Differentiation and development of human female germ cells during prenatal gonadogenesis: an immunohistochemical study

H.Stoop[1]*, F.Honecker[1,2]*, M.Cools[1], R.de Krijger[1], C.Bokemeyer[2] and L.H.J.Looijenga[1,3]

[1]Department of Pathology, Josephine Nefkens Institute, Erasmus MC-University Medical Center Rotterdam, Daniel den Hoed Cancer Center, Rotterdam, The Netherlands and [2]Department of Hematology/Oncology, University of Tübingen, Tübingen, Germany
*Stoop and Honecker contributed equally to the work.

[3]To whom correspondence should be addressed: Department of Pathology, Erasmus MC-University Medical Center Rotterdam, Josephine Nefkens Institute, Room 430b, P.O.Box 1738, 3000 DR Rotterdam, The Netherlands. E-mail: l.looijenga@erasmusmc.nl

BACKGROUND: In the development of the human ovary, the second trimester includes the transition from oogonial replication to primordial follicle formation. The present study was carried out to assess differentiation and proliferation of germ cells in a series of female gonads from 19 fetuses from the second and third trimester, and two neonates. METHODS: Using immunohistochemistry, the following markers were studied: placental/germ-like cell alkaline phosphatases (PLAP), the marker of pluripotency OCT3/4, the proliferation marker Ki-67, β-catenin and E-cadherin, the stem cell factor receptor c-KIT, and VASA, a protein specific for the germ cell lineage. RESULTS: PLAP and OCT3/4 were seen during oogenesis, but not in germ cells engaged in folliculogenesis. A similar pattern was observed for Ki-67. Loss of pluripotency occurs once oocytes engage in follicle formation, suggesting a role of cell–cell interactions in the process of germ cell maturation. VASA, c-KIT, β-catenin and E-cadherin were found in germ cells at all developmental stages of oogenesis and folliculogenesis. CONCLUSIONS: Immunohistochemically, two groups of germ cells can be distinguished. Germ cells that are predominantly found in the cortical region of the ovary before weeks 22–24 of gestation, showing an immature phenotype, are mitotically active, and express OCT3/4, a marker of pluripotency. On the other hand, germ cells undergoing folliculogenesis have lost their pluripotent potential and no longer proliferate.

Key words: differentiation/fetal ovary/germ cells/immunohistochemistry/proliferation

Introduction

In humans and rodents, amongst other species, the embryonic precursors of the gametes are known as primordial germ cells (PGC) (see Donovan, 1998, for review). These cells are set aside to an extra-embryonic location early during embryonic development, and migrate to the area where the genital ridge will be formed around weeks 5 and 6 of human development (Witschi, 1948). Subsequently, the gonads and sexual differentiation occur in weeks 6 and 7 (Falin, 1969). Between weeks 7 and 9 during ovarian development (referred to as prefollicular stage) the germ cells (oogonia) and granulosa cells characteristically arrange in cords and sheets without specific organization (Gondos, 1985). The population of oogonia increases by undergoing multiple divisions, after which they become oocytes, stop proliferating, and enter the first steps of meiosis. The first distinctive change in prenatal germ cell development in the ovary is therefore the onset of meiosis, which starts at 11–12 weeks of gestation and extends into the second tri-

mester (Rabinovici and Jaffe, 1990). Contradictory data exist with regard to the onset of folliculogenesis, i.e. the occurrence of primordial follicles, which has been reported to take place between 16 and 21 weeks of gestation (Kurilo, 1981; Konishi et al., 1986). Due to the proliferation of premeiotic oogonia on the one hand and the apoptotic elimination of both oogonia and germ cells at different stages of folliculogenesis during intrauterine maturation on the other hand, considerable changes in the total number of germ cells are seen (De Pol et al., 1997). Whereas at 5 weeks of gestational age an estimated 700–1300 germ cells are present, germ cell number peaks between weeks 16 and 20, reaching an estimated 6×10^6 cells per ovary (Baker, 1963). At birth, the total number of germ cells has been estimated to be $\sim 1 \times 10^6$ cells, and the number of follicles has been reported to be in the range of 1.3–3.8×10^5, with 95% being represented by primordial follicles (Baker, 1963; Forabosco et al., 1991). Maturation is a gradual process, as is demonstrated by the initiation of

Table I. Antibodies (source) and detection method used for immunohistochemistry

Antibody	Company	Code	Pre-treatment	Dilutions	Secondary antibody (1:200) (biotinylated)	Visualization
PLAP	Cell Marque, Hot Springs, AR, USA	CMC203	HIAR[a]	1:200	Rabbit anti-mouse (Dako-Cytomation, E0413)	ABCplx-ap[b]
c-KIT	Dako-Cytomation, Glostrup, Denmark	A4502	HIAR	1:500	Swine anti-rabbit (Dako-Cytomation, E0413)	ABCplx-ap
OCT3/4	Santa Cruz Bio-technology, Santa Cruz, CA, USA	sc-8629	HIAR	1:1000	Horse anti-goat (Vector Laboratories, Burlingame, CA, USA, BA9500)	ABCplx-hrp[c]
VASA	Provided by D.Castrillon[d]		HIAR	1:2000	Swine anti-rabbit	ABCplx-ap
β-Catenin	Zymed Laboratories, South San Francisco, USA	CAT-5H10	HIAR	1:2000	Rabbit anti-mouse	ABCplx-hrp[c]
E-Cadherin	Zymed Laboratories, South San Francisco, CA, USA	HECD-1	HIAR	1:300	Rabbit anti-mouse	ABCplx-hrp[c]
Ki-67	Dako-Cytomation, Glostrup, Denmark	A047	HIAR	1:50	Swine anti-rabbit	ABCplx-hrp

[a]Heat-induced antigen retrieval (Shi et al., 1991).
[b]ABC complex, alkaline phosphatase, DakoCytomation, Code: K0391.
[c]ABC complex, horse radish peroxidase, DakoCytomation, Code: K0377.
[d]Castrillon et al. (2000).
PLAP = placental/germ-like cell alkaline phosphatases.

certain stages at certain times, i.e. the temporal regulation of oocyte development. In humans, this process is not synchronized, as germ cells of different developmental stages can be detected at the same time in the same prenatal ovary. This indicates that unknown intracellular mechanisms govern the individual pace of maturation (Rabinovici and Jaffe, 1990).

In order to further investigate the distribution and timing of proliferation and differentiation, we undertook an extensive study on the presence of a number of markers involved in either proliferation or maturation of normal female germ cells spanning the intrauterine period between week 15.5 gestational age and the neonatal period. In the human ovary, this crucial period includes the transition from oogonial replication by mitosis to primordial follicle formation. In this study we investigated the maturation process seen in normal development of the ovary and assess its potential as a reference for future identification of pathological processes of maturation.

