No evidence for the presence of oogonia in the human ovary after their final clearance during the first two years of life

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Background: Conflicting results of studies on mouse and human have either verified or refuted the presence of oogonia/primordial germ cells in the post-natal ovary. The aim of this study was to trace whether oogonia recognized by immunohistochemical methods in the first trimester human ovary were present also in peri- and post-natal ovaries.

Methods: For this study, 82 human ovaries were collected: 25 from embryos from 5 to 10 weeks post conception (wpc), 2 at 18 wpc, 32 from 32 wpc to 2 years and 23 from 2 to 32 years. Of these, 80 ovaries were fixed and paraffin-embedded and 2 (8 year-old) ovaries were processed for plastic sections. Serial sections were prepared for immunohistochemical detection of markers for oogonia: tyrosine kinase receptor for stem cell factor (SCF)(C-KIT), stage-specific embryonic antigen-4 (SSEA4), homeobox gene transcription factor (NANOG), octamer binding transcription factor 4 (OCT4) and melanoma antigen-4 (Mage-A4), while noting that C-KIT also stains diplotene oocytes.

Results: Almost all oogonia exclusively stained for SSEA4, NANOG, OCT4 and C-KIT, whereas MAGE-A4 only stained a small fraction. At birth only a few oogonia were stained. These disappeared before 2 years, leaving only diplotene oocytes stained for C-KIT. From 18 wpc to 2 years, the medulla contained conglomerates of healthy and degenerating oogonia and small follicles, waste baskets (WBs) and oogonia enclosed in growing follicles (FWB). Medulla of older ovaries contained groups of primordial, healthy follicles.

Conclusions: We found no evidence for the presence of oogonia in the human ovary after their final clearing during the first 2 years. We suggest that perinatal medullary WB and FWB give rise to the groups of small, healthy follicles in the medulla.

Key words: human ovary / oogonia / immunohistochemistry

Introduction

When primordial germ cells (PGCs) arrive at the gonadal anlage, they are termed oogonia in the female and spermatogonia in the male. Oogenesis is the process that transforms the mitotically dividing oogonia to oocytes by initiation of meiosis. For decades it has been accepted that all oocytes are made before or around the time of birth in mammals and that no oogonia exist in the adult mammalian ovary. However, this concept was challenged by two reports dealing with the developing and adult mouse ovaries: one claiming that oogonia were present in the ovarian surface epithelium (OSE) (Johnson et al., 2004) and the other reporting that circulating cells from the bone marrow contribute to ovarian regeneration through de novo oogenesis in mice previously sterilized with chemotherapy (Johnson et al., 2005). A later study on parabiotic mice connected through a common circulation system failed, however, to support the notion that blood born progenitor germ cells can result in ovulated oocytes (Eggan et al., 2006). This does not necessarily exclude that circulating bone marrow stem cells may form oogonia and oocytes if they settle into the ovarian tissue. Recently, gene with germ like specific expression that encodes an ATP-dependent RNA helicase of the DEAD-box protein family (VASA) expressing cells isolated from...
neonatal and adult mouse ovaries and transplanted to sterilized mouse recipients were shown to generate oocytes that could be fertilized and produce viable offspring (Zou et al., 2009). Other studies used a transgenic mouse model that expressed green fluorescent protein under a germ-cell-specific Oct-4 promoter and isolated male and female germ-cell line stem cells from male and female mouse, respectively (Izadyar et al., 2008; Pacchiariotti et al., 2010). Most convincingly, mouse bone marrow transplanted to sterilized mice generated oocytes (Lee et al., 2007). Nevertheless, more evidence is needed to sustain the notion of new formation of oocytes in the normal adult mammalian ovary (Gosden, 2004; Greenfield and Flaws, 2004; Telfer, 2004; Byskov et al., 2005; Telfer et al., 2005; Sottili, 2007; de Felici, 2010). An extensive review of evidence for and against neo-ogenesis further discusses these ideas (Notaranni, 2011).

A recent study of normal adult human ovaries (28–53 years) showed no evidence for the presence of oogonia; neither early meiosis-specific or oogenesis-specific mRNAs nor immunohistochemical markers for oogonia or meiosis could be detected (Liu et al., 2007). In contrast, several studies of mammalian, including human, embryonic gonads have shown that PGCs as well as oogonia and spermatogonia express markers and often share similarities with embryonic stem (ES) cells that characterize pluripotency; such markers include octamer binding transcription factor 4 (OCT4) (member of the POU family of transcription factors) (Brehm et al., 1998; Pesce et al., 1998; Shamblott et al., 1998; Shamblott et al., 2001; Liu et al., 2004), NANOG (Cheville and Roche, 1999; Yakirevich et al., 2003) and the stage-specific embryonic antigens, SSEAs (Liu et al., 2004). OCT4 seems to be required for maintenance of cellular toti/pluripotency and PGCs survival (Kehler et al., 2004). NANOG, another transcription factor, has been proposed to be a gatekeeper of the pluripotency related to ES cells and PGCs (Hyslop et al., 2005) and is down-regulated in human fetal spermatogonia before OCT4 expression is lost (Hoei-Hansen et al., 2005; Stoop et al., 2005).

