Transferrin in the developing ovarian follicle: evidence for de-novo expression by granulosa cells

D.A.Briggs, D.J.Sharp, D.Miller1 and R.G.Gosden

Centre for Reproduction, Growth and Development, Division of Obstetrics and Gynaecology, D Floor, Clarendon Wing (Leeds General Infirmary), Leeds, LS2 9NS, UK

1To whom correspondence should be addressed

Introduction

Prior to oogenesis, a small number of primordial follicles first undergo co-ordinated spatial and temporal development. This process requires stage-specific stimulation by gonadotrophins to bring the follicle(s) to ripeness and ovulation. Yet, there is increasing evidence of the primacy of the autocrine and paracrine factors in controlling follicle development (Gougeon, 1996). Communication between cell types can occur directly via cell–cell contact at gap junctions, of which there are both homologous and heterologous types in the follicle. It can also be a result of diffusion and uptake of molecules from the extracellular fluid which is most abundant at antral stages. This follicular fluid is an exudate of plasma, water and solutes which cross the leaky follicular epithelium to which is added the secreted products of follicle cells (Gosden et al., 1988).

One of the key objectives of research in folliculogenesis is to identify secreted proteins that affect the fate of follicles by promoting growth and development or atresia. A number of specific proteins have been identified, e.g. zona pellucida protein 3 (ZP3) (Kinloch et al., 1988), which is a structural component of the rigid zona pellucida, and growth differentiation factor-9 (GDF-9) (McPherron and Lee, 1993; Dong et al., 1996) which is involved in oocyte–granulosa interactions. Other proteins, e.g. cytokines and insulin-like growth factor binding proteins (IGFBPs), that are not specific to the follicle, play crucial roles too. Transferrin, better known for its role as a circulating carrier of iron, may have an important role in follicles, which would not be surprising in view of the evidence in spermatogenesis where it is crucial for the development of the male germ cell (Griswold, 1995). Since transferrin is expressed by Sertoli cells we might expect to find it in the granulosa cells which are the homologous cells in the ovary. Within the ovary there is tentative evidence that transferrin concentrations in follicular fluid are correlated with the degree of follicular maturity and steroidogenesis (Entman et al., 1987; Mantzavinos et al., 1993) and transferrin is often included in culture media for ovarian follicles or granulosa-oocyte complexes, albeit as a precautionary measure (Eppig and Schroeder, 1989). The demand for iron is expected to increase given the enormous increase in mass of the oocyte and its associated granulosa cells. The presence of the transferrin receptor in human granulosa cells has been observed previously (Aleshire et al., 1989) using the OKT9 antibody implying that a receptor-mediated uptake mechanism may exist in these cells. Transferrin might perform functions other than iron transport in follicles, such as influencing development as a growth factor (de Jong et al., 1990) or act as an iron chelator to damp down the generation of reactive oxygen species, which may promote atresia in the follicle (Tilly, 1998). Despite the indications that transferrin may play an important role in the ovary by influencing folliculogenesis there is little data supporting this hypothesis.

Transferrin belongs to a family of metal-binding glycoproteins with molecular masses of 76–81 kDa consisting of a single polypeptide chain of ~680 amino acid residues...
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(Williams, 1982; Bowman et al., 1988). The amino acid chain is folded into two homologous globular domains by intragenic duplication, each domain having one high-affinity binding site for ferric ion. Like most plasma proteins, the principal site of transferrin production is the liver, and the cDNA sequence for human hepatic transferrin has been fully characterized (Schaeffer et al., 1987). Important extrahepatic sites of expression have also been discovered, including Sertoli cells (Skinner and Griswold, 1980), brain (Tu et al., 1991), muscle, lymphocytes, bone marrow, salivary gland, spleen, lymph nodes, placenta, heart and lung (Aldred et al., 1987). In addition to these members of the transferrin family, there is lactotransferrin, a distinct iron binding protein secreted into milk by the mammary gland (Mazurier et al., 1983), ovotransferrin, which is a major protein of avian egg white expressed and secreted by the oviduct (Jeltsch and Chambon, 1982), and melanotransferrin, which is a membrane-bound protein associated with melanocytes (Rose et al., 1986). It is thought that while the plasma variant from the liver supplies most body tissues with iron, other transferrins are produced locally and transport iron in sanctuary areas that cannot readily be reached by plasma.

