s Health, University of Helsinki, Finland;
[h]Department of Obstetrics and Gynecology, University of Helsinki, Finland;
[i]Biochemistry and Developmental Biology Group Institute of Biomedicine, University of Helsinki, Finland;
jDepartment of Pediatrics, University of Washington, St. Louis, USA

Department of Obstetrics and Gynecology, P.O. Box 5000 , 90014, University of Oulu, Finland
Tel: +358-8-3153172 Fax: +358-8-3154310, juha.tapanainen@oulu.fi

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Abstract:

WNT4 plays an important role in female sexual development and ovarian function. WNT4-deficiency leads disturbed development of the internal genitalia in mouse and human, and to a dramatic reduction of mouse oocytes. However, the expression and role of WNT4 in human ovaries is yet unknown. The expression of WNT4 mRNA and protein was studied in human adult and fetal ovaries (gestational ages 12–41 weeks), and the role of Wnt4 in oocyte apoptosis was investigated in Wnt4-deficient mice. WNT4 mRNA and protein were present in human ovaries throughout fetal development and in different follicular stages in adult ovaries. Compared with wild-type mice, Wnt4-deficient mice had a markedly enhanced rate of oocyte apoptosis, with the highest values at gestational ages of 14.5 and 16.5 days post-coitum. The current results support a view that WNT4 may have a role in oocyte selection and follicle formation and maturation in human ovaries.

Introduction:

The locally acting excreted glycoprotein WNT4 is a member of the WNT (wingless-type MMTV integration site family) family of extracellular ligands (Cadigan, Nusse 1997, Vainio et al. 1999). The action of the WNT4 ligand is transmitted through receptors located on the cellular membrane, most importantly those of the Frizzled family (Cadigan, Liu 2006). This canonical *Wnt* signaling pathway eventually leads to nuclear accumulation of β-catenin and transcriptional regulation of target genes (Cadigan, Liu 2006). *Wnt* genes have been highly conserved throughout evolution and *Wnt* signaling has been observed to play a role in a wide variety of physiological and pathological events from embryogenesis to the onset and development of cancer.

Wnt4 has an important role in female sexual differentiation (Vainio et al. 1999, Jeays-Ward et al. 2003, Kim et al. 2006, Ottolenghi et al. 2007). It is downregulated in male gonads after the initiation of testicular development (11.5 days post-coitum [dpc]) while it is persistent in the developing ovary (Vainio et al. 1999). Mice with homozygous mutation in the *Wnt4* gene have severely disturbed development of the internal genitalia and ovaries. *Wnt4*-deficient ovaries are masculinized, with a round and non-encapsulated form and they develop in close association with a fat body structure. Female mutant mice lack Müllerian ducts and have male-type Wolffian ducts instead (Vainio et al. 1999). In addition to their masculinized appearance, mutant ovaries express many Leydig and Sertoli cell markers and secrete testosterone and Müllerian-inhibiting substance (MIS) (Heikkila et al. 2001, Heikkila et al. 2005).

WNT4 plays an important role also in human sex determination. Female patients with loss-of-function mutation in the *WNT4* gene have developmental abnormalities mimicking the phenotype observed in *Wnt4* mutant mice. They are partially masculinized, with clinical features of primary amenorrhea, androgen excess, regressed Müllerian duct derivatives and anomalous location of the ovaries (Biason-Lauber et al. 2004, Biason-Lauber et al. 2007).

By the time of birth, the number of oocytes in *Wnt4* mutant mouse ovaries is abnormally low (Vainio et al. 1999). Wnt4 has not been shown to affect the number of primordial germ cells in the undifferentiated gonad, but it is thought to act as an oocyte survival factor during female embryogenesis (Vainio et al. 1999, Heikkila et al. 2001). The massive germ cell loss in *Wnt4*-deficient mice occurs most rapidly at 16.5 dpc, when more than 90% of the oocytes are being depleted (Yao et al. 2004). At this time, apoptotic oocytes can be detected in the central region of both wild-type and mutant ovaries, but in contrast to the normal ovary, rapid apoptosis takes place in the ovarian cortices of *Wnt4*-deficient mice (Yao et al. 2004).

During normal ovarian development, Bcl-2 family genes are central in the regulation of oocyte apoptosis. The family contains several anti- and pro-apoptotic factors which have been shown to be important in the regulation of apoptosis in mouse ovaries. Oocytes of $BclX_L$- deficient mice undergo massive apoptosis (Rucker et al. 2000), while knockout of pro-apoptotic *Bax* results in a decreased rate of oocyte apoptosis (Perez et al. 1999, Alton, Taketo 2007). In addition to the Bcl-2 family, numerous other factors are associated with the regulation of ovarian apoptosis. For instance, TRAIL, a tumor necrosis factor-related apoptosis-inducing ligand, has been shown to participate in the regulation of apoptosis in mammalian ovaries (Inoue et al. 2003, Jaaskelainen et al. 2009). Moreover, several transcriptional factors such as GATA-4 and its co-factor FOG-2 participate in the regulation of oocyte survival in mice and men (Heikinheimo et al. 1997, Vaskivuo et al. 2001, Anttonen et al. 2003).