Generally, expression of many genes for pluripotency is shared between ES cells and PGCs. However, when PGCs arrive at the gonads and become oogonia or spermatogonia they assume differentiation which, in the oogonia, is closely related to beginning of meiosis. At meiosis, there seems to be a permanent down-regulation of expression of these genes related to pluripotency, i.e. OCT4 (Pesce et al., 1998; Rajpert-De Meyts et al., 2004; Anderson et al., 2007), NANOG (Hoei-Hansen et al., 2005) and SSEA4 (He et al., 2006). Also the receptor for stem cell factor, tyrosine kinase receptor for stem cell factor (SCF)(C-KIT), is temporally downregulated in the human female germ line during the passage through the first meiotic prophase, but resumes expression at the diplotene stage in connection with folliculogenesis (Hoyer et al., 2005; Liu et al., 2007).

Melanoma antigens of the MAGE-A gene family, which encodes for a variety of different tumor antigens, have also been considered as pluripotent germ cell markers. They are expressed in spermatogonia of the human fetal testes before week 25 of gestation (Takahashi et al., 1995; Pauls et al., 2006), in the human oogonia prenatally (MAGE-A1) (Gjerris et al., 2007) and in some of migrating PGCs and early oogonia in female, human embryos (MAGE-A4) (Møllgaard et al., 2010). Interestingly, some of these genes are also expressed in different types of human gonadal germ cell tumors, e.g. MAGE-A4 in testicular germ cell tumors (Cheville and Roche, 1999; Aubry et al., 2001; Rajpert-De Meyts et al., 2003) and in rare ovarian germ cell neoplasms (Hoei-Hansen et al., 2007). The most well known cell types to express MAGE genes are spermatogonia and the trophoblast of the placenta (Cheville and Roche, 1999; Jungbluth et al., 2007). The pluripotent marker OCT4 is also expressed in human gonadoblastomas/seminomas (Looijenga et al., 2003; Rajpert-De Meyts et al., 2004) and NANOG is expressed in teratocarcinomas (Clark et al., 2004) and germ cell tumors (Hart et al., 2005; Hoei-Hansen et al., 2005).

Formation of the human gonads begins around day 30 after fertilization when the first PGCs reach the gonadal anlage at the ventral upper part of the mesonephros (Witschi, 1948), but it is not clear when the last PGCs reach the developing gonads. The oogonia multiply mitotically, but when meiosis is initiated and terminates mitotic proliferation, they are termed oocytes. The first oogonia enter meiosis by the end of the embryonic period, i.e. from Week 9 prenatally (Bendsen et al., 2006) and around the time of birth only a few oogonia seem to be present as almost all have been converted to oocytes (Linter-Moore et al., 1974). However, it is not known for how long oogonia remain in a girl’s ovary.

The aim of the present study was to trace oogonia in the cortex and medulla of the human ovary, from embryonic life to adulthood using histology and immunohistochemical staining for PGC and oogonia cell-specific markers: C-KIT, SSEA4, NANOG and OCT4 and the testis-tumor related MAGE-A4. The first trimester ovaries contained only oogonia and a few early meiotic oocytes and somatic cells and were therefore used as positive controls.

Materials and Methods

Human ovaries

For this study, 82 human ovaries were used. The control group consisted of 25 first trimester ovaries from legal abortions as well as two specimens from 18 wpc. The ages of the first trimester specimens were estimated by weeks post conception (wpc) and ranged from 5 to 10 wpc. For perinatal ovaries, there were 32 specimens aged from 32 wpc to 2 years. For ovaries from 2 years to 32 years old, there were 25 specimens. Ovaries from the legal abortions were all from women who gave informed consent to study the aborted material. Ovaries from all other patients were obtained for various medical reasons (Table I). These ovaries were archived pathological material.

Histology

There were 74 ovaries fixed in Bouin’s fixative (2–10 h), while two ovaries of 18 wpc and four first trimester ovaries were fixed in Lillie’s fixative (24 h). Two ovaries, from 8 years old girls, were cut into smaller pieces and fixed in 1% glutaraldehyde and embedded in araldit for preparation of 1 μm plastic sections and stained with toluidine blue. All other ovaries were paraffin-embedded and prepared for histology and immunohistochemistry (IHC). Larger post-natal ovaries were cut in halves at the longitudinal axis prior to fixation. Smaller ovaries were mostly fixed intact, then parts of smaller ovaries were serially sectioned at 5 μm and prepared for IHC or alternately stained with hematoxylin and periodic acid Shiff (PAS) (HP). In the larger ovaries of different post-natal ages, 50–100 serial sections, 30–40 μm thick, were cut at two to three different segments of the ovary randomly cut from the embedded material, and stained with HP or prepared for IHC.

