Tissue-specific knockout of E-cadherin (*Cdh1*) in developing mouse gonads causes germ cells loss

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Abstract

The normal course of gonad development is critical for the sexual development and reproductive capacity of the individual. During development, an incipient bipotential gonad which consists of unorganized aggregate of cells, must differentiate into highly structured testis or ovary. Cell adhesion molecules (CAMs) are a group of proteins crucial for segregation and aggregation of different cell types to form different tissues. E-cadherin (*Cdh1*) is one of the CAMs expressed in the developing gonads. We used tissue-specific knockout of *Cdh1* gene in OCT4⁺ germ cells and, separately, in SF1⁺ somatic cells of developing gonads. The knockout of E-cadherin in somatic cells caused decrease in the number of germ cells, while the knockout in the germ cells caused their almost complete loss. Thus, the presence of E-cadherin in both the germ and somatic cells is necessary for the survival of germ cells. Although the lack of E-cadherin did not impair cell proliferation, it enhanced apoptosis, which was a possible cause of germ cell loss. However, the somatic cells of the gonad differentiated normally into Sertoli cells in the testis cords, and into follicular cells in the ovaries. The testis and ovigerous cords maintained their integrity; they were covered by continuous basement membranes. The testicular interstitium with steroidogenic fetal Leydig cells did not show any noticeable changes. However, in the female gonads, because of the lack of germ cells, the ovarian follicles were absent. The sex determination and sexual differentiation of the gonad were not impaired. These results underscore an important role of E-cadherin in germ cell survival and gonad development.

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Introduction

Cell adhesion is crucial for development of the embryo. Every cell of a given type produces a set of cell type-specific adhesion molecules (CAMs) on its surface. Because most of the CAMs interact homotypically, the cells of the same type are able to recognize and adhere to each other and form a specific tissue. Because CAM gene family contains at least 220 genes (Piprek *et al.* 2017), this process has a very high degree of specificity and complexity.

Gonads, both testes and ovaries, are composed of several cell types. In the developing testes, the germ cells are enclosed by differentiating Sertoli cells within elongated structures termed the testis cords (Chen & Liu 2016, Piprek *et al.* 2016). The cords are enclosed by the basement membrane abutted by the peritubular myoid cells. The space between testis cords is occupied by the interstitial cells, including steroidogenic Leydig cells (Piprek *et al.* 2010). The developing ovaries contain

small groups of germ cells, called the ovarian cysts, which are enclosed by differentiating follicular cells. The space between the cysts is filled with the stromal cells. It is unknown how, during gonad development, these different cell types form, segregate and adhere to each other to establish different tissues. We identified expression patterns of 83 genes related to cell adhesion in the germ cells and somatic cells of developing mouse testes, and 68 genes in developing mouse ovaries (Piprek *et al.* 2017). We showed previously that there is a sex-specific difference in CAMs expression between male and female germ cells, and especially between male and female supporting cells (Sertoli and follicular cells respectively in testes and ovaries). These results prompted us to study in details the role of selected CAMs in gonad development.

E-cadherin (epithelial cadherin), encoded by *Cdh1* gene, is highly expressed in the germ cells of developing gonads (Mackay *et al.* 1999, Piprek *et al.* 2017). There is also low level of expression of E-cadherin in somatic

cells of developing mouse gonads of both sexes (Piprek *et al.* 2017). The developing mouse gonads cultured *in vitro* in the presence of anti-E-cadherin antibodies, which block E-cadherin-mediated cell adhesion, showed lower number and mislocalization of the germ cells (Bendel-Stenzel *et al.* 2000, Di Carlo & De Felici 2000).

Despite these significant findings pointing to the importance of E-cadherin in gonads development, the exact role of E-cadherin in gonad development, and its role in the germ cells vs somatic cells remain unknown. The main obstacle in answering these questions is that the conventional (whole-body) knockout of E-cadherin in mouse is lethal, and the embryos fail to develop past 32-cell stage (Larue *et al.* 1994, Ohsugi *et al.* 1997). To overcome the embryonic lethality, we studied the role of E-cadherin in developing mouse gonads using conditional knockout with cre-loxP system. We used three mouse strains to deplete E-cadherin in the germ cells or somatic cells of developing gonads. A mouse strain with Cre recombinase under the control of *Oct4* promoter (Greder *et al.* 2012) was used to delete E-cadherin specifically in the germ cells. A mouse strain expressing cre recombinase under the control of *Sf1* (*Nr5a1*) promoter (Dhillon *et al.* 2006) was used to induce cre expression in somatic cells of developing gonads. The octamer-binding transcription factor 4 (OCT4) is a marker of germ cells and the steroidogenic factor 1 (SF1) is a marker of coelomic epithelium, which gives rise to majority of somatic cell types in developing gonads (Ikeda *et al.* 1994). The SF1⁺ cells of coelomic epithelium give rise to the Sertoli cells and some interstitial cells in the testes, and the follicular cells and some theca cells in the ovaries (Albrecht & Eicher 2001, DeFalco *et al.* 2011, Liu *et al.* 2015). Both strains (OCT4-cre and SF1-cre) were crossed with a mouse strain, which had loxP sequences flanking a segment (from exon 6 to 10) of the *Cdh1* gene (Boussadia *et al.* 2002). We studied the effect of E-cadherin knockout using light microscope analysis, immunostaining and RT-qPCR.