Materials and methods

Tissue samples

Use of tissues for scientific reasons has been approved by an institutional review board (MEC 02.981). The samples are used according to the 'Code for Proper Secondary Use of Human Tissue in the Netherlands', as developed by the Dutch Federation of Medical Scientific Societies (FMWV) (version 2002). Human gonads of 19 females from the second and third trimester after spontaneous or induced abortions (gestational ages 15.5–40 weeks), or premature and term neonates that had died shortly after birth were obtained from post-mortem examinations in our department. Ovaries were dissected and fixed in 10% formalin and processed into paraffin. To assure satisfactory quality, poor preservation of tissue samples assessed by haematoxylin–eosin staining led to exclusion from this analysis. Cases showing conditions that can possibly affect gonadal development such as trisomy 18 and 21, hydrocephalus, maldeveloped kidneys, or gross intrauterine growth retardation, were excluded from the study. Gestational ages were calculated in relation to the mothers' last menstrual cycle and were in accordance with the foot length and the crown–heel length measurements at autopsy.

Histochemical and immunohistochemical stainings

Stainings were performed as described before (Stoop et al., 2001). For immunohistochemistry, sections were incubated with the primary antibody overnight at 4°C [placental/germ-like cell alkaline phosphatases (PLAP), c-KIT, VASA, β-catenin, E-cadherin] or 2 h at room temperature (OCT3/4, Ki-67). The primary and secondary antibodies used are indicated in Table I. All slides were counterstained with haematoxylin. For PLAP, β-catenin and E-cadherin, positive staining of the Fallopian tube, and for c-KIT, the presence of mast cells were used as an internal positive control. Negative controls were performed by omitting the primary antibody, resulting in complete absence of signal.

Double-stainings were performed by using a combination of the same detection methods but with different substrates: Fast Blue/-Naphtol ASMX phosphate (F3378 and N500; Sigma, Germany) for blue staining and 3-amino-9-ethyl-carbazole (A.5754 and D4254; Sigma)/H_2O_2 for red staining, without counterstaining. Endogenous

peroxidase activity and/or endogenous biotin was blocked using 3% H_2O_2 (for 5 min) and/or a blocking kit for endogenous biotin (Vector Laboratories, USA) to prevent background staining.

To semiquantitatively assess expression of the markers investigated, cells showing a positive signal were counted in representative visual fields (magnification $\times 200$) containing surface areas of both the medullary and the cortical region of the ovary in comparable proportions in all cases. In addition, cells positive for PLAP, OCT3/4 and Ki-67 were counted in three representative high power fields (magnification $\times 400$) of both the medullary and cortical regions of seven cases (gestational ages 15.5, 20, 23, 28, 33, 35 and 36 weeks) to assess regional differences. For quantitative analysis of OCT3/4, the percentage of positive cells among the total number of germ cells (as identified by histology) was assessed in three cases

(gestational ages 15.5, 28 and 36 weeks) counting three representative visual fields (magnification $\times 200$). The counting was performed independently by three different observers (H.S., F.H. and M.C.) who were unaware of the gestational age at the time-point of the investigation.

Results

The presence of a number of markers during normal fetal and neonatal ovarian development (week 15.5 gestational age until 6 weeks postnatally) was studied in 19 ovaries. Figure 1 summarizes the results of a semiquantitative assessment of the different markers. Each black dot represents one case.
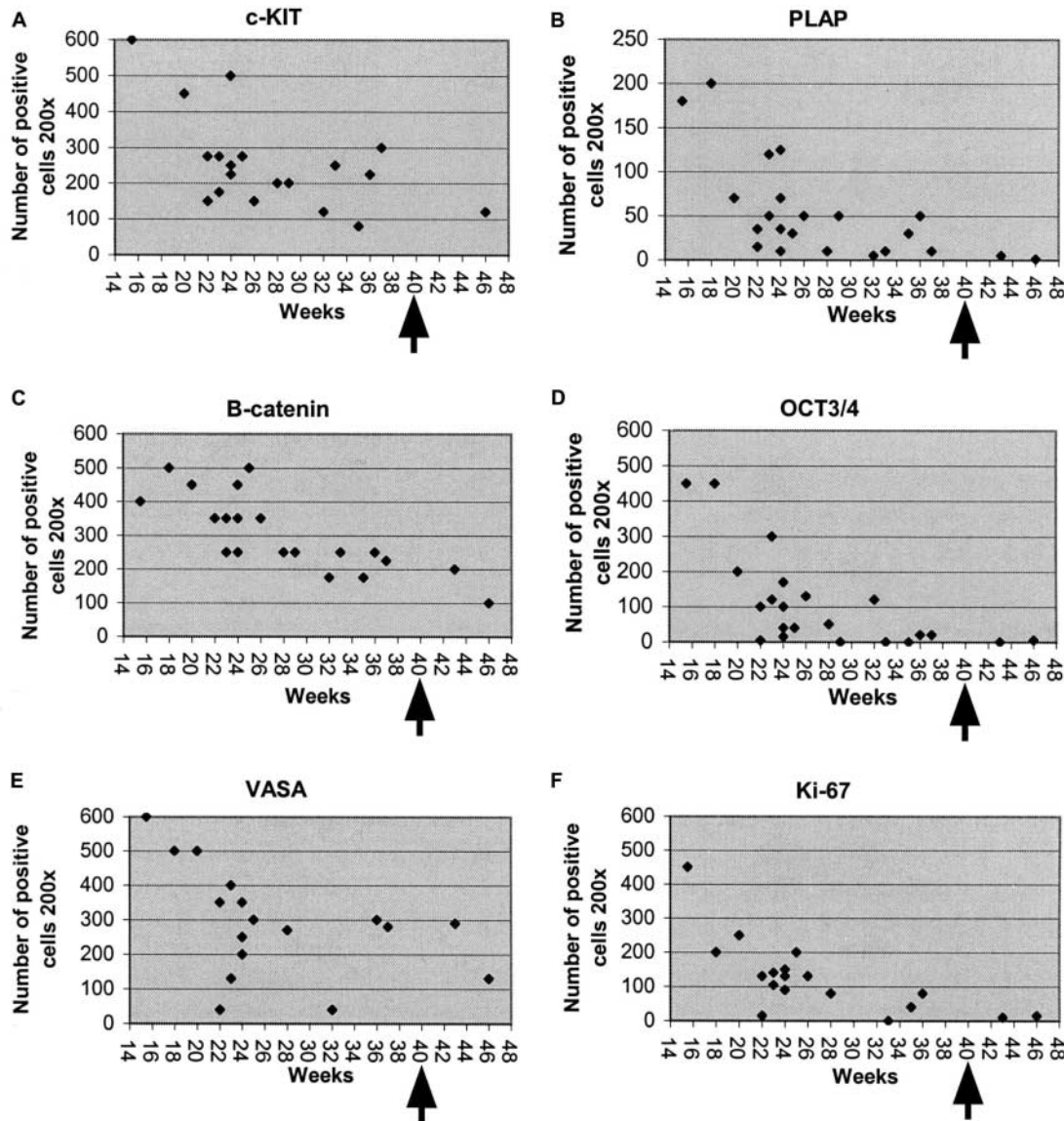