Immunohistochemical methods

Sections were de-paraffinized, rehydrated and washed in tris-buffered saline (TBS; 0.05 M Tris, pH 7.6, 0.15 M NaCl) with 0.01% Nonidet P-40 (TBS/
Nonidet). Antigen retrieval of NANOG, OCT4 and C-KIT was performed for 10 min in a microwave oven using citrate buffer, pH 6 (NANOG) or tris–EDTA–glycerol [(TEG buffer, pH 9 (OCT4 and C-KIT)]. After heat treatment, the sections were rested for 20 min at room temperature. To block endogenous peroxidase activity, sections were incubated in 0.45% H2O2 in TBS/Nonidet for 15 min, and then in 10% normal goat serum in TBS/Nonidet for 30 min at room temperature to block non-specific binding. Sections were then incubated overnight at 4 °C with the primary antibody. Details concerning primary antibodies and dilutions are shown in Table I. For detecting mouse and rabbit primary antibodies, anEnVision™System/Peroxidase, (DakoCytomation, code K1392) was used. For detecting goat primary antibodies, secondary antibody (Amerham Biosciences code RPN1025V, diluted 1:20 in 10% donkey serum/TBS) and StreptABComplex/HRP (DakoCytomation code K0377) were used.

As controls, sections were either incubated without primary or secondary antibodies, or incubated with matched isotype proteins (mouse IgG3 or IgG2a), or with irrelevant goat or rabbit antibodies instead of primary antibodies. As a further control for OCT4, the section was preincubated with the corresponding peptide at the proportion of 1–5 for 1 h before antibody incubation.

For double staining, antigen retrieval was performed in TEG buffer, and then sections from human fetal ovaries were incubated with polyclonal rabbit antibody OCT4 (dilution 1:250) overnight at 4 °C. They were then processed for 30 min with goat anti-rabbit rhodamin (1:100, Jackson 111-025-144) followed by mouse anti-SSEA4 (dilution 1:75) overnight at 4 °C. After incubation for 30 min with EnVision™ + System/HRP anti-mouse (K4007) followed by tyramid treatment (Invitrogen, TSATM kit # 12, T20922) according to the manufacturers instructions, sections were coverslipped.

A Carl Zeiss LSM 510 was used for confocal microscopy.

**Results**

**General pattern of marker distribution**

Immunostaining for the markers used resulted in a well-defined staining pattern, which could be a nuclear staining (NANOG, OCT4), or a combination of a uniformly or granular cytoplasmic and membrane staining (SSEA4, MAGE-A4) and/or a membrane-associated staining (C-KIT). This staining pattern was similar irrespective of the fixative used. The specificity and sensitivity of the IHC method was tested with two antibodies directed against different epitopes of human OCT4.

The control sections for the OCT4 antibody, which included preabsorption with the OCT4 peptide, showed a lack of reactivity (Fig. 1B). Control sections for mouse monoclonal antibodies SSEA4 and Mage-A4, which were incubated with the respective isotype IgG (IgG3 or IgG2a) or no antibody, were blank. Control sections for polyclonal rabbit and goat antibodies, which were incubated with irrelevant polyclonal antibodies or without the primary antibodies, showed no reactivity.

The distribution of marker immunoreactivity is described in relation to three stages in ovarian development: (i) first trimester ovaries characterized by oogonia and lack of diplotene oocytes. This group serves as a control group for the ICH detection of oogonia. Moreover, two ovaries from 18 wpc served as controls for immunohistochemical reactions; (ii) perinatal ovaries, from 32 wpc until 2 years characterized by fewer or none oogonia, many diplotene oocytes and follicle development; (iii) post-natal ovaries from 2 years to 32 years where previous investigators were unable to recognize oogonia and all oocytes were enclosed in follicles.

Figure 2 shows a schematic overview of the time-related presence of oogonia and oocytes expressing IHC markers, and specifically also those present in OSE and in waste baskets (WBs) and follicle waste baskets (FWBs) (as seen in the perinatal ovaries from 32 wpc to 2 years).

**The control group: first trimester ovaries**

**Histology based on HP staining**

In ovaries from 5 to 7 wpc, oogonia were equally distributed within most of the ovarian tissue. Thereafter the concentration of oogonia gradually increased in the peripheral cortex compared with the central region thus forming an ovarian cortex and medulla toward the end of 8 wpc. In 9 and 10 wpc, the first few oocytes in leptotene and zygotene stages (the two first transitory stages of the first meiotic prophase) were recognized in the ovarian cranial inner cortex. The OSE of the first trimester ovaries was one to three cell layers thick often without a distinct coherent basement membrane. It changed between cuboidal and squamous, as the shape of the ovary changed from having a smooth surface from 5 to 8 wpc, followed by invaginations into the ovarian cortex around 9–10 wpc. Oogonia were often found in OSE throughout the first trimester ovaries (Fig. 3).