In the present work the expression of *WNT4* mRNA and protein was studied for the first time during human fetal development and human adult follicular maturation. In addition, apoptosis and the expression of key apoptosis-regulating factors in *Wnt4*-deficient and in wild-type mouse ovaries were analyzed in detail to evaluate the contribution of *Wnt4* signaling to survival of the maturing oocytes.

Materials and methods:

Human ovarian tissue samples

Fetal ovarian tissue samples were obtained from 7 fetuses (aged 12–23 wk) after spontaneous or therapeutic abortion and from 9 fetuses (aged 22–41 wk) after intrauterine death followed by spontaneous or induced delivery or cesarean section. In addition, ovaries from 6 neonates (gestational age 23, 26, 27, 31, 35 and 38 weeks) who died because of perinatal asphyxia or infection within 30 min–3 days after birth were studied. The obduction data of all female fetuses during the years 2000–2007 at Oulu University Hospital were examined and on the basis of the information all samples with chromosomal abnormalities, significant anomalies or autolysis were excluded. Thereafter, a pediatric pathologist analyzed the morphology of the remaining samples, and samples with signs of autolysis in HE-staining were further excluded. Three fetuses included in the study were diagnosed as having chorioamnionitis. However, their ovaries were morphologically intact and did not have any signs of infection. All fetuses and neonates had normal karyotypes. Adult ovarian tissues were obtained from 19 premenopausal patients aged 22–49 years undergoing ovariectomy because of endometriosis. In addition, human adrenal gland and testis were used as control tissues. All samples were fixed in 4% buffered formaldehyde for 24 hours and embedded in paraffin. Histological sections (4 µm) were cut and processed for immunohistochemistry and *in situ* hybridization. The study was approved by the Ethics Committees of Oulu University Hospital and the University of Oulu. A permit to study human autopsy tissues and resection material was obtained from the Finnish National Authority for Medicolegal Affairs.

Generation of Wnt4 mutant mice:

Wnt4 +/- mice in SV129 background were crossed to obtain -/- embryos and the genotypes were analyzed as described earlier (Heikkila et al. 2005, Stark et al. 1994). Female wild-type and *Wnt4*-deficient mouse embryos aged 12.5, 14.5 and 16.5 dpc and newborn animals were studied.

Immunohistochemistry:

Immunohistochemistry for WNT4 was performed on human ovarian paraffin-embedded tissues. Paraffin sections were first incubated at 37 ºC for 30 min and then at 60 ºC for 30 min. Deparaffinization was carried out in xylene and the tissues were hydrated gradually through a series of graded alcohols. Pretreatment in Tris-HCl in a microwave oven (700 W for 2 min and 300 W for 15 min) was performed. Tissues were then cooled at RT for 20 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol. The sections were then washed in dH_2O and PBS. Diluted normal serum provided with the immunostaining kit (Vectastain Elite ABC Kit, Vector laboratories, Burlingame, CA) was used to block nonspecific binding sites. Primary antibody against WNT4 protein (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; cat. no. sc-5214) at a concentration of 1:50 in background-reducing antibody diluent (Dako, Glostrup, Denmark) was added to the samples and they were incubated under plastic cover slips in a humidified chamber overnight at +4 ºC. A commercially available avidin-biotin immunoperoxidase system was used to visualize bound antibodies (Vectastain Elite ABC Kit, Vector laboratories, Burlingame, CA).

Immunohistochemistry involving mouse ovarian tissues was performed following a standard protocol. Primary antibodies against Bcl-X_L (1:800), Bok (1:100), TRAIL (1:50), GATA-4 (1:200) and FOG-2 (1:200) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; cat. nos. sc-7122, sc-11424, sc-6079, sc-1237 and sc-10755, respectively) and aromatase (1:500) (Acris Antibodies, Hiddenhausen, Germany, cat. no. BP278), diluted in PBS, were used. A commercially available avidin-biotin immunoperoxidase system was used to visualize bound antibodies (Vectastain Elite ABC Kit, Vector laboratories, Burlingame, CA).

In situ hybridization

Probes for *WNT4 in situ* hybridization were prepared from a commercially available *WNT4* cDNA clone in pBluescriptR vector (Image clone 30330746, Genbank accession no **BC057781**, MCR geneservice, Cambridge, UK).