Figure 1. Absolute numbers of cells per representative visual field (magnification factor $\times 200$) showing a positive staining with immunohistochemistry for the markers at different gestational ages (weeks). The earliest case studied in this series was 15.5 weeks, the oldest was the ovary of a neonate of 6 weeks. The arrow indicates partus (median 40 ± 2 weeks). The left panel (**A**, **C**, **E**) shows markers that remain positive throughout pre- and perinatal oogenesis and formation of primordial follicles. The right panel (**B**, **D**, **F**) illustrates factors associated with germ cell differentiation and proliferation. Each black dot represents one case. Note smaller scale of the *y*-axis (lower overall numbers) in **B** (placental/germ cell alkaline phosphatases, PLAP). As β-catenin and E-cadherin were expressed in the same cells in adjacent slides with similar localization, cell numbers for β-catenin (**C**) are also representative for E-cadherin.

Table II. Differences in the expression of placental/germ-like cell alkaline phosphatases (PLAP), OCT3/4 and Ki-67 between the cortical and the medullary region of fetal ovaries aged 15.5–36 weeks

	Gestational age (weeks)						
	15.5	20	23	28	33	35	36
PLAP							
Cortex	94 (72–115)	35 (23–44)	73 (54–85)	9 (3–12)	11 (9–13)	0	14 (12–17)
Medulla	34 (12–52)	4 (2–6)	4 (1–6)	5 (1–9)	0	2 (1–3)	3 (2–4)
OCT3/4							
Cortex	131 (106–157)	57 (56–57)	122 (99–162)	32 (29–33)	2 (0–3)	0	9 (1–20)
Medulla	49 (36–58)	12 (1–21)	26 (14–43)	8 (2–14)	0	2 (0–5)	4 (1–6)
Ki-67							
Cortex	234 (221–252)	136 (112–148	110 (95–118)	77 (62–98)	4 (2–7)	15 (9–18)	60 (46–75)
Medulla	163 (152–172)	86 (70–110)	61 (55–65)	53 (36–75)	0	26 (16–32)	45 (24–69)

The average and the range of cell numbers found positive for the indicated markers in three independent high power magnification fields ($\times 400$) is given. Note that expression of the early markers PLAP and OCT3/4 and the proliferation marker Ki-67 is predominantly found in the cortical region. Overall, the number of positive cells decreases between 15.5 and 28 weeks, but shows some inter-individual variation during the third trimester.

Table II shows the results of a differential analysis, comparing expression of PLAP, OCT3/4 and Ki-67 in the medullary and cortical regions of the ovaries of seven cases, aged 15.5, 20, 23, 28, 33, 35 and 36 weeks. Table III gives both the absolute numbers and the percentage of cells positive for OCT3/4 in three of these cases, aged 15.5, 28 and 36 weeks. Illustrations of representative stainings are shown in Figures 2 and 3. Results of single- and double-stainings are described below. The data in Figure 4 (grey bars) were compared to previously published findings (black bars). Figure 5 shows a model of the progression of markers from oogonia to primordial follicles.

Immunohistochemical detection of Ki-67, PLAP and OCT3/4

Ki-67, PLAP and OCT3/4 were all found much more frequently at earlier fetal ages, and expression decreased with advancing gestational age (Figure 1). The various regions of the ovary showed different stages of germ cell maturation: cells positive for Ki-67, PLAP and OCT3/4 were mainly observed in the cortical region of the developing ovaries, i.e. the region where most immature germ cells are located during the process of oogenesis (Tables II and III). In the medulla, where the majority of maturing germ cells are found during ovarian development, positive cells were much less frequent.

The proliferation index was assessed using Ki-67, which showed a nuclear staining in both early germ cells and interstitial cells throughout the whole period of ovarian development investigated in this series. A marked decrease in the

number of positive cells was observed after weeks 22–24 of gestational age, mainly due to the cessation of mitosis of germ cells. This indicates that proliferating germ cells contribute significantly to the overall number of Ki-67 positive cells in fetal ovaries. In germ cells, Ki-67 was observed mainly in oogonia (see Figure 2A). Yet, whereas the majority of oocytes engaged in folliculogenesis did not show Ki-67 expression, it was occasionally observed in oocytes of primordial follicles, where it was strictly confined to the nucleolus. Perinatally, only a few cells, mainly interstitial cells or granulosa cells, were positive.

The highest number of germ cells positive for PLAP was seen in the earliest stages of fetal development examined, and decreased sharply after 25 weeks gestational age (Figure 1). PLAP was detected in early germ cells predominantly located in the cortical region (Figure 2B and Table II). After birth, PLAP positive germ cells were hardly ever seen, with a maximum of one to three positive cells per visual field. PLAP expression was not restricted to germ cells, but was also seen in the Fallopian tube and occasionally in the epithelial lining of the ovary.

Nuclear staining for OCT3/4 was restricted to germ cells and the overall staining pattern was comparable to PLAP, yet total numbers of germ cells positive for OCT3/4 were somewhat higher (Figure 1 and Table II). In addition to oogonia, OCT3/4 was occasionally seen in early oocytes, but was never detectable in cells involved in folliculogenesis (Figure 2C). Similar to the expression pattern of Ki-67 and PLAP, a decline in the expression of OCT3/4 in fetal ovaries was seen at ~ 24 weeks of gestation. At term and in ovaries of neo-

Table III. Absolute numbers compared to total present (and percentage) of cells positive for OCT3/4 in three ovaries of 15.5, 28 and 36 weeks gestational age

OCT3/4	Gestational age (weeks)		
	15.5	28	36
Cortex	231/681 (34%; range 32–38)	5/324 (1.5%; range 0–4)	13/242 (5.4%; range 0–7)
Medulla	35/271 (13%; range 6–18)	2/201 (1%; range 0–2)	0/188 (0)