**Immunohistochemistry**

In the first trimester ovarian samples, the plasma membrane of all oogonia was stained for C-KIT, whereas the somatic cells were unstained (Fig. 3B). The oogonia of the first trimester ovaries exhibited a strong cytoplasmatic staining for SSEA4 (Fig. 3C). The nuclei of oogonia were stained for NANOG, whereas somatic cells were unstained (Fig. 3D). The nucleus of all germ cells stained for OCT4 resembling the staining pattern of NANOG (Fig. 3E). Only a small fraction of the oogonia exhibited MAGE-A4-stained cytoplasm and no or faint nuclear staining (Fig. 3F). The staining intensity of the cytoplasm varied from faint to heavy (Fig. 3F). The MAGE-A4-stained oogonia

<table>
<thead>
<tr>
<th>Table I</th>
<th>Number of human ovaries and diagnosis.</th>
</tr>
</thead>
<tbody>
<tr>
<td>First trimester (25) and 18 wpc (2)</td>
<td>32 weeks pc to 2 years (32)</td>
</tr>
<tr>
<td>Congenital heart disease (8)</td>
<td>Congenital heart disease (2)</td>
</tr>
<tr>
<td>Pneumonia (3)</td>
<td>Pneumonia (1)</td>
</tr>
<tr>
<td>Hyaline membrane (1)</td>
<td>Hodgkin’s lymphoma (6)</td>
</tr>
<tr>
<td>Traffic accident (3)</td>
<td>Traffic accident (1)</td>
</tr>
<tr>
<td>Premature birth (3)</td>
<td>Sarcoma (2)</td>
</tr>
<tr>
<td>Meningitis (2)</td>
<td>Breast cancer (2)</td>
</tr>
<tr>
<td>Encephalitis (2)</td>
<td>Spina bifida (1)</td>
</tr>
<tr>
<td>Leukemia (2)</td>
<td>Leukemia (6)</td>
</tr>
<tr>
<td>Hydrocephalus (2)</td>
<td>Nephritis (1)</td>
</tr>
<tr>
<td>Cystic lung (2)</td>
<td>Ovariecotomy (1)</td>
</tr>
<tr>
<td>Gastroenteritis (2)</td>
<td>Oesophagus atresia (1)</td>
</tr>
<tr>
<td>Goitre (1)</td>
<td></td>
</tr>
</tbody>
</table>
were sometimes located in small groups equally distributed in the cortex.

The perinatal ovaries aged 18 wpc and from 32 wpc to 2 years

Histology based on HP staining

In ovaries aged 18 wpc, any germ cells of the inner cortex have entered meiosis and many small and growing follicles are present in the medulla and the inner part of cortex. A considerable number of cortical placed oogonia are still seen (Fig. 1). In ovaries of this group as well as in ovaries from 32 wpc and to 2 years, two remarkable features were observed. First, in the medulla, irregular conglomerates of oogonia and oocytes in transitory stages of meiosis as well as small, normal looking diplotene oocytes (the resting stage of the first meiotic prophase) mixed with somatic cells and surrounded by a fragmented basement membrane were prominent (Fig. 4E–H and Fig. 5A). The germ cell number (oogonia and oocytes) may reach around 50 in the largest cross section of such clusters. Many of these germ cells were healthy looking, whereas others showed signs of degeneration with vacuolized cytoplasm and pyknotic nuclei. These structures have been termed WBs (Hoyer et al., 2005). They disappeared before the age of 2 years. Second, in the inner part of cortex and in medulla larger, abnormal follicles containing a degenerating larger oocyte and oogonia in the granulosa layer, sometimes 10–30 in the largest cross section, were often seen (Fig. 4C, D, I). These follicles are termed FWBs. In 32 wpc ovaries, many oogonia of the medulla and the inner part of cortex have entered meiosis, but a thin cortical rim with oogonia are still present. By the time of birth, only a few oogonia were still present. Some of these were in OSE-connected cords in ovarian cortex up to a few months after birth (Fig. 4A and B). Such cords occasionally also contained oocytes in leptotene stages but these were absent after the third month.

Immunohistochemistry

All oocytes were stained by C-KIT. The number of SSEA4-stained germ cells decreased prenatally. By the time of birth, only the few

Table II Primary antibodies and dilutions.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Company</th>
<th>Code nr.</th>
<th>Antibody-species</th>
<th>Pretreatment</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSEA4</td>
<td>Chemicon</td>
<td>MAB 4304</td>
<td>Mouse</td>
<td>None</td>
<td>1:75</td>
</tr>
<tr>
<td>NANOG</td>
<td>R&amp;D</td>
<td>AF 1997</td>
<td>Goat</td>
<td>Citrate pH 6</td>
<td>1:50</td>
</tr>
<tr>
<td>OCT4</td>
<td>Abcam</td>
<td>Ab 19857</td>
<td>Rabbit</td>
<td>TEG</td>
<td>1:250</td>
</tr>
<tr>
<td>Mage-A4</td>
<td>Zymed invitrogen</td>
<td>6C1</td>
<td>Mouse</td>
<td>None</td>
<td>1:100</td>
</tr>
<tr>
<td>C-KIT/CD117</td>
<td>DakoCytomation</td>
<td>A4502</td>
<td>Rabbit</td>
<td>TEG</td>
<td>1:300</td>
</tr>
</tbody>
</table>