Human tissue sections were hybridized with single-stranded antisense and sense RNA probes of human *WNT4*. The plasmid containing *WNT4* cDNA was linearized with EcoRI for the antisense and BamHI for the sense RNA probes. The RNA probes were labeled with digoxigenin-UTP by *in vitro* transcription with T3/T7 polymerase using a DIG RNA Labeling Kit (Roche Diagnostics GmbH, Mannheim, Germany). 200 ng of the probe was used in the hybridization, and the sense probe was used as a negative control. In situ hybridization was carried out with Ventana Discovery automatic staining instrument using commercial buffers (Roche Diagnostics GmbH, Mannheim, Germany). The DIG labelled probe was detected with monoclonal biotinylated anti-digoxigenin antibody (Jackson ImmunoResearch Laboratories Inc., PA) used at 1:2000 dilution and with the BlueMap kit (Roche Diagnostics GmbH, Mannheim, Germany).

In situ DNA 3′ end-labeling:

Apoptosis was studied by using an Apoptag® Peroxidase *In Situ* Apoptosis Detection Kit (Millipore, Billerica, MA) as previously described (Vaskivuo et al. 2001). The instructions of the manufacturer were followed. Briefly, paraffin-embedded tissues were deparaffinized in xylene and rehydrated through a series of graded alcohols. The tissues were pretreated in proteinase K (4 mg/ml) and 3% hydrogen peroxide before applying the enzyme deoxynucleotidyl transferase (TdT) to the samples. Incubation took place under plastic cover slips in a humidified chamber at 37 ºC for one hour. Bound enzyme was visualized with anti-digoxigenin antibody and diaminobenzidine tetrahydrochloride (15 µl/ml) (DAB, DakoCytomation Ltd., Ely, UK). The samples were counterstained with hematoxylin.

Sample analysis:

Immunohistochemical and *in situ* hybridization stainings were analyzed by two independent observers.

For analysis of apoptosis the ovarian samples from *Wnt4*-deficient and wild-type animals were photographed using Qimaging MicroPublisher 5.0 RTV and Qcapture-Pro software. The size of each tissue section was then measured (ImageJ program) and apoptotic cells were counted and the results adjusted to section size. The data was analysed by using SPSS 15.0 software (SPSS, Chicago, IL, USA). The data is presented as arithmetic mean plus SD. Student's *t* test were used for analyses of signicance and a value of $p < 0.05$ was considered significant.

Results

WNT4 mRNA and protein are expressed in human fetal ovaries

The *in situ* hybridization analyses showed strong expression of *WNT4* mRNA in human ovaries at all fetal ages studied. Both granulosa cells and oocytes expressed *WNT4* mRNA (Fig. 1 a). Similarly, immunostaining of WNT4 protein was detected in human ovaries throughout fetal development (Fig. 2A a-h). During early ovarian development (12–18 weeks), moderate WNT4 protein expression was mainly localized to pregranulosa and granulosa cells and only weak protein expression could be detected in oocytes, recognized by their round appearance and large amount of cytoplasm (Fig. 2A, a and b). As follicle formation proceeded, staining intensity tended to increase, but more samples would be needed to confirm this observation (Fig 2A c-f). During the latter half of fetal development strong *WNT4* mRNA and protein expression was observed mainly in oocytes, but granulosa cells in primordial and primary follicles were also moderately stained (Figs. 1 a; 2A, e-h).

WNT4 mRNA and protein are present during follicular maturation in human adult ovaries

WNT4 mRNA was expressed in granulosa cells and oocytes of primordial/primary and secondary follicles (Fig. 1 c) and in granulosa and theca cells of the antral follicles (Fig. 1 e). Immunohistochemical staining of WNT4 protein was observed at all stages of follicular maturation. In small primordial/primary follicles the oocytes were strongly positive for WNT4 (Fig. 2B, a). Greater magnification showed that granulosa cells in these follicles were also positively stained (Fig. 2B, c). Interestingly, in antral follicles, moderate WNT4 staining was detected in theca cells while granulosa cells were stained weakly (Fig. 2B, e). In all samples staining was located mainly in the cytoplasm.
Human adrenal gland and testis were used as control tissues in immunohistochemistry and *in situ* hybridizations. Adrenal gland showed strong *WNT4* mRNA and protein expression (Fig. 1 g; 2B, g and h). Only weak expression was observed in human adult testis, mainly localized to Leydig cells (Fig. 1 i; 2B, i and j). Similar *Wnt4* expression pattern has been previously detected in marsupial adult testis (Yu et al. 2006).