The total number of germ cells, identified by histology, and the number of germ cells expressing OCT3/4 immunohistochemically was assessed in three independent low power fields (magnification $\times 200$) in both the cortical and the medullary region of three fetal ovaries. In parentheses, the percentage of germ cells expressing OCT3/4 is given, together with the range (as percentage) of three different counts of three different fields per case. Whereas a significant number of germ cells express OCT3/4 at 15.5 weeks, predominantly in the cortex, the percentage of positive cells decreases markedly during the third trimester. Note the decrease in overall numbers of germ cells.
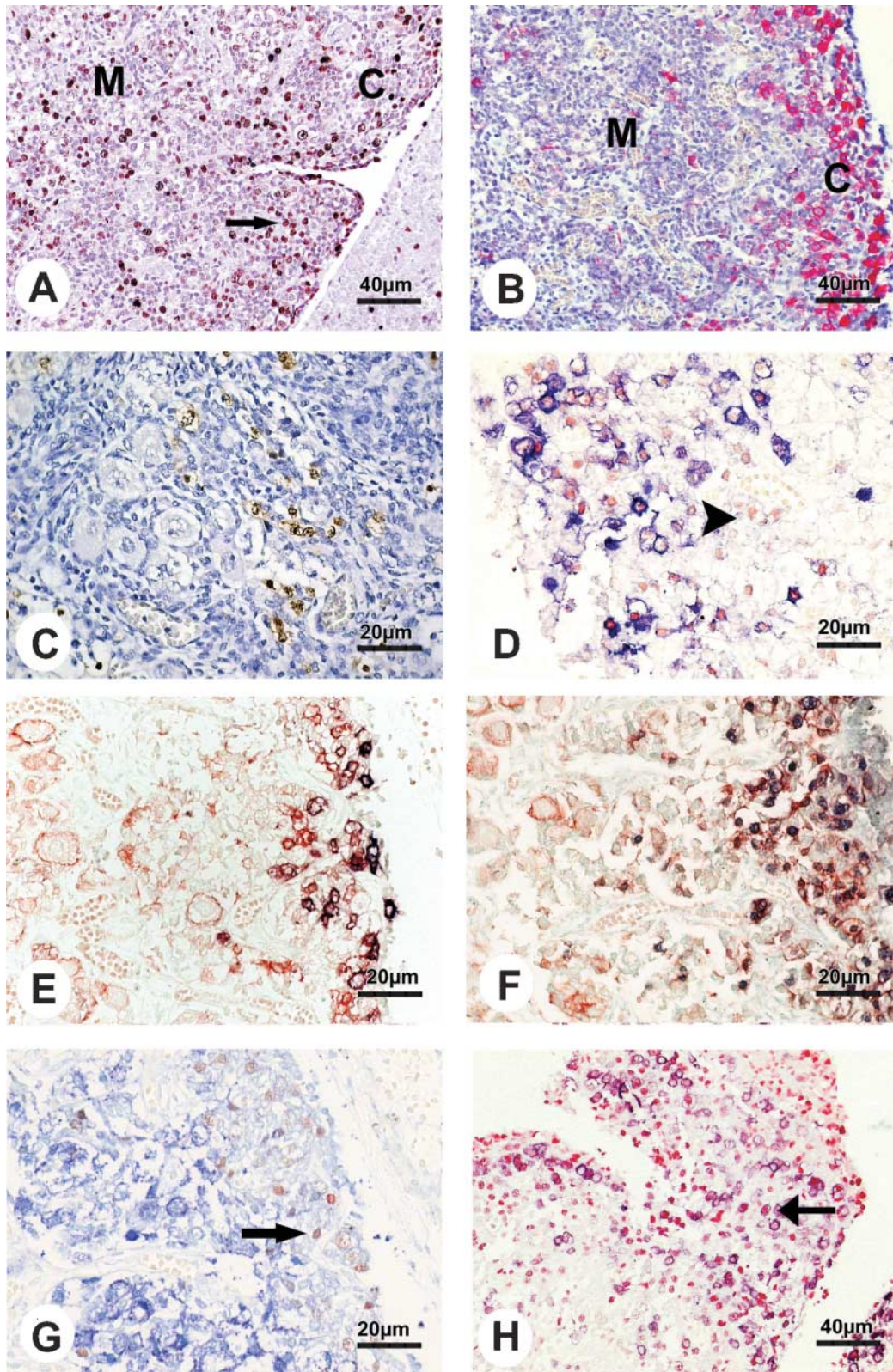
Figure 2. Results of immunohistochemical analysis of different markers in fetal germ cells. The cortex (C) and medulla (M) are indicated. (**A**) Fetal ovary (15.5 weeks of development) (magnification ×200); Ki-67 (brown nuclear signal) seen both in germ cells and cells of non-germ cell origin (e.g. stromal cells, endothelial cells, granulosa cells). Positive cells are mainly seen in a cortical localization (indicated by an arrow). (**B**) Fetal ovary (same case) (magnification ×200); placental/germ cell alkaline phosphatase (PLAP); red cytoplasmic signal. Note high number of positive cells (oogonia) at a cortical localization. (**C**) Fetal ovary (22 weeks of development) (magnification ×400); OCT3/4 (brown nuclear signal). Oogonia and immature oocytes, mainly cortically localized, are positive, whereas primordial follicles are negative. (**D**) Fetal ovary (same case) (magnification ×400); double-staining for OCT3/4 (red nuclear signal) and PLAP (blue cytoplasmic signal). OCT3/4 and PLAP are co-expressed in the majority of immature germ cells, and occasionally cells are positive for OCT3/4 but negative for

nates, hardly any positive germ cells were detectable.

Immunohistochemical detection of c-KIT, β-catenin/E-cadherin and VASA

A comparable staining pattern was seen for c-KIT, β-catenin and E-cadherin (Figure 3A–F). Both in early, immature germ cells (oogonia) and at all later stages of folliculogenesis, these factors were predominantly localized at the membrane, but sometimes also showed a cytoplasmic localization (see also Figure 1). In primordial follicles, all three factors showed strong signal intensity at sites of cell–cell interaction (Figure 3A–F). As β-catenin and E-cadherin were expressed in the same cells in adjacent slides in similar localization, these factors could be linked, forming an E-cadherin–catenin complex in germ cells. In addition to their presence in germ cells, these factors were also seen in epithelial cells of the Fallopian tube, serving as an internal positive control.