Figure 1 Expression of OCT4, SSEA4 and OCT4 + SSEA4 in sections from a human ovary, 18 wpc. Expression of OCT4 (A) and the neighboring control section (B) which was preincubated with the corresponding peptide (pa) before staining. The black stained oogonia, single (arrows) or in clusters (arrowhead), are mainly confined to a peripheral rim of the ovary. The white ‘holes’ are mainly unstained oocytes. A close-by section of the same ovary showed the peripheral rim with the oogonial cytoplasm stained by SSEA4 (C, green) and the nuclei stained by OCT4 (D, red), with both shown simultaneously (E). Two oogonia are marked by circles and rectangles, respectively. A Carl Zeiss LSM 510 was used for confocal microscopy (C–E). The bar of A and B corresponds to 500 μM. The bar of C–E corresponds to 20 μM.
remaining oogonia in the cortex and oogonia contained in follicles and WB were stained (not shown). NANOG-stained germ cells were rare or absent from after birth (not shown). The number of OCT4-stained germ cells was also much reduced, as more and more oocytes differentiated during prenatal life. In the last prenatal, month only single peripherally situated oogonia and a few oogonia confined to the centrally placed follicles containing oogonia and the WB were stained (Fig. 4E), whereas all somatic cells remained unstained. Similarly, MAGE-A4-stained oogonia were only present in the follicles containing oogonia and in WB from the time of birth (Fig. 4I).

### The post-natal ovaries from 2 years to 32 years

**Histology based on HP staining**

No oogonia were recognized in ovaries after the age of 2 years. During this period, the medulla contained growing antral follicles, but until puberty antral follicles remained smaller than \( \sim 5 \text{ mm} \) in diameter. After puberty also larger antral follicles were present in all ovaries. From the second year of life, the medulla contained isolated groups of 5–25 closely placed primordial follicles often in connection with the ovarian cortex, but no oogonia were present. During the first postnatal year, follicles containing oogonia and antral follicles were present in the medulla, but no oogonia were recognized. Later, all follicles became antral and oogonia were no longer present. The medulla contained mostly antral follicles, and the cortex contained small antral follicles and growing antral follicles. No oogonia were present in any of the ovaries studied postnatally. The medulla also contained growing granulosa cells in postnatal ovaries, but no oogonia were present. The cortex also contained growing granulosa cells, but no oogonia were present.

### Table: Time-related presence of oogonia and oocytes in OSE, WBs and follicles, and their expression of pluripotency markers

<table>
<thead>
<tr>
<th>Marker</th>
<th>Oogonia - Oocytes</th>
<th>Prenatal (2nd year (month))</th>
<th>Postnatal (2 nd year</th>
<th>&gt; 2 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-KIT</td>
<td>Oogonia in OSE</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SSEA4</td>
<td>Oogonia in OSE</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>OCT4</td>
<td>Oogonia in OSE</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MAGE-A4</td>
<td>Oogonia in OSE</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Figure 2** Overview of the time-related presence of oogonia and oocytes in OSE, WBs and follicles, and their expression of pluripotency markers C-KIT, SSEA4, NANOG, OCT4 and MAGE-A4. A contiguous line symbolizes that almost all oogonia (and oocytes for C-KIT) are stained. The dashed line symbolizes that a low number of oogonia are stained. The punctuated line concerning staining by MAGE-A4 symbolizes that only a smaller fraction of the oogonia are stained (relevant for MAGE-A4). Abbreviations: OSE, ovarian surface epithelium; WB, waste baskets, including oogonia, oocytes, small and growing follicles, degenerating as well as healthy looking. FWB, follicle waste baskets, including growing follicles with oogonia inclosed in their granulosa layer.
with a few primary and secondary follicles, sometimes close to an
antral follicle (Fig. 6). These medullary small follicles appeared
morphologically normal (Fig. 6B and C). Up to the age of around 6
years, follicles with two or more oocytes were common.

Immunohistochemistry
After the age of 2 years, the only C-KIT-positive cells were the normal
follicle-enclosed oocytes (Fig. 5C–E). No cells were stained with
SSEA4, NANOG or MAGE-A4 after the age of 2 years (Fig. 5G–J).
The control sections were unstained as well. One exception was an
occasional faint OCT4 staining of the oocyte cytoplasm of small,
growing follicles. The medullary-placed groups of small follicles
exhibited the same staining pattern of the oocyte as other follicles.