Increased oocyte apoptosis in Wnt4-deficient mice

Apoptosis was detected in the ovaries of fetal and newborn wild-type and mutant mice (Fig. 3, A-H). Most of the apoptotic cells presented typical morphological features of germ cells including large size and round cytoplasm. In both genotypes the apoptosis rate increased after mid-pregnancy, reaching a maximum at 16.5 dpc (Fig. 3, E and F) and then declining towards birth. When comparing the numbers of apoptotic cells in *Wnt4* mutant and wild-type mice, a significantly higher number of apoptotic cells was observed in *Wnt4*-deficient ovaries at 14.5 dpc (Fig. 3, C and D) and the difference was most pronounced at 16.5 dpc (Fig. 3, E and F). In the ovaries of newborn mice the rate of apoptosis was similar in *Wnt4* wild-type and mutant mice (Fig. 3, G and H).

Apoptosis regulating factors in Wnt4-deficient mouse ovaries

Immunohistochemical staining of Bcl-$X_{L,}$ Bok, TRAIL, GATA-4, FOG-2 and aromatase showed that these proteins were similarly expressed in the ovaries of mutant and wild-type mice during fetal development (Fig 4). Expression of the pro-apoptotic Bcl-2 family member Bok was restricted mainly to oocytes (Fig 4, e and f). In both genotypes Bok was observed in the oocytes of the medullar and cortex regions of the ovary. The cytokine-like apoptosis-inducing ligand TRAIL (Fig 4, a and b), the anti-apoptotic mitochondrial membrane protein Bcl-X_L (Fig 4, c and d) and aromatase (Fig 4, k and l) were expressed in oocytes and somatic cells in both *Wnt4*-deficient and wild-type ovaries throughout fetal development. GATA-4 (Fig 4, g and h) and its cofactor FOG-2 (Fig 4, i and j) were expressed mainly in pregranulosa and granulosa cells of mutant and wild-type ovaries.

Discussion:

In the present study we demonstrate the expression of *WNT4* mRNA and protein in human ovaries during fetal development and fertile life. These results support the role of *WNT4* in human ovarian development and its importance in normal ovarian function (Biason-Lauber et al. 2004, Biason-Lauber et al. 2007). The expression of WNT4 protein in human fetal ovaries was high during mid-pregnancy, when new follicles are also rapidly being formed. This implies that *WNT4* may have a substantial role in regulation of the follicle formation in human ovaries, as also demonstrated in *Wnt4*-deficient animals, which lack proper follicle structures (Vainio et al. 1999). Moreover, *WNT4* mRNA and protein were present in ovaries later during fetal life and in adult ovaries, supporting a view that *WNT4* may also have a role in follicular development and maturation in human ovaries (Philibert et al. 2008).

Wnt4 mRNA has previously been shown to be expressed in rodent ovaries during fetal development (Vainio et al. 1999, Barrionuevo et al. 2006) and its expression increases in young females after the primordial follicles are formed (Hsieh et al. 2002). In human ovaries a somewhat similar tendency was observed. *WNT4* was detected throughout the fetal development and the strongest expression of *WNT4* took place after follicular formation when oocyte apoptosis starts to decrease (Vaskivuo et al. 2001). The observed *WNT4* expression pattern in human ovaries and the markedly increased apoptosis rate in *Wnt4*-deficient mouse ovaries suggest that *WNT4* may be an important factor in the selection of human follicles that either survive or become deleted from the ovary.

In adult human ovaries *WNT4* mRNA and protein were expressed in the small primary and growing secondary follicles. Antral follicles expressed *WNT4* variably and in addition to granulosa cells the theca cells surrounding the follicles were also positive. This suggests that *WNT4* may be involved in the regulation of all cell compartments. Similarly to our finding in human ovaries, *Wnt4* mRNA is expressed in small growing follicles and in preovulatory and ovulatory follicles in mouse ovaries (Hsieh et al. 2002).

In adult and fetal human ovaries *WNT4* mRNA and protein were detected both in the granulosa cells and in the oocytes. There has not been general agreement on the cellular localization of *WNT4* in the ovary. The results of previous studies on mice suggest that *Wnt4* is expressed in granulosa cells rather than germ cells (Vainio et al. 1999, Yu et al. 2006). On the other hand, *Wnt4* mRNA has been detected in oocytes in postnatal chick ovaries (Oreal et al. 2002). Although oocytes are known to show nonspecific immunostaining, the present results raise the possibility that *WNT4* expression also takes place in human germ cells. However, comparison of results from mice and humans might also point towards species-specific differences in the expression pattern of *Wnt4*.

The expression of apoptosis-regulating factors was studied in the ovaries of *Wnt4*-deficient and wild-type mice by immunohistochemistry. Several analyses were performed with primary antibodies against TRAIL, Bok, Bcl-X_L, GATA-4, FOG-2 and aromatase. No significant differences were observed in the expression patterns of these proteins between *Wnt4*-deficient and wild-type animals. Thus our results suggest that increased apoptosis in the absence of *Wnt4* action is not directly caused by a significant change in the expression levels of the factors known to regulate apoptosis in ovaries and other organs. Further studies are, however, needed to comprehend fully the mechanisms of WNT4 in regulating apoptosis in ovaries.