VASA was observed in the cytoplasm of germ cells, both oogonia and oocytes, at all gestational ages and after birth (Figure 1G and H). In addition to oogonia and early oocytes, all stages of follicle maturation showed presence of VASA. Signal intensity varied with maturation of germ cells: early germ cells showed only weak staining for VASA, and signal intensity increased with maturation and was strongest in primordial follicles (Figure 3G). The total number of germ cells, determined by VASA, decreased with gestational age: whereas at 15.5 weeks ∼600 germ cells, mostly oogonia and early oocytes, were seen per visual field, the average number of germ cells at term was ∼100–200 per visual field.

Results of double-stainings

Double-stainings were performed to assess correlations or differences of markers at different stages of maturation. A total of nine cases of 15.5, 18, 24 (three different cases), 28, 33, 36 weeks gestational age, and a neonate of 2 weeks, born at term, were stained for six different combinations: OCT3/4 and PLAP, PLAP and c-KIT, OCT3/4 and c-KIT, OCT3/4 and VASA, OCT3/4 and β-catenin, and PLAP and Ki-67 (Figure 2D–H respectively). The results of all double-staining experiments were in accordance with the results of the respective single-staining experiments. Staining for OCT3/4 and PLAP revealed that a higher number of germ cells were positive for OCT3/4 than for PLAP, with a constant ratio of 1.4:1 observed for all ages investigated (Figure 2D). PLAP was never detected in germ cells negative for OCT3/4, and expression of OCT3/4 was still observed at later stages of

development, when PLAP was already undetectable. Double-staining for PLAP and c-KIT showed that at all gestational ages more germ cells were positive for c-KIT than for PLAP. PLAP was never detected in oogonia without expression of c-KIT, but in contrast to PLAP, c-KIT remained positive throughout maturation, including different stages of folliculogenesis (Figure 2E). In accordance with these findings, OCT3/4 and c-KIT were found in comparable numbers only in early germ cells, whereas during folliculogenesis, c-KIT remained positive in germ cells that had already lost OCT3/4 expression (Figure 2F). Similar staining patterns were observed for the combinations OCT3/4 and VASA, and OCT3/4 and β-catenin (not shown), where co-expression was restricted to early germ cells, whereas expression of OCT3/4 was no longer seen at later stages of maturation. Interestingly, an inverse correlation was found between the presence of OCT3/4 and the staining intensity of VASA. Oogonia were positive for OCT3/4, but only weakly positive for VASA, whereas primordial follicles staining strongly for VASA no longer showed OCT3/4 (Figure 2G). Double-staining for PLAP and Ki-67 revealed that significantly more cells were positive for Ki-67 than for PLAP at all ages. Furthermore, not all early germ cells (identified by PLAP expression) were proliferating at the same time (Figure 2H). A proportion of germ cells of between 50% at earlier and 37% at later gestational ages showed co-expression of both markers. This indicates that in addition to a decrease in the absolute number of early germ cells, these cells are also less likely to proliferate at later stages of ovarian development. During the third trimester, expansion of the pool of early germ cells (indicated by co-expression of Ki-67 and PLAP) has basically ceased.

Discussion

The aim of this study was to examine the differential expression of a number of factors involved in proliferation and differentiation of human fetal germ cells. The findings give an indication of the temporal scale as well as the regional differences during the maturation of normal female germ cells in the human ovary. The overall picture of the presence of the different factors in fetal and neonatal ovaries is shown graphically in Figure 4 (grey bars) and compared to findings in humans published so far in the literature (black bars). Figure 5 gives a scheme of marker progression during maturation from oogonia to primordial follicles.