Distribution pattern of C-KIT-stained cells
in ovarian cortex from girls and women
Around 32 wpc, almost all germ cells had entered meiosis and had
reached diplotene stage and were stained with C-KIT (Fig. 5A).
However, after birth small oogonia-like cells could still be seen
stained with C-KIT close to OSE (Fig. 5A and B). During the first 2
years of life, the cortical rim with oocytes gradually retracted from
the ovarian surface as the tunica albuginea increased in thickness.
This pattern is seen in Fig. 5A–E. The age-related increase in thickness
of the tunica albuginea is clearly visualized by the lack of C-KIT-stained
cells in tunica albuginea. It is therefore also rather easy to scan the
C-KIT-stained sections for the presence of oogonia/oocytes close
to, or within, the OSE or in the tunica albuginea. No C-KIT-positive
stained cells (small or large) were seen in tunica albuginea of ovaries
after the age of 2 years.

Discussion
The present study demonstrates that no cells in post-natal human
ovaries older than 2 years could be recognized as oogonia by

Figure 3 Expression of C-KIT, SSEA4, NANOG, OCT4 and
MAGE-A4, and morphology of first trimester human ovaries. (A)
HP-stained section of an ovary (OV) and mesonephros (M), 7 wpc.
(Aa) shows the peripheral part of cortex from (A) with two
oogonia in the OSE, the basement membrane of which is stained
red with PAS. (B) Section of an ovary, 8 wpc, stained for C-KIT.
The medulla with fewer oogonia than in the cortex is being formed.
(Bb) shows the peripheral part of cortex with C-KIT-stained plasma
membrane of oogonia in OSE and below. (C) Ovary, 10 wpc,
stained for SSEA4. The cytoplasm of almost all oogonia are stained,
exhibiting a forming cortex with many stained germ cells and a
medulla with fewer stained germ cells. (Cc) shows the peripheral
part of cortex from (C) with one SSEA4-stained oogonium in the
OSE and two below. (D) An adjacent section to (C) stained for
NANOG. Almost all oogonia are stained visualizing the similar distri-
bution pattern of germ cells as in (B) and (C). (Dd) shows the periph-
eral part of cortex from (D) with one NANOG-stained oogonium in
the OSE and several below. (E): Section of an ovary, 10 wpc,
stained for OCT4. The cytoplasm of almost all oogonia are stained,
exhibiting a forming cortex with many stained germ cells and a
medulla with fewer stained germ cells. (Ee) shows the peripheral
part of cortex from (E) with a cluster of at least four OCT4-stained
oogonia in OSE and several below. (F) The adjacent section of the
ovary seen in (A) stained for MAGE-A4. Only a fraction of the
oogonia are stained, with a few exhibiting a heavy staining, (Ff)
shows the peripheral part of cortex from (F) with two oogonia in
OSE; one heavily and one faintly stained for MAGE-A4. Below the
OSE one heavily and three faintly stained oogonia are seen. Abbrevi-
ations: OSE, ovarian surface epithelium with its basement membrane
indicated by a stippled line. OV, ovary; M, mesonephros. The bar of
all figures labeled with capital letters corresponds to 650 μM. The bar
of all figures labeled with capital plus small letters corresponds to
15 μM.
Figure 4 Expression of C-KIT, MAGE-A4 and OCT4, and morphology of perinatal human ovaries. (A) High magnification of peripheral part of a 13 day old ovary stained with HP. Three large diplotene oocytes and two smaller oogonia-like cells (arrows) appear to be enclosed in common cell cords. (B) Section close to (A) stained for C-KIT. The arrows point at the nucleus (no staining) of three small oogonia with stained plasma membranes. Stained plasma membranes of oocytes are seen in the lower part of the figure in connection with the oogonia. (C) Section of a 6 day old ovary stained with HP showing part of a medullary placed follicle containing many oogonia (arrows) in the granulosa layer. (D) Section adjacent to the one seen in (C) stained for C-KIT. The plasma membrane of the oogonia in the granulosa layer are all stained, whereas the granulosa cells are unstained. (E) Part of a WB from the medulla of an ovary of a fetus close to term with four oogonia stained for OCT4. (F) HP-stained section from medulla-cortex transition of a 32 wpc ovary with a large WB (marked by a stippled line) in the medulla. (G) A higher magnification of part of the WB in (F) with somatic cells, a diplotene oocyte partly enclosed in a follicle and abnormal oocytes at the pachytene stage (arrows). (H) Section adjacent to (G) stained for C-KIT showing a small follicle, in which the plasma membrane of the oocyte is stained, and smaller stained cells, probably oogonia, as well as an oocyte possibly in pachytene stage of the meiotic prophase (arrow) with unstained plasma membrane. (I) A tangential section of a follicle from a 5 week old ovary showing a few small oogonia and a larger cell stained for MAGE-A4. Abbreviations: WB, waste basket; The bars of A–D correspond to 18 μM. The bar of F corresponds to 25 μM. The bar of G corresponds to 12 μM. The bars of E, H and I correspond to 10 μM.