To conclude, *WNT4* mRNA and protein are present in human fetal and adult ovaries and ovarian apoptosis is significantly enhanced in *Wnt4* mutant mice when compared with wild-type animals. The results support a view that *WNT4* may have a role of in the regulation of oocyte survival, follicle formation and follicle maturation in human ovaries. More robust studies on the molecular mechanisms are needed to understand the protective role of *WNT4* in oocyte demise.

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Figure Legends

Figure 1. *WNT4* mRNA expression in human fetal and adult ovaries. *WNT4* mRNA expression was observed in all of the fetal ovaries studied (a). Sense control (b).
In adult human ovaries *WNT4* mRNA was expressed in oocytes and granulosa cells of primordial/primary follicles (c). In growing antral follicles strong *WNT4* labeling was detected in granulosa and theca cells (e). Sense controls (d and f).
Human adrenal gland tissue was used as positive control and showed strong *WNT4* expression (g). Weak WNT4 mRNA expression could also be detected in Leydig cells of adult human testes (i). Sense controls (h and j).
Blue color indicates mRNA expression. *= Oocyte, arrows indicate granulosa cells. Scale bar 100 µm.

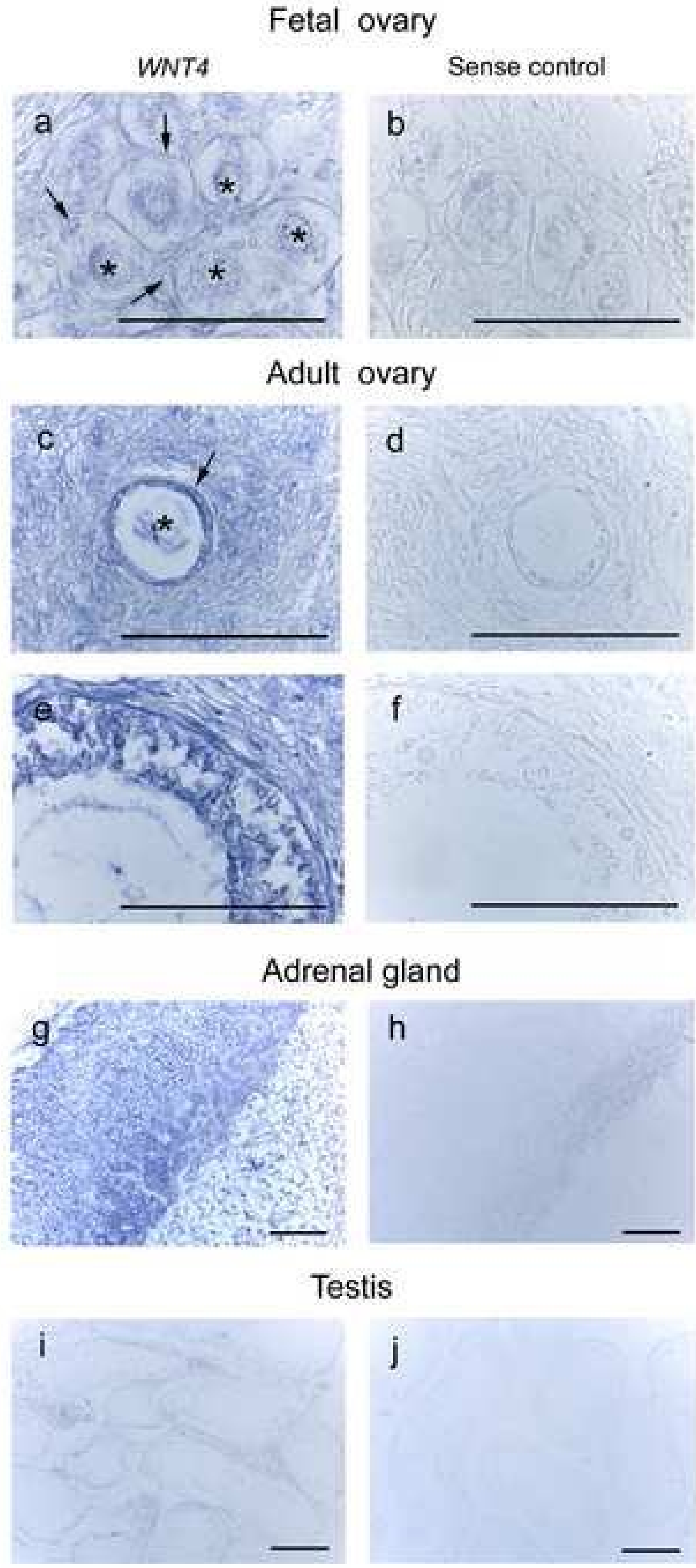

Fetal ovary
WNT4
Sense control
a
b
Adult ovary
c
d
e
f
Adrenal gland
g
h
Testis
i
j