Materials and methods

Animals

The study was approved by the I Local Commission for Ethics in Experiments on Animals. The animals were bred in the Animal Facility at the Jagiellonian University (Krakow, Poland). Three transgenic mouse lines used in this study were from Jackson Laboratory.

Mouse strain B6(SJL)-Pou5f1^{tm1.1(cre/Esr1*)Yseg/J} injected with tamoxifen was used for the induction of Cre recombinase expression in the germ cells expressing *Oct4* (Greder *et al.* 2012). Pregnant mice were injected with tamoxifen three times (2 mg/40 g body mass dissolved in sunflower oil) at embryonic days E10.5, E11.5 and E12.5. The sunflower oil injection was used as a control. *Cdh1* gene coding for E-cadherin was deleted in B6.129-*Cdh1*^{tm2Kem/J} mouse strain, which has loxP sites flanking exons 6–10 (Boussadia *et al.* 2002). Mouse strain

Tg(Nr5a1-cre)7Lowl/J (Dhillon *et al.* 2006) was used for Cre recombinase expression in the somatic cells (which express *Sf1*) of the gonads. The number of studied individuals is shown in Supplementary Table 1 (see section on supplementary data given at the end of this article).

Timed matings were performed by placing a male with two females overnight. The following morning, females were checked for the presence of the vaginal plug, and the pregnancies were estimated as E0.5 (embryonic day). Females were killed by spinal dislocation at embryonic days: E10.5, E11.5, E12.5, E13.5, E14.5, E16.5 and E18.5, and the newborns were killed at 1 and 2 dpp (days post partum).

Genotyping

The sex of all studied individuals was established by the genotyping using primers to detect *Sly* (Y chromosome) and *Xlr* (X chromosome) (McFarlane *et al.* 2013). Primers used to genotype cre⁺ and floxed specimens are listed in Supplementary Table 2. A standard PCR protocol was used for genotyping.

Gonad, RNA isolation and real-time quantitative PCR

Total RNA was isolated from fetal or newborns gonads using TRIzol, and further purified with RNeasy Mini kit according to the manufacturer's protocol (Qiagen). Total RNA in RNase-free water was stored at -80°C and used for multigene qPCR analysis. 50 ng RNA was reverse-transcribed into cDNA using random primers and SuperScript III Reverse Transcriptase (Invitrogen, 18080044) according to the manufacturer's protocol. A list of primers is shown in Supplementary Table 2. RT-qPCR was performed in 5 µL reactions using SYBR Green Master Mix (Life Technologies, 4312704) on a 7500 Fast Real-Time PCR System (Applied Biosystems) with universal cycling parameters and analyzed as previously described (Svingen *et al.* 2009). *Actb* was used as a reference gene. Statistical analysis was performed using initially the nonparametric ANOVA Kruskal–Wallis test followed by the Tukey's test. Statistica 7.0 software was used for the analyses.

Gonadal cell isolation and sorting

In order to check the effectiveness of the knockout, we analyzed the expression of cre and *Cdh1* genes separately in isolated SSEA-positive germ cells and Pcdh18-positive somatic cells. We previously showed that protocadherin 18 (*Pcdh18*) is highly expressed in the supporting and interstitial/stromal cells, but not in the germ cells of developing mouse gonads of both sexes (Piprek *et al.* 2017). SSEA1 is a marker of germ cells previously used for their isolation (Durcova-Hills *et al.* 1999). The gonads were dissected at E10.5, E11.5, E13.5, E16.5, 2 dpp and were incubated in 250 µL 0.25% Trypsin–EDTA (Sigma, #T4049) at 37°C for 5–10 min as previously described (Piprek *et al.* 2017). After tissue dissociation, the enzyme solution was replaced with 400 µL PBS. Cells were centrifuged for 10 min at 9,500g, and the cell pellet was resuspended in 3% BSA/PBS containing antibodies (10 µg/mL DyLight650-conjugated anti-SSEA1, Invitrogen #MA1-022-D650, and