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morphy or by markers characteristically staining pluripotent premeiotic germ cells prenatally, i.e. SSEA4, OCT4, NANOG or MAGE-A4. It therefore seems unlikely that oogonia could play a significant role in human reproduction after birth. In a study of the adult human ovary, Liu et al. (2007) also failed to identify oogonia using IHC and RT–PCR for detecting different oogenesis-associated proteins and RNAs. In a recent review, de Felici (2010) discussed whether the oocyte pool might be renewed under specific physiological conditions or perhaps be activated after ovarian damage. It certainly seems that in vitro purified large cells from mouse ovaries, which proliferate and express VASA, can generate oocytes after transplantation to sterilized mice and produce viable offspring (Zou et al., 2009). Perhaps the mammalian ovary contains cells with stem-cell-like characteristics that can be provoked to enter a differentiation pathway toward oocytes, at least in vitro.

We found that no cells were stained by the germ-cell-specific cancer (testis) antigen MAGE-A4 in ovaries of children older than 3 months. If oogonia are present in the post-natal human ovary, the risk of ovarian germ cell tumors might be expected. However, gonadoblastomas of the ovary occur almost exclusively in patients presenting intersex disorders (Pauls et al., 2006). This observation is in accordance with the fact that gonadoblastomas of the ovary, in contrast to the testis, is a very rare disease and indicates that oogonia giving rise to germinomas are absent or seldom in the human ovary as previously reported (Rajpert-De Meyts et al., 2004). In parallel, the absence of OCT4-positive oogonia in ovaries of girls in the present study also supports the notion that pluripotent germline stem cells characterizing dysgerminoma are very rare in the human ovary (Cheng et al., 2004; Patterson and Rustin, 2006). Thus, it seems unlikely that new oocytes are generated from stem germ cells in the post-natal human ovary as suggested by Bukovsky et al. (2005a,b).

The present study of ovaries of young girls and women failed to reveal any oogonia as characterized by pluripotent IHC markers, except in the follicles containing oogonia and in WB, both of which disappeared before the age of 2 years. This is in accordance with Liu et al. who identified no oogonia in their study of the adult human ovary and could not detect any meiotic-specific or oogenesis-associated mRNAs (Liu et al., 2007). This, however, does not exclude that a very low number of cells with such mRNA might still be present but not detectable with these methods, as massive amounts of other mRNAs are present in the ovaries. In fact, a recent study of isolated OSE cells from human ovaries without follicles or oocytes indicates that OSE-produced oogonia-like cells expressing SSEA4, OCT4, C-KIT, NANOG and ZP2 after a week of culture (Virant-Klun et al., 2008). Thus, although we found no evidence that OSE of normal post-natal ovaries contains cells that stained for SSEA4, OCT4, C-KIT and NANOG, the in vitro condition may allow for conversion of OSE cells into a multipotent stem-like cell type.

Since previous studies have suggested a role of OSE in germ cell/oocyte formation (Kingery, 1917; Allen, 1923; Bukovsky et al., 2004; Johnson et al., 2004), we also focused on the presence of oogonia/oocytes in the outer ovarian cortex, in particular the OSE. We found in concordance with others (Motta and Makabe, 1986; Wordinger et al., 1990) that oogonia are present in OSE during prenatal life but are rare or absent in the ovaries of newborn girls. The presence of oogonia/oocytes in OSE and in particular in the bursa of the
neonatal mouse has prompted the assumption that such germ cells are in the process of being eliminated from the ovary (Byskov and Rasmussen, 1973; Motta and Makabe, 1986; Wordinger et al., 1990). The disappearance of oogonia from OSE of the human ovary shortly after birth does not support the idea that germ stem cells of OSE participate in neo-oogenesis in vivo.

Although not quantified in the present study, oocytes and a rather large number of oogonia-like cells positively stained for C-KIT, OCT4 and MAGE-A4 are trapped in follicles (FWB) and WB during prenatal life as previously reported (Hoyer et al., 2005). Such structures are often still seen in the neonate’s ovary up to around the age of 2 years. As the FWB and WB grow in size and number toward birth, a large number of oogonia/oocytes become isolated in the medulla. However, during the first or second year of life, the enclosed oogonia gradually disappear as visualized by loss of staining for OCT4, SSEA4, NANOG and MAGE-A4, whereas at the same time the groups of small and early growing follicles are seen in the medulla. It has recently been described that a small fraction of oogonia of human fetal ovaries can be stained with antibodies for MAGE types cancer/testis-antigens, peaking mid-gestationally and with only few stained cells present at birth (Gjerstorff et al., 2007; Nelson et al., 2007). Nelson et al. described that these immuno-reactive oogonia, and also occasionally oocytes, formed cords with cells exhibiting autolytic changes, in the cortex as well as in the ovarian centre (Nelson et al., 2007). These structures might be similar to the pre- and perinatal FWB and WB described in the present study. We suggest that some of healthy follicles of the FWB and WB in medulla may transform into the clusters of healthy looking primordial follicles present in the medulla of the post-natal ovary as recently described (Kristensen et al., 2011). Bukovsky et al. suggested that follicular renewal de novo takes place in the deeper ovarian cortex where small clusters of primordial and early growing follicles have been described (Bukovsky et al., 2005a,b). However, we have no evidence of de novo formation of these oocytes/follicles in the post-natal ovary older than 2 years. The isolation of the numerous oogonia and oocytes in the FWB and WB, many of which are degenerating, may also explain the decrease in the number of germ cells during fetal life (Baker, 1963).

