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Zona pellucida components are present in human fetal ovary before follicle formation

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Abstract

The *zona pellucida* is a glycoprotein matrix surrounding oocytes and early-stage embryos in mammals. To elucidate the roles of the zona pellucida glycoproteins ZP1 and ZP3 and their key regulatory factor FIGLA in human ovarian development and folliculogenesis, their expression and localization was studied in human fetal and adult ovaries. FIGLA mRNA and ZP3 mRNA/protein were localized mainly in the oocytes, and during fetal development their maximal expression was observed around the 20th week, the time of follicle formation. The expression of ZP1 mRNA was low both in fetal and adult ovaries. Present findings demonstrate that ZP3 and FIGLA transcripts are expressed in the oocytes from early ovarian development. The function of ZP proteins during early fetal life is not clear, but the simultaneous expression of FIGLA and ZP3 suggests, that they may have a role in the development of primordial follicle before *zona pellucida* formation.

Keywords: Fetal ovary; FIGLA; Folliculogenesis; ZP1; ZP3

1. Introduction

The *zona pellucida* (ZP) is an extracellular matrix that surrounds the mammalian oocyte and early-stage embryo. The human ZP consists of at least four glycoproteins, zona pellucida glycoprotein 1–4 (ZP1–4) (Sacco et al., 1981; Shabanowitz and O'Rand, 1988; Hughes and Barratt, 1999; Lefievre et al., 2004). Current understanding of the structure and function of the *zona pellucida* is mainly based on studies in mice, in which the ZP is composed of three glycoproteins, Zp1–3 (Bleil and Wassarman, 1980b). Mouse Zp3 functions as a primary sperm receptor and plays an important role in binding of spermatozoa to the *zona pellucida* and in initiation of the acrosome reaction (Bleil and Wassarman, 1980a; Wassarman, 1988, 2005), while Zp1 functions as a key structural component maintaining the three-dimensional structure of the zona matrix (Greve and Wassarman, 1985; Bleil et al., 1988; Wassarman, 1988). Zp2 serves as a secondary receptor for sperm during the fertilization in mice (Bleil et al., 1988). However, there are several well-known species-specific differences in the function of ZP proteins (Prasad et al., 1996; Yurewicz et al., 1998; Bedford, 1977; Rankin et al., 1998, 2003) and it is therefore difficult to apply these findings directly to humans. We have previously shown that in humans, sequence variations in the genes for ZP1 and ZP3 but not in ZP2 and ZP4 can be associated with fertilization failure in *in vitro* fertilization (Mannikko et al., 2005).

The expression of ZP genes is under direct regulation of a transcription factor termed Factor in the Germ Line Alpha (FIGLA) (Liang et al., 1997; Bayne et al., 2004), which is essential for the formation of primordial follicles and survival of the oocyte in mice (Soyal et al., 2000). FIGLA is expressed in human fetal (Bayne et al., 2004) and adult (Huntriss et al., 2002; Bayne et al., 2004) ovaries, but its role is still not well understood.

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The aim of the study was to elucidate the roles of ZP1 and ZP3, the key candidate components of the *zona pellucida* in humans (Mannikko et al., 2005), and their regulatory factor FIGLA during human ovarian development by analysing the expression and localization of ZP1, ZP3 and FIGLA mRNA, and ZP3 protein in human fetal and adult ovaries.

2. Materials and methods

2.1. Tissue samples

Ovarian samples from 10 fetuses (fetal age 11–21 weeks) were obtained after spontaneous or therapeutic abortions. Ovarian samples from 6 fetuses (23–37 weeks) were obtained after intrauterine fetal death followed by spontaneous or induced deliveries or Caesarean sections. In addition, ovaries from two neonates (23 and 31 weeks) who died because of perinatal asphyxia or infection within 8 h after birth were studied (Table 1). All fetuses and neonates had normal karyotypes. Samples with visible autolysis were excluded from the study. Adult ovarian tissue was obtained from seven patients undergoing ovariectomy due to endometriosis. All samples were fixed in 4% buffered formaldehyde for 24 h, dehydrated, and embedded in paraffin. Histological sections (4 μm) were cut and processed for *in situ* hybridization and immunohistochemistry. The study was approved by the Ethics Committee of Oulu University Hospital and a permit to study human autopsy tissue was obtained from the National Authority for Medicolegal Affairs.

2.2. In situ hybridization

Expressed sequence tags (EST) clones (image clone 1837179 for ZP1, 5724267 for ZP3 and 5744748 for FIGLA) were acquired from MRC geneservice (Cambridge, UK). Plasmid DNA preparations were made using PhoenIX™ Maxiprep Kits (Qbiogene, Morgan Irvine, CA, USA) according to the instructions of the manufacturer. Templates were linearized by digestion with suitable restriction enzymes. DNA was purified according to the instructions in the QIAquick® PCR Purification Kit (QIAGEN, Hilden, Germany).