10 µg/ml FITC-conjugated anti-Pcdh18, Biorbyt, #orb3038) and incubated for 30 min at RT. Subsequently, cells were centrifuged for 10 min at 9,500g and resuspended in PBS. For cell sorting the MoFlo XDP cytofluorimeter with a sorter (Beckman Coulter) was used. Cells were sorted depending on DyLight 650 and FITC signal. The cells were collected into lysis buffer (DNA/RNA Shield, Zymo Research #R1100). Pooled gonads from three fetuses were used for each time point and experiments were repeated three times. Total RNA was isolated using TRIzol, purified with RNeasy Mini kit according to the manufacturer's instructions (Qiagen), and RT-qPCR was performed as described above. A list of primers is shown in Supplementary Table 2. The purity of isolated cells was confirmed by analysis of expression of germ cell markers (*Oct4* and *Mvh*) and somatic cell markers (*Sox9*, *Foxl2*) (Supplementary Figs 1E, F and 2E, F).

Histology and immunohistochemistry

Dissected gonads were fixed in Bouin's solution, dehydrated and embedded in paraffin (Paraplast, Sigma, P3683). Histological staining was performed according to Debrieuill's trichrome method as previously described (Kiernan 1990, Piprek et al. 2012). For immunochemistry, heat-induced epitope retrieval was conducted in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6) at 95°C for 20 min. Subsequently, the sections were blocked with 3% H₂O₂ and 10% goat serum (Sigma, G9023), and incubated with primary antibodies (rabbit polyclonal anti-AMH, Santa Cruz Biotechnology, sc-166752; anti-collagen I, Abcam, ab34710; anti-Cyp17a1, Abcam, ab125022; anti-PCNA, Abcam, ab18197; anti-cleaved caspase 3, Assay BioTech, L0104; anti-cre recombinase, Abcam, ab190177; anti-E-cadherin, Abcam, ab152102; anti-TRA98, Abcam, ab82527) at 4°C, overnight, followed by the UltraVision Quanto Detection System (ThermoFisher, TL-125-QHD). Mayer's hematoxylin was used as a counterstain. Sections were examined under Nikon Eclipse E600 microscope. The germ cells were recognized due to their bigger size and the presence of larger and hematoxylin pale-stained nuclei as compared to the somatic cells.

Cell counting

The sections of all gonads from the animals killed at E18.5 were immunostained (PCNA, caspase 3, Cyp17a1 and TRA98), and the number of PCNA-, caspase 3-, Cyp17a1- and TRA98-positive cells was calculated in the 10000 µm² area in five cross-sections from each gonad using ImageJ software. Average values and standard deviation were calculated using Microsoft Excel software. The number of cells in the knockout gonads was compared to the control using χ^2 test. Statistical data were analyzed using Statistica 6 PL Software (Krakow, Poland).

Results

The effectiveness of genetic knockout

First we checked the effectiveness of the knockout using RT-qPCR to assess the level of expression of *cre*

recombinase and *Cdh1* separately in germ and somatic cells isolated from XY and XX mouse gonads. A high expression of *cre* gene under the control of *Oct4* promoter was detected in embryonic XY and XX gonads from E11.5 (Supplementary Fig. 1A and B). The expression of *cre* mRNA decreased after E16.5 (Supplementary Fig. 1A and B). The expression of *Cdh1* was vestigial at E13.5 and absent from E16.5 (Supplementary Fig. 1C and D), which proved the effectiveness of the knockout in the germ cells.

The expression of *cre* recombinase under the control of *Sf1* promoter was observed from E11.5 onward (Supplementary Fig. 2A and B). The expression of *Cdh1* was visibly lower at E11.5, and absent from E13.5 in XY gonads and from E16.5 in XX gonads (Supplementary Fig. 1C and D), which proved the effectiveness of the knockout in the somatic cells.

Knockout of E-cadherin (*Cdh1*) in OCT4-positive germ cells of developing mouse gonads

Knockout of *Cdh1* (E-cadherin) in germ cells (*Oct4-cre⁺ Cdh1^{fl/fl}*) caused a dramatic decrease in germ cell number (Fig. 1 and Supplementary Tables 3, 4). The majority of studied gonads of both sexes (86%) were completely deprived of germ cells. The loss of germ cells (first noticeable at stage E16.5) correlated with a decrease in the expression of germ cell marker *Mvh* (Fig. 2A and B) and a decrease in the size of the gonads. The testes of knockout males at E18.5 were smaller (the diameter of cross section at the widest area was $587 \pm 102.95 \mu\text{m}$) than the control testes ($668 \pm 72.65 \mu\text{m}$). The ovaries of knockout females at E18.5 were also smaller ($323 \pm 98.45 \mu\text{m}$) than the control ovaries ($456 \pm 64.08 \mu\text{m}$). The effects of

Figure 1 Germ cell number in the control and E-cadherin knockout gonads at E18.5. The number of germ cells is significantly lower in knockout XY and XX gonads than in the control. * $P < 0.05$ (by χ^2).