PLAP (indicated by an arrowhead). (**E**) Fetal ovary (same case) (magnification ×400); double-staining for PLAP (blue cytoplasmic signal) and c-KIT (red membranous signal). Primordial follicles express only c-KIT, and only a minority of cortically located immature germ cells is positive for both markers. (**F**) Fetal ovary (same case) (magnification ×400); double-staining for OCT3/4 (blue nuclear signal) and c-KIT (red membranous signal). Similar staining pattern as seen in E, but note that more germ cells are positive for OCT3/4 than for PLAP. (**G**) Fetal ovary (same case) (magnification ×400); double-staining for OCT3/4 (red nuclear signal) and VASA (blue cytoplasmic signal). Primordial follicles, mostly located in the medullary region, show a strong intensity of VASA and are negative for OCT3/4, whereas immature germ cells, mostly found in a cortical localization, are positive for OCT3/4 but show only weak VASA staining. (**H**) Fetal ovary (15.5 weeks of development) (magnification ×200); double-staining for Ki-67 (red nuclear signal) and PLAP (blue cytoplasmic signal). Significantly more cells are positive for Ki-67 than for PLAP. The majority of proliferating cells are of non-germ cell origin, as demonstrated by positive staining for Ki-67 but absence of PLAP; however, a few oogonia (identified by PLAP expression) show co-expression of Ki-67, indicating that these immature germ cells are mitotically active (indicated by an arrow).
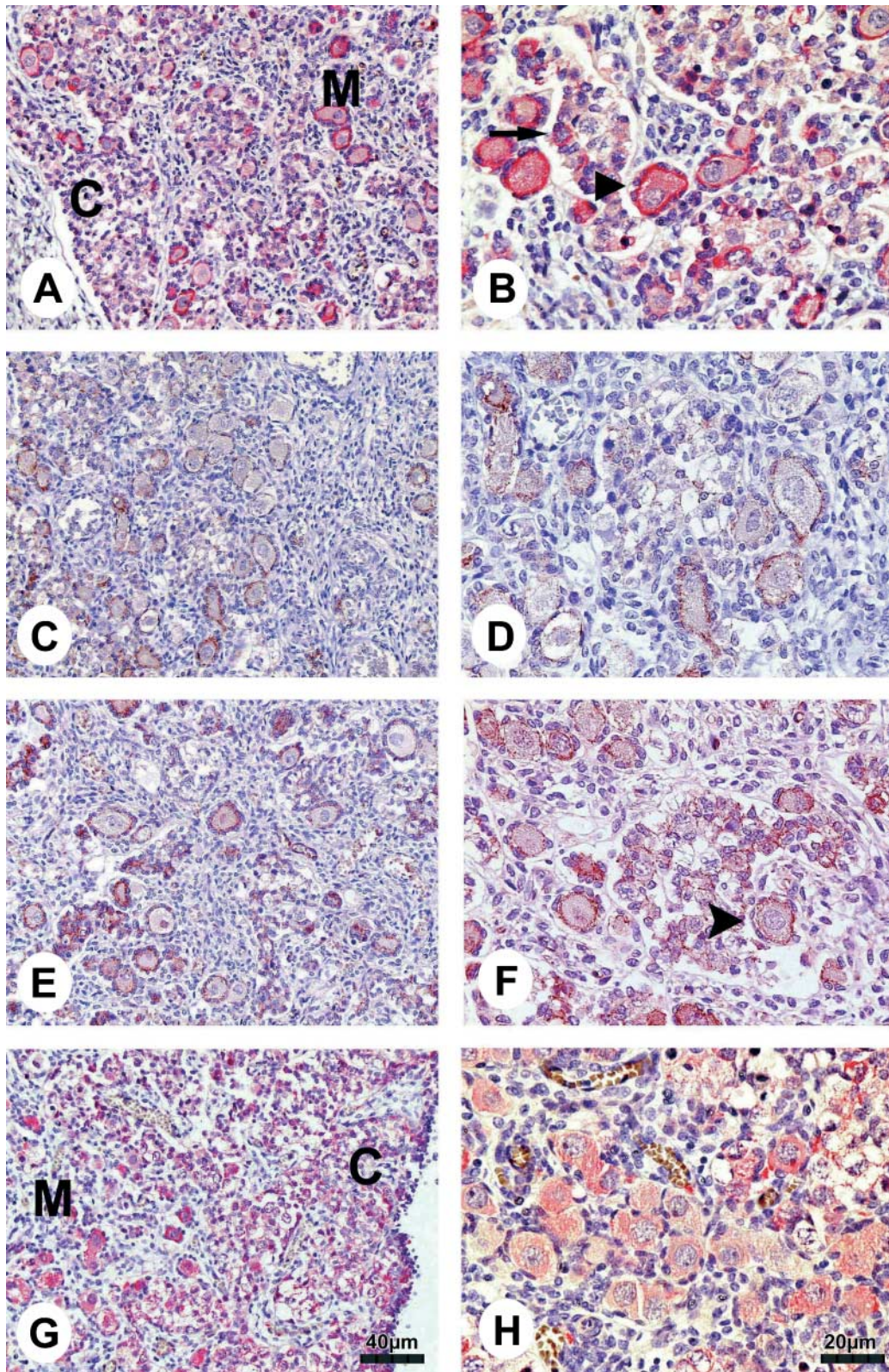
Figure 3. Results of immunohistochemical analysis of different markers in fetal germ cells. The cortex (C) and medulla (M) are indicated. (**A**) Fetal ovary (28 weeks of development); c-KIT (red cytoplasmic and membranous signal), positive in both immature oocytes and oocytes involved in folliculogenesis. (**B**) Fetal ovary (same case); c-KIT. Note staining of both immature oocytes (arrow) and primordial follicles (arrowhead). (**C**) Fetal ovary (same case); E-cadherin (brown-reddish membranous signal). (**D**) Fetal ovary (same case); E-cadherin. (**E**) Fetal ovary (same case); β-catenin (brown-reddish signal) is seen in oocytes at different stages of folliculogenesis. (**F**) Fetal ovary (same case); β-catenin. Note strong submembranous signal intensity in the primordial follicles (arrow), suggesting formation of an E-cadherin–catenin complex. (**G**) Fetal ovary (22 weeks of development); VASA (red cytoplasmic signal), seen both in germ cells of all stages of development. (**H**) Fetal ovary (same case); VASA. Magnifications: left panels ×200, right panels ×400.
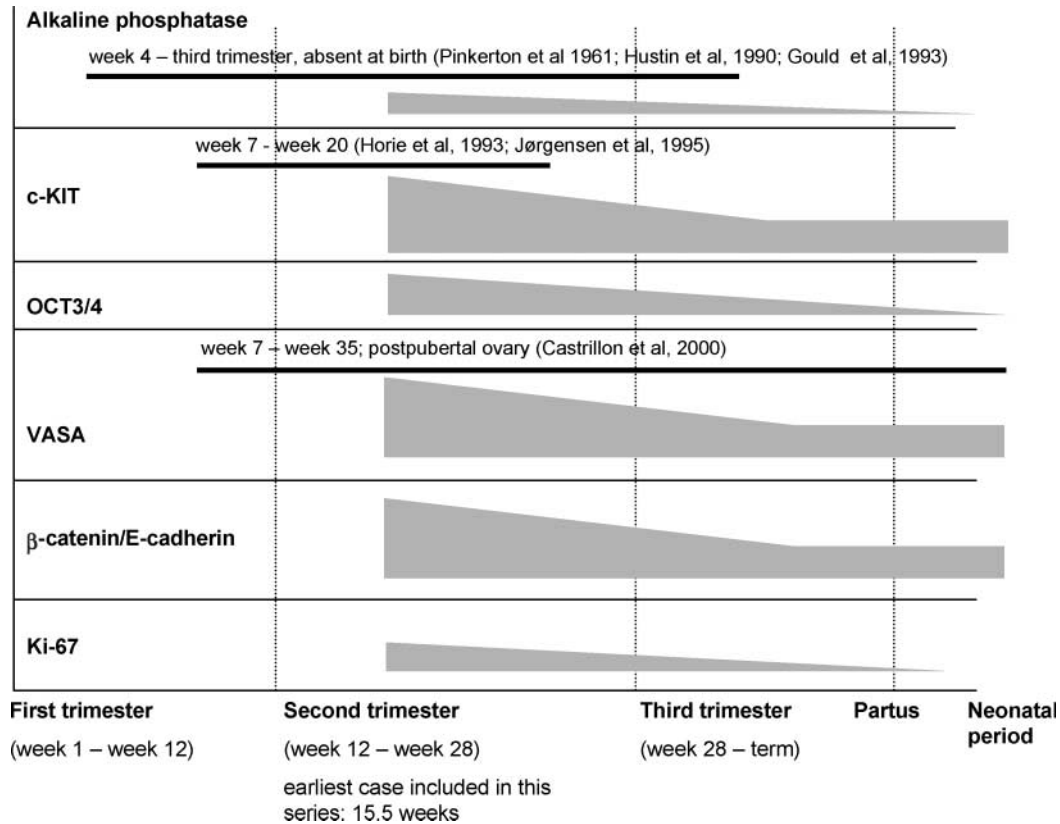
Figure 4. Overview of the data currently available from the literature on the expression of different antigens in germ cells during female intrauterine development (black bars), compared with findings of this study (grey bars). Heights of grey bars represent numbers of germ cells expressing the individual factor at different developmental ages.
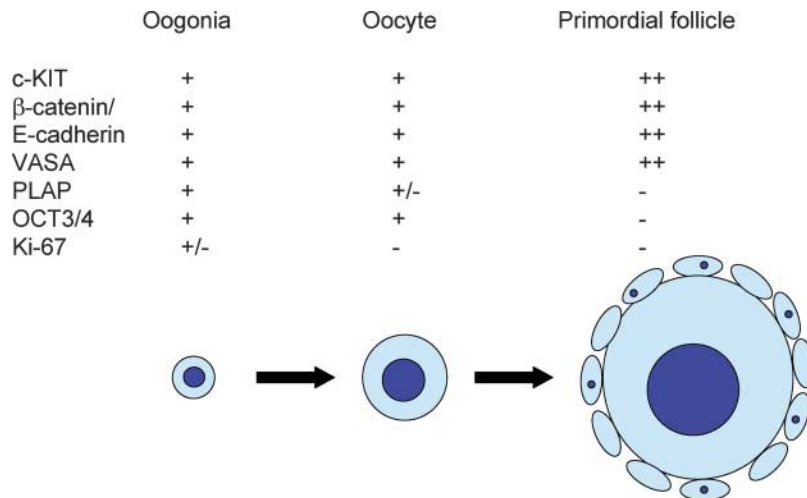

Figure 5. Progression of markers during germ cell maturation from oogonia to primordial follicles during the pre- and perinatal periods.