Figure 2. WNT4 protein expression in human fetal (A) and adult (B) ovaries. WNT4 protein was detected in human ovaries at all fetal ages studied. During early development, staining was mainly present in granulosa cells, while the oocytes (with round shape and a large cytoplasm) showed weak expression (A: a and b). From fetal age 19 weeks onwards, staining intensity in oocytes tended to increase (A: c and d). After midgestation strong/moderate WNT4 protein expression was detected in most of the follicle-encapsulated oocytes, and granulosa cells were also moderately stained (A; e-h). Negative control using PBS instead of primary antibody (A: i and j).
Staining of WNT4 was intense in primary and secondary follicles of adult human ovaries and was detected mainly in oocytes, but also granulosa cells were moderately stained (B: a and c). In antral follicles granulosa cells were weakly stained and moderate staining was observed in theca cells (B: e). Negative control using PBS instead of primary antibody (B: b, d and f).
Human adrenal gland (B: g and h) and testis (B: i and j) were used as control tissues. Strong staining was detected in adrenals while in human testis tubular cells showed only negligible and Leydig cells moderate staining.
Brown color indicates protein expression. *= Oocyte, arrows indicate granulosa cells. Scale bar 100 µm.

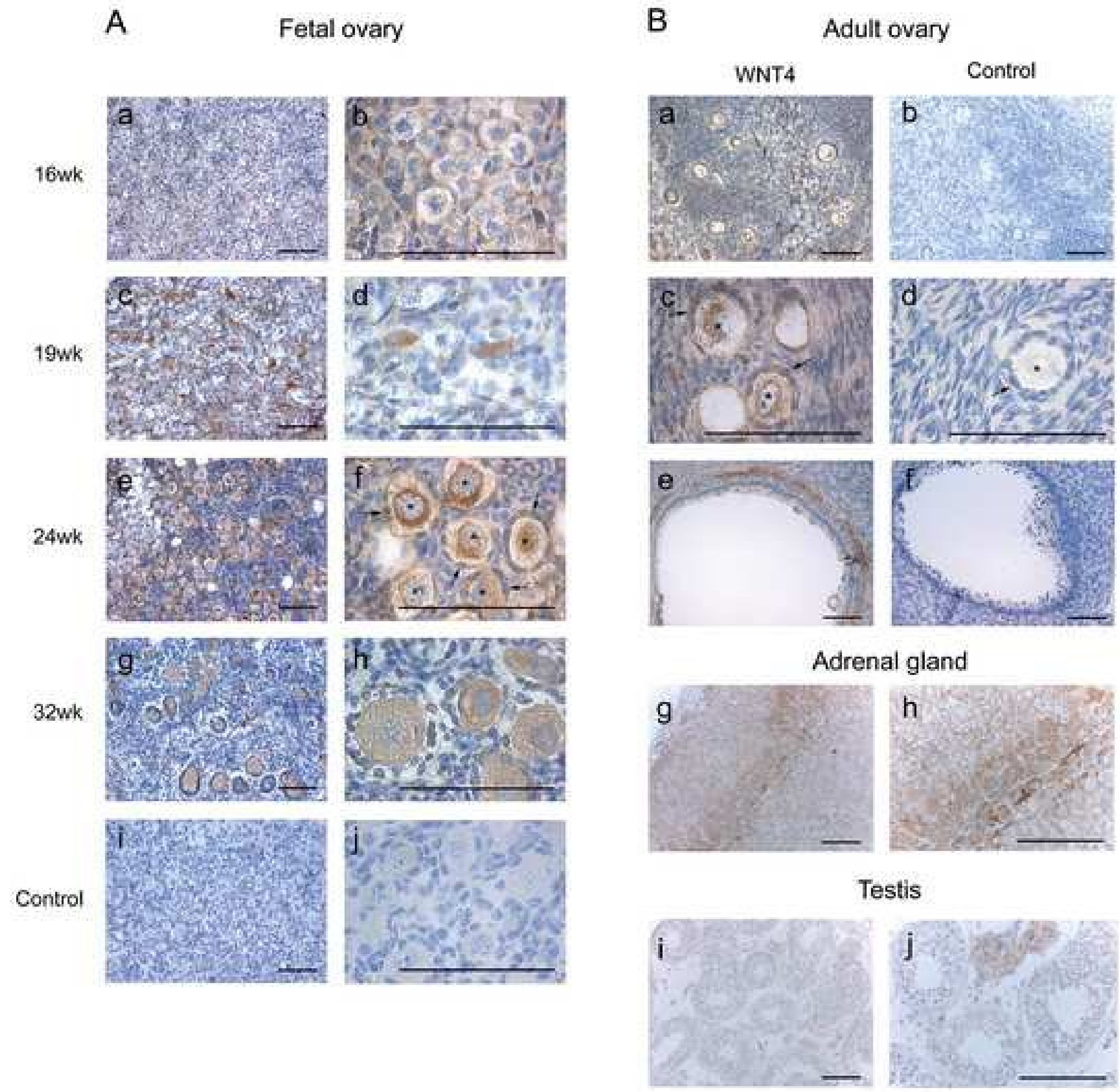


Figure 3. Apoptosis in *Wnt4*-deficient and wild-type mouse ovaries. Apoptotic cells were counted in sections of ovaries of different gestational ages: 12.5 dpc (A and B), 14.5 dpc (C and D), 16.5 dpc (E and F) and newborn (G and H), and the numbers of cells were adjusted according to the size of the section. The numbers of apoptotic cells were highest at 16.5 dpc in both genotypes. However, a markedly enhanced rate of apoptosis was observed in *Wnt4*-deficient animals at gestational ages of 14.5 dpc and 16.5 dpc. Scale bar 100 µm.