Figure 2 Gene expression in control and E-cadherin knockout in the germ cells at E18.5. The expression of *Mvh* is significantly lower in knockout XY (A) and XX (B) gonads than in the control. * $P < 0.05$ (by χ^2).

E-cadherin knockout in the testes and in the ovaries are summarized in Tables 1 and 2, respectively.

To check if the loss of germ cells resulted from the changes in the proliferation or apoptotic cell death, we studied the expression of PCNA (proliferating cell nuclear antigen) and caspase 3, respectively. The PCNA immunostaining and the number of proliferating cells did not reveal any significant differences between control and knockout female and male gonads (Fig. 3A, B, E, F

and Supplementary Tables 5, 6). However, the number of caspase 3-positive (apoptotic) cells was much higher both in the knockout testes and the knockout ovaries, than in the control (Fig. 3C, D, G, H and Supplementary Tables 7, 8). This suggested that the lower number of germ cells observed in the knockout gonads possibly resulted not from the decreased proliferation but from the increased apoptotic cell death.

Histological analysis showed that the control testes were composed of numerous convoluted and tubular testis cords, in which the germ cells were enclosed by a monolayer of differentiating Sertoli cells (pre-Sertoli cells); the basement membranes surrounded the testis cords, and the interstitium was located between the cords (Fig. 4A, C, G). The control ovary was filled with the irregularly shaped ovigerous cords in which germ cells were enclosed by differentiating follicular cells (pre-follicular cells); the basement membranes enclosed the ovigerous cords, and the stromal cells were located between the cords (Fig. 5A, C and E). The ovarian follicles (each follicle containing a single diplotene oocyte surrounded by follicular cells), which had separated from the ovigerous cords, were located near the medullary region of the ovary as early as at E18.5 (Fig. 5C).

The histological analysis of developing male gonads showed the presence of testis cords in both the knockout and control testes (Fig. 4A, B, C and D). Importantly, in the knockout testis cords no germ cells were visible (sterile cords) or only singular germ cells were present (Fig. 4F), while in control testis the testis cords were filled with numerous germ cells (Fig. 4E). To assess the changes in the testis cords, we performed immunostaining with Sertoli cells marker AMH (anti-müllerian hormone) and the basement membrane marker collagen I. AMH immunostaining showed the presence of Sertoli cells filling the center of the testis cords in both knockout and control testes (Fig. 4G and H). However, in the knockout testes, because of the lack of germ cells, the Sertoli cells did not form protrusions, which, normally, enclose the germ cells. The collagen I immunostaining showed that the knockout of E-cadherin in the germ cells did not

Table 1 Testes – effects of E-cadherin KO in Oct4⁺ germ cells and Sf1⁺ somatic cells.

	Control	Mutant phenotype E-cadherin KO in germ cells	Mutant phenotype E-cadherin KO in somatic cells
Testis size	Normal	Smaller*	Smaller*
Testis cords	Normal	Smaller*	Smaller*
Germ cells	Normal number of germ cells	Lower number of germ cells or sterile gonad*	Lower number of germ cells*
Sertoli cells	Continuous monolayer of Sertoli cells	Continuous monolayer of Sertoli cells	Continuous monolayer of Sertoli cells
Basement membrane	Continuous	Continuous	Continuous
Interstitial	Normal	Normal	Normal
Fetal Leydig cells	Normal number	Normal number	Normal number
Proliferation	Normal (high level)	Normal (high level)	Normal (high level)
Apoptosis	Normal (low level)	Increased in the germ cells*	Increased in the germ cells*

*Indicates affected cells and structures.

Table 2 Ovaries – effects of E-cadherin KO in Oct4⁺ germ cells and Sf1⁺ somatic cells.