Stem cell surface markers such as SSEA4 are often used to characterize human ES cells. In fact, a similarity between pluripotent stem cells and PGCs seems evident as the two cell types can differentiate into each other (Shamblott et al., 1998; Shamblott et al., 2001). The expression of SSEA4 in human PGCs in the prenatal ovary was recently described along with expression of C-KIT, OCT4 and NANOG (Kerr et al., 2008). The observed membrane as well as cytoplasmic staining by SSEA4 is not unusual; the cytoplasmic staining may be related to a specific stage of the cell cycle. For example, in the early neuroepithelium a similar staining by SSEA4 has been described in vivo.

Figure 5 Comparison between C-KIT-stained cells in ovaries of different ages and expression of C-KIT, SSEA, NANOG, OCT4 and MAGE-A4 of human post-natal ovaries. (A) Section of the cortex from a 32 wpc ovary stained for C-KIT showing the cortex full of oocytes and part of the medulla with WBs packed with germ cells. (B) C-KIT staining of the cortical part of a 13 day old ovary with many oocytes in the cortex, but fewer than in the 32 wpc ovary. The oocyte-free tunica albuginea appears to be developing although some C-KIT-positive cells are seen in, or close to, the OSE. (C) C-KIT-stained cortex of a 2 year old ovary with fewer oocytes in the cortex than in the perinatal ovary (B) and a developing tunica albuginea (double-arrow) without stained cells. Part of an antral follicle is seen below the cortex. (D) The cortex of a 16 year old ovary with no C-KIT-stained cells in the broad tunica albuginea (double-arrow). (E) The cortex of a 32 year old ovary with no C-KIT-stained cells in the broad tunica albuginea (double-arrow) and apparently fewer oocytes below the tunica than in (D). (F) HP-stained cortex of a 16 year old ovary without oocytes in the tunica albuginea. (G) Adjacent section to (D) stained for SSEA4. No staining is seen. (H) Section close to (C) stained for NANOG. No cells are stained. The oocytes can be noticed by the light scatter of their membranes. A peripheral cut of a small antral follicle can be distinguished in the lower part of the section. (I) Section close to (D) stained for OCT4. The oocytes can be distinguished by their light scatter. No cells are stained. (J) Section close to (E) stained for MAGE-A4 and a faint hematoxylin staining. No cells are stained for MAGE-A4. A close-up of two small follicles is seen in the lower right corner. The bar of all figures (except the inset) corresponds to 100 μM. Inset: The bar of the inset corresponds to 40 μM.
Kerr et al. also found expression of SSEA4 in somatic cells surrounding germ cell clusters and cords in the human ovarian cortex in the first half of pregnancy (Kerr et al., 2008). In the present study, we did not succeed in finding somatic cells stained for SSEA4 in prenatal ovaries. The lack of cells with OCT4-stained nuclei in the ovary after the early post-natal stage indicates that oogonia only survive a short time after birth. OCT4 seems to be required for survival of PGCs and loss of expression leads to germ cell apoptosis (Kehler et al., 2004). However, OCT4 is re-expressed at low levels in the oocyte from the time when the oocyte begins to grow (Parfenov et al., 2003).

In conclusion, we found no evidence for the presence of germ stem cells or oogonia in the post-natal human ovary after the final clearing of these cells during the first 1 or 2 years of life. However, the small follicles gathered in groups in the medulla at all post-natal ages indicate pre- or perinatal re-arrangement of the developing oocyte pool.

Authors’ roles

A.G.B. played a role in conception and design, financial support, administrative support, provision of study material or patients, collection and/or assembly of data, data analysis and interpretation, manuscript writing and final approval of manuscript. P.E.H. was involved in conception and design, data analysis and interpretation, manuscript writing and final approval of manuscript. Dr Høyer died 29 March 2011. C.Y.A. took part in conception and design, provision of study material or patients, data analysis and interpretation, manuscript writing and final approval of manuscript. S.G.K. played a role in provision of study material or patients, collection and/or assembly of data, data analysis and interpretation, and final approval of manuscript. Å.J. was responsible for conception and design, data analysis and interpretation, manuscript writing and final approval of manuscript. K.M. was involved in conception and design, provision of study material or patients, collection and/or assembly of data, data analysis and interpretation, manuscript writing and final approval of manuscript.

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