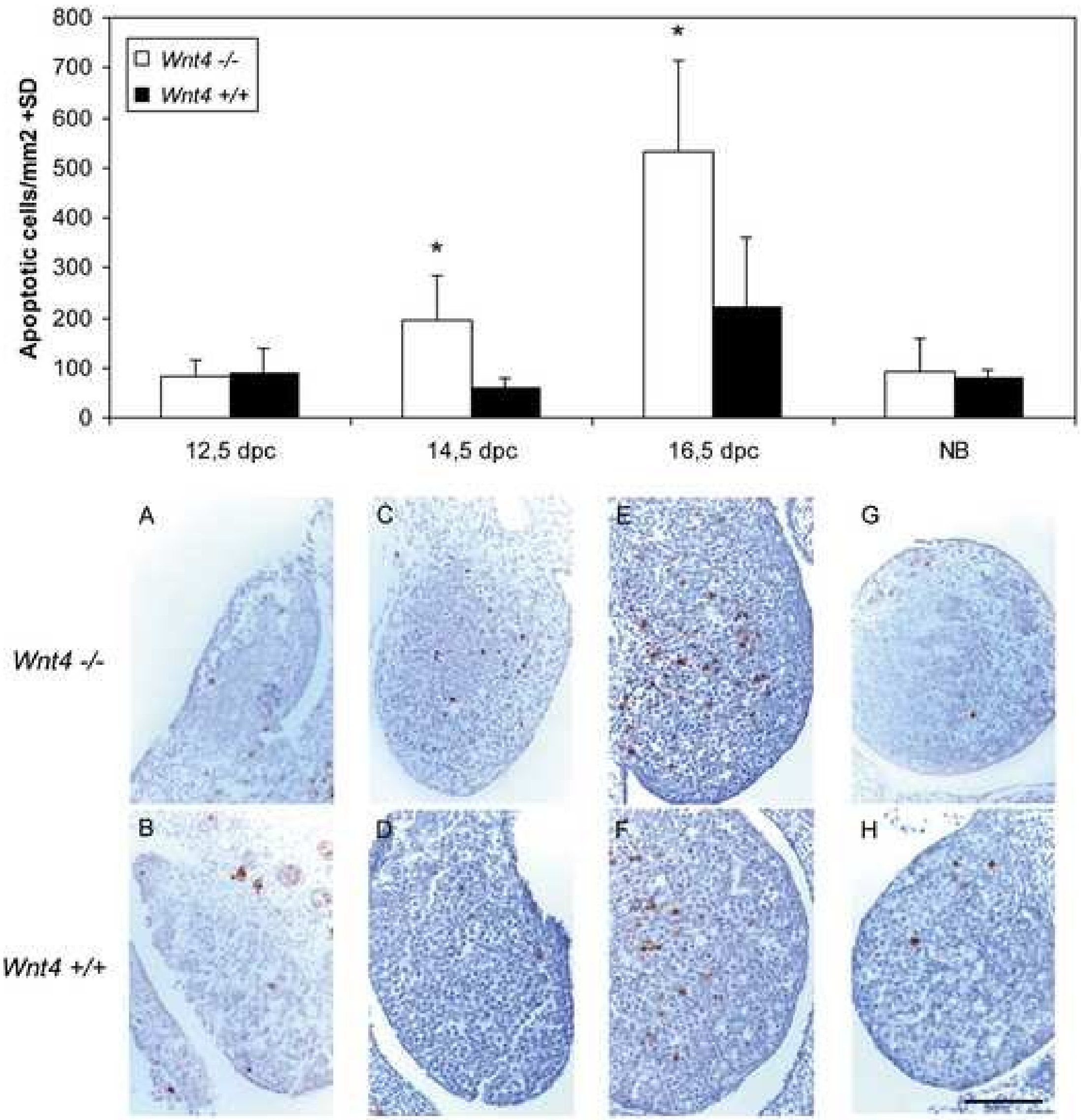
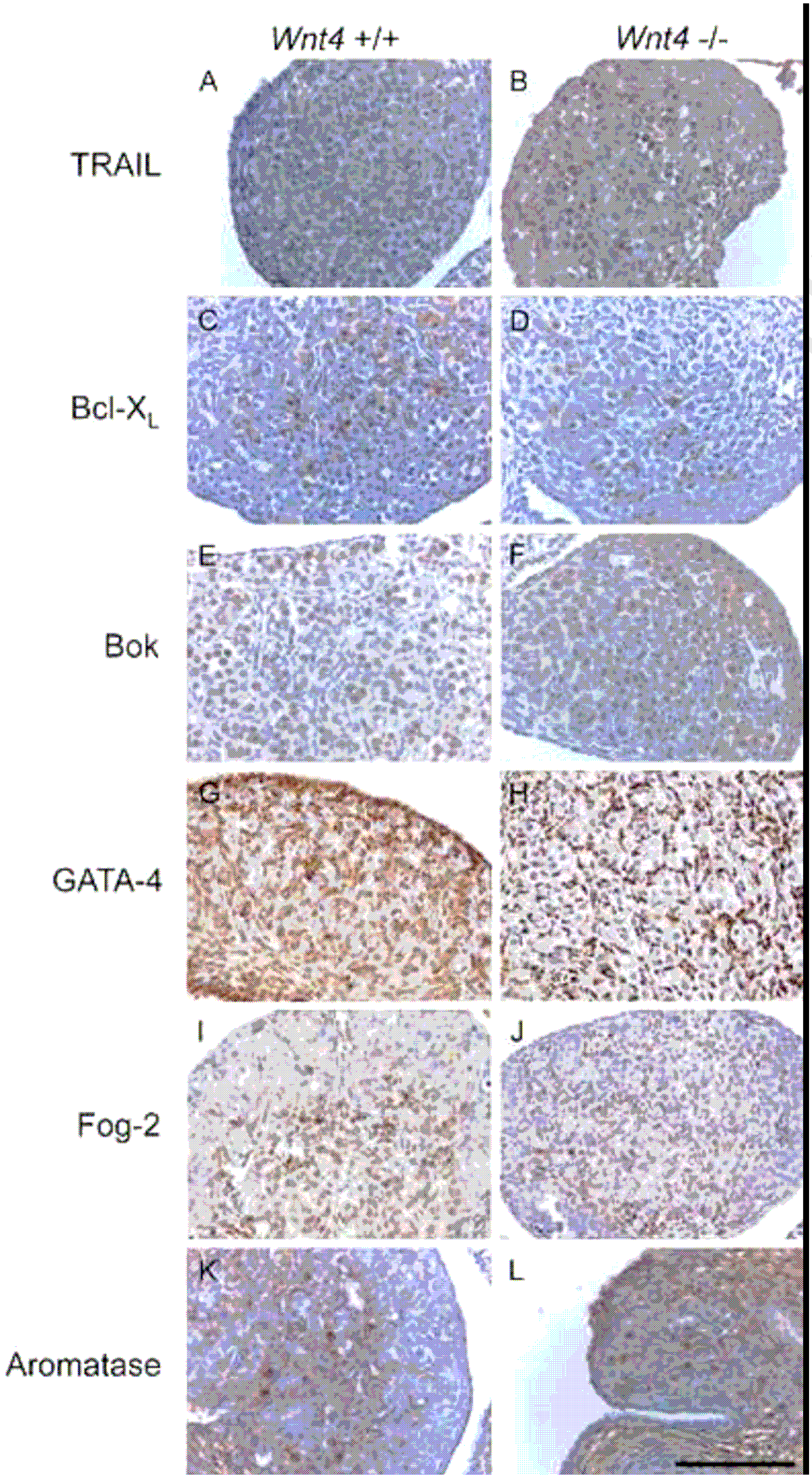


Figure 4. Immunohistochemical staining of Bcl-$X_{L,}$ Bok, TRAIL, GATA-4, FOG-2 and aromatase in *Wnt4*- deficient and wild-type mouse ovaries. All the studied proteins were present in both the *Wnt4* - deficient and wildtype ovaries and no marked differences were detected between the two genotypes.


Wnt4 +/+
Wnt4 -/-
A
B
TRAIL
C
D
Bcl-X$_L$
E
F
Bok
G
H
GATA-4
I
J
Fog-2
K
L
Aromatase

F