	Control	Mutant phenotype E-cadherin KO in germ cells	Mutant phenotype E-cadherin KO in somatic cells
Ovary size	Normal	Smaller*	Smaller*
Ovigerous cords	Normal	Smaller*	Smaller*
Germ cells	Normal number of germ cells	Lower number of germ cells or sterile gonad*	Lower number of germ cells*
Follicular cells	Present	Present	Present
Ovarian follicles	Present	Absent*	Absent*
Basement membrane	Continuous	Continuous	Continuous
Proliferation	Normal (high level)	Normal (high level)	Normal (high level)
Apoptosis	Normal (low level)	Increased*	Increased*

*Indicates affected cells and structures.

affect the integrity of the testis cords, which remained enclosed by continuous basement membrane (Fig. 4I and J). To check if the organization of the interstitium located between the testis cords was affected we studied immunolocalization of Cyp17a1 – a marker of fetal Leydig cells located in the interstitium. We did not find significant differences in number of steroidogenic cells between the knockout and control testes interstitium (Fig. 4K, L and Supplementary Table 9).

In the developing ovaries, the histological analysis showed the presence of ovigerous cords, whose borders were clearly visible in collagen I immunostaining (Fig. 5A, B, C, D, E and F). The most striking difference between the knockout and the control ovaries was a lower number or complete lack of the germ cells, and smaller ovigerous cords in the knockout (Fig. 5G and H). In the control ovaries, singular oocytes enclosed by a monolayer of follicular cells were present near the ovarian medulla as early as at E18.5, which indicated the formation of the ovarian follicles. In contrast, in the knockout ovaries, which lacked the germ cells, the ovarian follicles were absent (Fig. 5C and D). However, the knockout ovaries had the ovigerous cords. In order to exclude the effect of tamoxifen (used to induce *cre* expression) on gonadal development, we performed histological analysis of gonads from tamoxifen-treated animals. We did not observe any structural/histologic changes in the gonads from the tamoxifen-treated animals (data not shown).

In order to find out if/how the knockout of E-cadherin influenced gene expression in developing testes and ovaries, we studied the expression of male sex determination genes, that also are the markers of Sertoli cells (*Amh*, *Sox9* and *Dhh*), and the expression of female sex determining genes, which are the markers of ovarian follicular cells (*Rspo1*, *Wnt4*, *Foxl2*). These genes are involved in male and female sex determination and testis and ovary differentiation. This analysis showed that the E-cadherin knockout did not affect the expression of these genes (Fig. 2A and B) and did not affect sex determination or sex ratio (the changes in the sex ratio could indicate sex reversal) (Supplementary Table 1). This shows that the lack of E-cadherin in the gonadal

germ cells did not disrupt sex determination and did not cause sex reversal.

Knockout of E-cadherin (*Cdh1*) in SF1-positive somatic cells of mouse gonads

Knockout of *Cdh1* (E-cadherin) in somatic cells (*Sf1-cre⁺ Cdh1^{fl/fl}*) caused a decrease in germ cell number in both XY and XX gonads starting from E16.5 onward. However, the this decrease in the germ cell number was much less severe than in the E-cadherin knockout in the germ cells (Fig. 1 and Supplementary Tables 3, 4). This germ cell loss was correlated with a decrease in the expression of the germ cell marker *Mvh* (Fig. 6A and B) and a decrease in the size of the gonads. The testes of knockout males at E18.5 were smaller (the diameter of cross section at the widest area was $618 \pm 97.98 \mu\text{m}$) in comparison to the control ($653 \pm 78.23 \mu\text{m}$). The ovaries of knockout females at E18.5 were also smaller ($390 \pm 76.34 \mu\text{m}$ versus $452 \pm 54.05 \mu\text{m}$). The effects of E-cadherin knockout in the testes and ovaries are summarized in Tables 1 and 2, respectively.

PCNA immunostaining showed the unchanged cell proliferation in the knockout gonads (Fig. 7A, B, E, F and Supplementary Tables 5, 6). However, the immunostaining of caspase 3 showed more apoptotic cells inside the testis and ovigerous cords in the developing knockout testes and ovaries in comparison to the controls (Fig. 7C, D, G, H and Supplementary Tables 7, 8). This indicates that the lower number of germ cells resulted not from a decline in the proliferation but from the enhanced apoptosis.