In the following paragraphs, the most interesting findings of the individual markers included in this study will be discussed in more detail.

To assess proliferation, we used Ki-67, a nuclear protein that is present in all phases of the cell cycle, but is absent in G_0-phase cells (Gerdes *et al.*, 1984). Overall expression of Ki-67 in both germ cells and cells of non-germ cell origin (mostly interstitial cells and granulosa cells) was highest in the earliest case investigated (15.5 weeks), and decreased steadily throughout the second and third trimester. Around term, almost all germ cells (mostly oocytes in primordial follicles) and the majority of cells of non-germ cell origin have entered a quiescent phase. Expression of Ki-67 was markedly higher in the cortex than in the medulla at earlier ages, whereas no differences between the two regions were found during the third trimester (Table II). To assess the proportion of germ cells of overall Ki-67 positive cells and examine the percentage of proliferating germ cells, we performed double-

stainings using a combination of PLAP and Ki-67. Of all cells positive for Ki-67, ~50% were early germ cells. The percentage of proliferating versus quiescent germ cells was 45 to 50% at 15.5 and 20 weeks, and between 37 and 40% of the few early germ cells found at week 28 gestation and 3 weeks after birth respectively. This indicates that immature germ cells at early stages show strong proliferation around weeks 15.5 to 20, whereas at later ages both the number and the fraction of proliferating germ cells decreases. At term, the expansion of germ cells has almost ceased. This finding is in line with earlier reports on germ cell numbers during the prenatal period (for review, see Rabinovici and Jaffe, 1990). Interestingly, a small number of oocytes in primordial follicles showed presence of Ki-67 in the nucleolus. Similar findings have been reported previously, suggesting a role of this factor not only during mitosis, but also during meiosis (Wrobel *et al.*, 1996; Traut *et al.*, 2002). The number of positive meiotic cells was too small to influence the overall conclusion, and did not allow conclusions regarding possible physiological meaning.

Alkaline phosphatases are regarded as archetypal oncofetal proteins. They are detectable at the mammalian blastocyst stage and have been described in germ cells of human embryos of <4 weeks gestational age (Pinkerton *et al.*, 1961). PLAP expression has been described in primordial germ cells at 8–13 weeks and at later ages in human oogonia not yet engaged in cell–cell interactions with somatic cells, whereas ovaries of neonates and infants were devoid of any immunopositivity (Hustin *et al.*, 1990; Gould *et al.*, 2000).

Table IV. Comparison of gene expression of female human and mouse germ cells during maturation

Marker	Early postmigratory germ cells (pre-meiotic)		Maturing germ cells		Late germ cells (post-meiotic prophase I)	
	Human	Mouse	Human	Mouse	Human	Mouse
OCT3/4	++	++*,[a]	+/−†	−[a,b]	?†	+[b]
c-KIT	+	+*,[a]	++	−*,[a]	++	+[c]
VASA	+	+*,[a]	++	++*,[a]	++[d]	++[e]

*Results are based on RT–PCR (expression of mRNA); all other results are based on immunohistochemistry.
†Note that the exact time-point of down-regulation of OCT3/4 with regards to onset of meiosis has not yet been established in human oocytes. We could not detect re-expression of OCT3/4 in primary and secondary follicles of 34 adult women (data not shown). However, the observation that OCT3/4 mRNA is not present in earlier stages of folliculogenesis but can be detected both in bovine and human mature oocytes and preimplantation embryos (Daniels *et al.*, 2000; Huntriss *et al.*, 2002) warrants investigation at what stage this protein is re-expressed in mature/ovulating oocytes or during fertilization.
[a]References: Hubner *et al.* (2003);
[b]Pesce *et al.* (1998);
[c]Horie *et al.* (1991);
[d]Castrillon *et al.* (2000);
[e]Toyooka *et al.* (2000).
The expression of a number of markers in human female germ cells (early postmigratory cells and cells at different stages of maturation), investigated in this analysis, is compared to data from the literature on the expression of these markers in mouse germ cells at comparable stages of maturation. The most striking difference between human and mouse germ cells is seen in expression of c-KIT, which is down-regulated in the mouse during oocyte maturation, but remains expressed in human oocytes throughout maturation.

Our finding that PLAP expression is restricted to early germ cells, most likely oogonia, is in line with these previous reports. The highest expression was seen in the cortex, where the number of positive cells decreased steadily with advancing age. In the medulla, differences at different ages were less pronounced after week 20. In our study, PLAP was still occasionally detected in germ cells of neonates shortly after birth, albeit at very low numbers.

OCT3/4 is a transcriptional regulator, expressed exclusively in pluripotent human embryonic stem cells and germ cells, including PGC (Goto *et al.*, 1999; Hansis *et al.*, 2000). In addition, presence of OCT3/4 has been described in germ cell tumour cells with pluripotent potential such as embryonal carcinoma and seminomas (Looijenga *et al.*, 2003). In fetal human testes, OCT3/4 has been found to be highly expressed in PGC between weeks 17 and 24 and to a lesser extent at later stages (Looijenga *et al.*, 2003). Expression of OCT3/4 in normal and dysgenetic human ovaries has been reported recently (Rajpert-De Meyts *et al.*, 2004). In our analysis, OCT3/4 was occasionally seen in early oocytes, but never in germ cells involved in folliculogenesis, indicating that human female germ cells irreversibly lose pluripotency once they progress to meiosis and engage in a close cell–cell interaction with granulosa cells. It is therefore tempting to speculate that both the processes of cell–cell interaction and down-regulation of OCT3/4 are important for germ cell maturation. It should be noted that this is different from the situation found in mouse follicles, where germ cells after completion of meiotic prophase I express OCT3/4 (see Table IV) (Pesce *et al.*, 1998). However, the exact mechanisms of interaction are not yet understood, and deserve further investigation. In this context it is interesting that ovarian teratomas originate from germ cells at different stages of meiosis (Surti *et al.*, 1990). The finding that these tumours show a restricted potential to differentiate and exclusively demonstrate somatic differentiation is in line with previous reports that the presence of OCT3/4 in embryonic stem cells is crucial to keep these cells in an undifferentiated state (Niwa *et al.*, 2000). We therefore speculate that ovarian teratomas arise from germ cells that have already lost expression of OCT3/4. Our findings demonstrate the value of both PLAP and OCT3/4 for the characterization of immature germ cells in the female gonad and indicate that these markers can be useful for the identification of developmentally arrested germ cells in pathological conditions, e.g. in dysgenetic gonads, as has been described recently (Rajpert-De Meyts *et al.*, 2004).