Table 1
Expression of ZP1, ZP3 and FIGLA mRNA in human fetal ovaries

Age (weeks + days)	Source of fetuses	ZP1	ZP3	FIGLA
11 + 3	s.a.	+/−	+	+
12	i.a.	+/−	+	+
13	i.a.	+/−	na	+
15	i.a.	+/−	+	+
16	s.a.	+/−	++	+
17 + 5	s.a.	+/−	++	++
18 + 1	s.a.	+/−	na	na
19 + 1	s.a.	+	+++	+
20 + 6	i.a.	+/−	na	+
21 + 6	i.a.	na	+++	++
23 + 1	s.d.	+/−	+++	++
25 + 6	i.d.	+	na	++
27	c.s.	+	+++	++
31 + 2	s.d.	na	+++	++
32 + 3	i.d.	+	++	++
33 + 4	i.d.	+	++	++
36 + 2	c.s.	na	na	++
37 + 6	i.d.	+	+	na

s.a.: spontaneous abortion; i.a.: induced abortion; s.d.: spontaneous delivery; i.d.: induced delivery; c.s.: Caesarean section; na: specimen not available.

In situ hybridization was performed as previously described (Vaskivuo et al., 2005). Briefly, the antisense and sense probes were transcribed from the linearized templates by T7, SP6 or T3 RNA polymerases (Promega Corporation, Madison, WI, USA) and labelled with ^{35}S-UTP (GE Healthcare, UK). Before hybridization the ovary samples were treated with proteinase K (10 mg/mL). The probe (45 μL) was applied to the tissue sections (1.5–2 million cpm/slide), sealed with a plastic coverslip and hybridized in a humidified chamber at 56 °C for 16 h. The sections were digested with RNAse, vacuum-dried and coated with photographic emulsion Kodak NTB (Kodak, Rochester, NY, USA) diluted 1:1 with 1% glycerol. The slides were exposed for 11–25 days and developed using Kodak Professional D-19 developer (Kodak, Rochester, NY, USA) for 3.5 min and fixed using Kodak Professional fixer (Kodak, Rochester, NY, USA) for 2 min. The slides were counterstained with haematoxylin.

All samples were evaluated by two independent observers (R-M.T. and T.E.V.) by using light and dark field microscopy. The mRNA expression levels were semi-quantified by scoring the samples into degrees of intensity from +/− (lowest) to +++ (highest) (Table 1).

2.3. Immunohistochemistry

Immunohistochemistry was performed using Vectastain Elite ABC Kits (Vectorlaboratories, Burlingame, CA), as previously described (Vaskivuo et al., 2001). Briefly, paraffin sections were deparaffinized in xylene and hydrated gradually through a series of graded alcohols. For adult tissue, standard sodium citrate epitope retrieval was used. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol. Polyclonal ZP3 antibody obtained from Aviva Systems Biology (San Diego, CA, USA) was used at 1:200 and incubated for 2 h at RT. For adult tissue, fetal calf serum was added to the primary antibody (final concentration 5%) in order to block non-specific binding and incubation was overnight at +4 °C. A colour reaction was produced with diaminobenzidine.

3. Results

3.1. ZP3 is abundantly expressed during mid-gestation

In situ hybridization analyses revealed that ZP3 mRNA was detected at a fetal age of 11 weeks (Table 1, ZP3) (Fig. 1a: A and B) and it was markedly increased towards mid-term, reaching its maximum around the 20th week, coinciding with the formation of follicles (Fig. 1a: C–F). Thereafter its levels remained high until the 30th week (Table 1, ZP3) (Fig. 1a: G and H). Localization of expression was difficult to determine before follicle formation, but after that all oocytes clearly expressed ZP3 mRNA. As a result of high expression inside the follicles, it was difficult to exclude possible expression in the granulosa cells. During the last few weeks of pregnancy, ZP3 mRNA levels clearly decreased and at birth low mRNA expression, equal to that observed in the first trimester, was detected (Fig. 1a: I and J).

Similarly to mRNA, immunohistochemical analysis demonstrated that ZP3 protein was already present at the 11th week of fetal life (Fig. 2A and B). High expression could be seen at the age of 17 weeks, but its localization was difficult to determine (Fig. 2C and D). The strongest immunostaining coincided with the high mRNA expression between 20th and 30th weeks (Fig. 2E–H). During this stage and thereafter (Fig. 2I and J), the most intensive staining was observed in the follicles, especially in the oocytes, and minimal or negligible staining was observed in the stroma.