Histological analysis of control and knockout testes showed the presence of typical testis cords with the Sertoli cells forming a continuous monolayer epithelium (Fig. 8A, B, C and D). The number of germ cells was visibly lower than in the control (Fig. 8E and F). Immunostaining for AMH (marker of Sertoli cells) did not show any changes in the Sertoli cells (Fig. 8G and H). Immunostaining for collagen I showed a continuous basement membrane enclosing the testis cords in the control and the knockout testes (Fig. 8I and J). This indicates that the integrity of Sertoli cell epithelium was not affected by the lack of E-cadherin. There was also

Figure 3 Proliferation (PCNA immunostaining) and apoptosis (caspase 3 immunostaining) in testes and ovaries in E-cadherin knockout in the germ cells at E18.5. Similar effect of PCNA immunostaining is visible in control (A) and knockout testis (B) indicating similar level of proliferation (Supplementary Table 5). The number of caspase 3-positive (arrowheads) is higher in the knockout testis (D) than in the control testis (C) (Supplementary Table 7). The knockout (F) and control ovaries (E) show similar level of PCNA-positive cells number, however, the number of caspase 3-positive cells is visibly higher in the knockout (H) than in the control (G) ovaries (Supplementary Tables 6 and 8). Scale bar: 25 μ m.

no significant change in the intersitium (which contains Cyp17a1-positive fetal Leydig cells) present between testis cords (Fig. 8K, L and Supplementary Table 9). However, the knockout testes had lower number of germ cells.

The developing knockout ovaries had lower number of germ cells, smaller ovigerous cords and lacked the ovarian follicles (Figs 1 and 9A, B, C, D, E, F, G, H).

The analysis of the expression of selected genes in the somatic cells specific knockout showed that the expression of testis specific genes (*Sox9*, *Amh*, *Dhh*) and ovarian genes (*Rspo1*, *Wnt4*, *Foxl2*) agreed with the

Figure 4 Histology of the testis in the control and E-cadherin knockout in the germ cells. (A and C) Testes of control individual (*Oct4-cre- Cdh1+/+*) at stage E18.5; germ cells (arrowheads) are enclosed by the Sertoli cells (Sc); the whole testis cord (ts) is encapsulated by the basement membrane (arrow); interstitium (i) is located between the cords; trichrome staining. (B and D) The same testis structure is visible in the knockout testis (*Oct4-cre+ Cdh1fl/fl*), however, in the knockout testis only few germ cells are present (arrowheads); trichrome staining. (E and F) The immunostaining of TRA98 shows that the number of germ cells (arrowheads) is significantly lower in the knockout than in control testis. The immunostaining of AMH in the control (G) and knockout testis (H) is similar, however, in the knockout testis cords, only singular germ cells (arrowheads) are present. Immunostaining of collagen I shows continuous basement membranes (arrows) enclosing each testis cord in control (I) and knockout (J) testes. Numerous Cyp17a1-positive cells are visible in control (K) and knockout (L) testes. Cyp17a1-positive cells are fetal Leydig cells present in the interstitium. Scale bar: A and B: 100 μ m; C, D, E, F, G, H, I and J: 25 μ m.

genetic sex (XY and XX) (Fig. 6A and B). Also the sex ratio was not affected in the knockout gonads (Supplementary Table 1). Altogether, these results showed that the lack of E-cadherin in the gonadal somatic cells did not alter sex determination and did not cause sex reversal.

Discussion

We used targeted knockout technique, with Cre recombinase expression under the control of and *Sf1* and *Oct4* promoters, which allowed us to silent E-cadherin expression in the selected types of cells (germ cells or gonadal somatic cells) during the early stage of gonad development. Our experiment showed that (1) E-cadherin plays a role in gonad development, (2) E-cadherin expression in both germ and somatic cells is necessary for the survival of germ cells (and thus for the fertility of an individual), (3) E-cadherin is crucial for the formation of the ovarian follicles, (4) E-cadherin is not necessary for Sertoli cells and follicular cells differentiation or the formation of testis cords or ovigerous cords (and thus is not necessary for the sexual differentiation), (5) E-cadherin is not necessary for the formation of interstitium and steroidogenic cells and (6) E-cadherin is not necessary for sex determination of the gonad.

We can not exclude the possibility that in our experiment the E-cadherin was also deleted in other tissues expressing *Sf1* such as the pituitary gland and hypothalamus. Nevertheless, previous studies showed that deletion of gene encoding FSH (follicle-stimulating hormone, produced by pituitary gland and influencing gonads) did not influence gonad structure or number of germ cells, but rather caused development of gonad tumors and blocked primary ovarian follicle development from primordial follicles during maturation (Kumar *et al.* 1999). Similarly a lack of another pituitary hormone – LH (luteinizing hormone) did not influenced gonad development during fetal life, however, was crucial for stimulation of spermatogenesis during maturation (Themmen & Huhtaniemi 2000). This indicated that phenotypes observed in our study are effects of E-cadherin ablation in the gonads, however, not in the hypothalamus or pituitary gland.