VASA is a member of the DEAD box family of RNA helicases and is specifically expressed in the germ cell lineage (Castrillon *et al.*, 2000). Furthermore, VASA expression has been detected by immunohistochemistry in migratory PGC at gestational week 7 and in germ cells both before and after birth (Castrillon *et al.*, 2000). It is most abundantly expressed in postmeiotic germ cells, i.e. in males in spermatocytes and in females in mature oocytes. VASA has also been described in testicular germ cell tumours retaining germ cell characteristics (Zeeman *et al.*, 2002). Interestingly, in murine embryonic stem cells, expression of Mvh, the mouse VASA homologue, has been described as an early event in the com-

mitment of stem cells to the germ cell lineage (Toyooka *et al.*, 2000, 2003). In our study, expression of VASA has been found both in germ cells at all gestational ages. In accordance with previous reports (Castrillon *et al.*, 2000), staining intensity was stronger in maturing oocytes than oogonia. Our results illustrate the usefulness of this protein as a marker of germ cells at all stages of development and allow an estimation of germ cell numbers at different ages.

c-KIT, β-catenin, and E-cadherin are all involved in cell signalling and cell–cell interaction. c-KIT is a type III receptor tyrosine kinase found on early germ cells, whereas the ligand stem cell factor (SCF or KIT-L) is present in granulosa cells (Matzuk *et al.*, 2002, for review). The c-KIT/SCF system has been found to be involved in survival and proliferation of migrating germ cells in mice (McLaren, 1992). In human intrauterine gonadogenesis, c-KIT expression has been described in female PGC at the period of arrival of PGC at the gonadal ridges at week 7 and later between 13 and 21 weeks of gestational age (Horie *et al.*, 1993; Jørgensen *et al.*, 1995; Robinson *et al.*, 2001). During folliculogenesis, c-KIT has been postulated to play a role in germ cell survival, possibly by up-regulating Mcl-1, an anti-apoptotic member of the Bcl-2 family (Hartley *et al.*, 2002).

Whereas some data are available on the cadherin–catenin complex in mouse PGC development and oocyte maturation (Ohsugi *et al.*, 1999; Di Carlo and De Felici, 2000), little is known about the role of these factors in human germ cell development. In an earlier analysis, the presence of E-cadherin on human oocytes has been demonstrated (Campbell *et al.*, 1995). Here we show that c-KIT, β-catenin and E-cadherin are all present in germ cells throughout all stages of female intrauterine development. The presence of these factors not only in maturing oocytes and germ cells during folliculogenesis, but also in oogonia could be demonstrated by double-stainings combining markers for early germ cells such as OCT3/4 and PLAP with c-KIT and β-catenin. As oogonia are often found in clusters, these factors seem to be involved in signalling between neighbouring immature germ cells. In primordial follicles, expression of these factors was predominantly seen in oocytes at the site of cell–cell interaction with granulosa cells. This suggests that cell–cell contacts play an important role, both for immature germ cells and later between oocytes and surrounding granulosa cells. In fact, E-cadherin has been reported to be required for germ cell determination in PGC (Okamura *et al.*, 2003), and in oocyte growth and acquisition of meiotic competence in mice (Carabatsos *et al.*, 2000). The functional importance of E-cadherin has further been demonstrated by dissociation/reassociation experiments in a mouse model: dissociated cells from gonads were unable to reform their initial contacts when cultured in the presence of an antibody to E-cadherin (Mackay *et al.*, 1999).

The total numbers of germ cells seen in one visual field, as assessed by either c-KIT, β-catenin or VASA at different gestational ages (see Figure 1), are in accordance with previous reports, where a steady decrease of germ cells from around weeks 18–20 gestational age resulted in numbers that were only one-sixth of peak values at term (Baker, 1963). We therefore conclude that assessment of cell numbers in one representative visual field at magnification ×200 and including comparable surface areas of cortical and medullary areas is a reliable method for semiquantitative analyses. Our results allow some interesting comparisons with data on mouse germ cell maturation (see Table IV).

Taken together, our data provide a number of interesting findings regarding the development and differentiation of human germ cells in the fetal ovary. Immature germ cells are characterized by expression of PLAP and show pluripotent potential, as is illustrated by the expression of OCT3/4. During the first part of the second trimester included in our study (gestational ages of weeks 15.5–20), the number of immature germ cells is high, and the pool of these cells decreases significantly after weeks 22–24. In accordance with previous reports, regional differences in the number of immature germ cells were found when comparing the cortex and medulla of the ovary. This finding supports the model of compartmentalization, in which the cortex is the area where expansion of immature germ cells take place, the medulla providing the environment needed for germ cell maturation. Accompanying the gradual shift from oogonia and early oocytes to germ cells engaged in formation of primordial follicles, loss of expression of first PLAP and consecutively OCT3/4 can be observed. Interestingly, in male fetal germ cells loss of pluripotency as judged by loss of OCT3/4 is observed at the time when gonocytes become attached to the basal membrane of the seminiferous tubules and come into close contact with nurturing Sertoli cells (Honecker *et al.*, 2004). As down-regulation of OCT3/4 in female germ cells occurs at a time when oocytes become surrounded by granulosa cells and start to form primordial follicles, a major role of cell–cell interactions in the maturation of germ cells in both sexes can be postulated. Expression of c-KIT, β-catenin, and E-cadherin in germ cells at all stages of germ cell development points towards a role of these factors for germ cell survival and maturation. Our findings help to define normal germ cell development and maturation in the human fetal ovary and will serve as a reference for further analyses investigating both normal and pathological processes in germ cell maturation, i.e. in dysgenetic gonads or in individuals showing chromosomal aberrations (Kersemaekers *et al.*, 2005, M.Cools *et al.*, unpublished data).

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