The E-cadherin is a calcium-dependent cell–cell adhesion molecule believed to be crucial for the formation and maintenance of the epithelium (van Roy & Berx 2008). E-cadherin is expressed very early during mammalian development. Already in the two-cell stage embryo, it is responsible for the adhesion of the blastomers. It is also crucial for the embryo compaction, which takes place in the late eight-cell stage mouse embryo (Larue *et al.* 1994, Ohsugi *et al.* 1997). It has been shown that E-cadherin is present on the surface of primordial germ cells (PGCs) from the moment they leave the hindgut and migrate toward the gonadal primordium and on the surface of germ cells in

developing gonads (Mackay *et al.* 1999, Bendel-Stenzel *et al.* 2000, Piprek *et al.* 2017). In adults, E-cadherin is critical for the integrity of the epithelium in various organs (van Roy & Berx 2008).

Mutations in E-cadherin are linked to the development and metastasis of breast and gastric carcinomas (Beavon 2000, Polyak & Weinberg 2009). Depletion of E-cadherin usually leads to a decrease in cell adhesion strength, triggering cell motility, which is crucial for the metastasis. So far, there were no studies on the role of E-cadherin in gonad development in the mouse. There were two *in vitro* studies in which mouse gonadal primordia were

Figure 5 Structure of the ovary in control and E-cadherin knockout in the germ cells at E18.5. Histology (A and C) of the control ovary (*Oct4-cre⁻ Cdh1^{+/+}*) shows the presence of numerous diplotene oocytes (arrowhead), however, oocytes are not visible in the knockout ovaries (B and D, *Oct4-cre⁺ Cdh1^{fl/fl}*). Ovarian follicles (arrow) form near the medullar region of the ovary; trichrome staining. Immunostaining of collagen I in the control (E) and knockout (F) ovaries shows significant differences in the ovarian structure; the ovigerous cords in the control ovary are thicker. (G and H) The immunostaining of TRA98 shows that the number of germ cells (arrowheads) is significantly lower in the knockout than in control ovary. Scale bar: A and B: 100 μ m; C, D, E and F: 25 μ m.

Figure 6 Gene expression in control and E-cadherin knockout in the somatic cells at E18.5. The number of germ cells is significantly lower in knockout XY (A) and XX (B) gonads than in the control. * $P < 0.05$ (by χ^2).

cultured in the medium supplemented with anti-E-cadherin antibody (Bendel-Stenzel *et al.* 2000, Di Carlo & De Felici 2000). These studies showed that the anti-E-cadherin antibody disrupted PGC-PGC aggregation, led to the ectopic localization of PGCs and decreased the number of germ cells in the gonadal primordia.

In our studies, the knockout of E-cadherin in germ cells led to a significant reduction or complete elimination of the germ cells. This indicates that E-cadherin is important not only for the migration and settlement of the primordial germ in the genital ridges (Bendel-Stenzel *et al.* 2000, Di Carlo & De Felici 2000), but also at the later stages of gonadogenesis.

We also showed that the number of germ cells was reduced when E-cadherin was deleted in the SF1⁺ somatic cells. This indicates that the presence of E-cadherin at the surface of germ cells and adjacent somatic cells (Sertoli and follicular cells) is important for their proper adhesion, correct signaling between somatic and germ cells, and, consequently, for the germ cell fate. We did not detect any significant differences in cell proliferation between knockout and control gonads. Our observation indicates that the germ cell loss in the knockout gonads did not result from impaired proliferation, but rather from

Figure 7 Proliferation (PCNA immunostaining) and apoptosis (caspase 3 immunostaining) in testes and ovaries in E-cadherin knockout in the somatic cells at E18.5. Visibly similar number of PCNA-positive cells is visible in control (A) and knockout testis (B) indicating similar level of proliferation (Supplementary Table 5). Apoptotic (caspase 3 positive) cells (arrowheads) are present in the knockout within the testis cords (D). The control testis cords (C) lack apoptotic cells. The knockout (F) and control ovaries (E) show similar number of PCNA-positive cells, however, caspase 3-positive cells are visibly more numerous in the knockout (H) than in the control (G) ovaries (Supplementary Tables 6 and 8). Scale bar: 25 μ m.

enhanced apoptosis, which we detected in both sexes and both knockouts. The impact of E-cadherin knockout on the adhesion between the somatic and germ cells may potentially induce apoptosis of germ cells. Indeed, it has been shown that E-cadherin-mediated contacts prevent apoptosis in rat granulosa cells (Peluso *et al.* 2001). Moreover, E-cadherin, either directly or indirectly activates Akt kinase (protein kinase B) activity, which in turn, inactivates caspase 3 and prevents apoptosis (Peluso *et al.* 2001). In contrast, the loss of E-cadherin inactivates Akt kinase, which leads to the activation of caspase 3 and promotes apoptosis (Peluso *et al.* 2001).

Figure 8 Histology of the testis in control and E-cadherin knockout in the somatic cells. (A and C) Testes of control individual (*Sf1-cre⁻ Cdh1^{+/+}*) at stage E18.5; germ cells (g) are enclosed by the Sertoli cells (Sc); the whole testis cord (ts) is encapsulated by basement membrane (arrow); interstitium (i) is located between the cords; trichrome staining. (B and D) The knockout testis (*Sf1-cre⁺ Cdh1^{fl/fl}*) have similar to control structure however, in the knockout testis the number of germ cells (g) is visibly lower; trichrome staining. (E and F) The immunostaining of TRA98 shows that the number of germ cells (arrowheads) is significantly lower in the knockout than in control testis. The immunostaining of AMH in the control (G) and knockout testis (H) is similar, however, the knockout testis cords contain only the Sertoli cells and the germ cells (g) cells are very rare. Immunostaining of collagen I shows integrity of the testis cords and the presence of continuous basement membranes (arrows) enclosing each testis cord in control (I) and knockout (J) testes. No significant differences are present in Cyp17a1 immunostaining in control (K) and knockout (L) testes. Cyp17a1-positive cells are fetal Leydig cells visible in the interstitium. Scale bar: A and B: 100 μ m; C, D, E, F, G, H, I and J: 25 μ m.

Figure 9 Structure of the ovary in control and E-cadherin knockout in the somatic cells at E18.5. Histology (A and C) of the control ovary (*Sf1-cre⁻ Cdh1^{+/+}*) shows presence of numerous diplotene oocytes (arrowheads), however, in the knockout ovaries (B and D, *Sf1-cre⁺ Cdh1^{fl/fl}*) the oocytes are absent; trichrome staining. Immunostaining of collagen I in the control (E) and knockout (F) ovaries shows borders between the ovigerous cords. In the knockout ovaries the cords are smaller. (G and H) The immunostaining of TRA98 shows that the number of germ cells (arrowheads) is significantly lower in the knockout than in control ovary. Scale bar: A and B: 100 μ m; C, D, E and F: 25 μ m.

The loss of E-cadherin was also shown to be involved in the induction of apoptosis in mouse skin epithelial cells and enterocytes (Fouquet *et al.* 2004, Galaz *et al.* 2005). Further studies are necessary to resolve whether the loss of E-cadherin in the germ cells induce apoptosis via inactivation of Akt kinase or by a different mechanism.

We showed that the germ cell loss in E-cadherin knockout in both germ or gonadal somatic cells started at stage E16.5. This suggests that the presence of E-cadherin around the embryonic day 16.5 is critical for germ cell survival. In developing female gonad, the germ cells are located within the germ cell cysts. The majority of germ cells enter meiosis at stage E15.5. At stage E17.5 some of the germ cells enter diplotene and the germ cysts brake

down into the ovarian follicles (Borum 1961, Pepling & Spradling 2001, Pepling *et al.* 2010). We also observed that the germ cells loss in the male gonads also occurred at the same time that is at stage E16.5. In the testes around E16.5, the germ cells enter G0 mitotic arrest (McLaren 2003, Töhönen *et al.* 2003). Further studies are needed to understand why the proper cell adhesion at this particular period of development is critical for the survival of germ cells in the male and female gonad.

We also showed that the depletion of E-cadherin in SF1⁺ somatic cells did not alter the formation of the testis cords or the ovigerous cords. This indicates that E-cadherin-mediated adhesion is not important for the differentiation and formation of the somatic component of the gonads or for the maintenance of testis cords integrity. Other studies showed that certain extracellular matrix components such as collagen IV seem to be crucial for the integrity of the testis cords. Chen *et al.* (2013) showed that a deletion of *Wt1* (Wilms tumor 1 gene) in Sertoli cells of developing mouse testes led to the disruption of testis cords due to the decreased expression of *Col4a1* and *Col4a2* genes (the expression of E-cadherin and other CAMs remained unchanged) (Chen *et al.* 2013). It is known that cadherins are involved in WNT/ β -catenin pathway that is pivotal for female sex determination in the mouse (reviewed by Piprek 2009a,b). Thus, the knockout of E-cadherin in developing gonads could possibly disrupt sex determination. Surprisingly, in the knockout gonads in our study, the expression of male and female sex-determining genes agreed with the genetic sex, and the sex ratio was not altered. This indicates that the lack of E-cadherin did not affect sex determination.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-18-0621>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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