The present study was based on a hypothetical role for transferrin in follicle development and the assumed need for iron by the developing oocyte. Our investigations followed three lines of enquiry. Initially, immunocytochemical techniques were used to investigate the protein in human granulosa cells, granulosa-cells and oocytes. Secondly, the existence of a de-novo potential for the production of transferrin was investigated using reverse transcription–polymerase chain reaction (RT–PCR) to detect its mRNA in both human and mouse cells and tissues. Finally, in order to assess the physiological significance of such a mechanism, transferrin levels were determined in follicular fluid from patients undergoing oocyte collection for in-vitro fertilization (IVF) treatment.

Materials and methods

**Tissue source and granulosa cell recovery**

The collection of specimens for research was approved by the Research Ethics committee of the Leeds Teaching Hospitals NHS Trust, and patients gave informed consent. Whole ovaries and ovarian biopsies were obtained from adult patients aged <35 years undergoing routine gynaecological surgery for conditions not involving ovarian pathology. Granulosa–lutein cells and follicular fluid were collected during oocyte retrieval for IVF treatment following a conventional long protocol (Ron-El et al., 1991; Van der Elst et al., 1996). This procedure involves down-regulation of the pituitary with a 750 µg daily dose of the gonadotrophin-releasing hormone (GnRH) analogue, buserelin (Hoechst, Middlesex, UK) commencing on day 21 of the previous luteal phase. Recombinant follicle stimulating hormone (FSH) (Gonal-F; Serono, Bari, Italy) was administered at doses ranging from 150–400 IU daily for an average of 12 days. Human chorionic gonadotrophin (HCG) (Pregnyl; Organon, New Jersey, USA) was then administered when the leading three follicles had reached ~17 mm in diameter and the values for circulating oestradiol were consistent with the number and sizes of the follicles observed. All aspirates were judged to be free of contaminating blood based on the clearly unpigmented presentation of the samples. Murine ovarian and liver tissues were dissected from 14 day old F1 hybrid mice (C57BL/6×CBA/Ca) from the University of Leeds Animal House, and frozen at ~80°C.

**Immunocytochemical analysis of transferrin and its receptor in human ovarian tissue**

The distribution of transferrin was examined in 4 µm serial sections of formalin-fixed, paraffin embedded human ovarian tissues from six patients. Additionally, transferrin and its receptor were localized in human granulosa and granulosa-lutein cells obtained from five patients during IVF cycles. The cells were pooled for each patient and purified by centrifugation at 350 g for 10 min through 45% (v/v) Percoll gradients pre-equilibrated in Dulbecco’s minimal essential medium (Gibco-BRL, Paisley, UK). Air-dried preparations were made on glass slides using a CytoSpin®3 cytocentrifuge (Shandon, Runcorn, UK) and fixed in ice cold acetone. All the specimens were incubated at room temperature in rabbit serum diluted 1:5 in phosphate-buffered saline (PBS; Sigma Chemical Company, Poole, UK) for 20 min to minimize non-specific antibody binding. All antibodies were diluted in PBS containing 1% (v/v) bovine serum albumin (BSA; Sigma).

Transferrin was localized by 1 h incubation in diluted goat anti-human transferrin polyclonal antiserum (Sigma; 1:100 dilution). The specimens were then treated with biotinylated goat anti-rabbit immunoglobulin G (IgG) (1:200 dilution) for 30 min, followed by preformed avidin biotinylated horseradish peroxidase complex (Vector Laboratories, Peterborough, UK) for 30 min, which was detected by 0.02% (w/v) 3,3-diaminobenzidine. Acetone fixed, cytospan granulosa cell preparations were processed for immunocytochemistry as above except that the antibody was used at 1:800 dilution. The transferrin receptor was localized on cytospan preparations as above except that an anti-mouse monoclonal antibody (mAb; Sigma) was used in conjunction with the APAP detection system (1:25 dilution; Dako) and alkaline phosphatase activity localized using the fast red substrate (1 mg/ml). Human liver tissue sections and omission of the primary antibody were included as positive and negative controls respectively.

**Detection of transferrin gene expression**

RNA extraction followed by RT–PCR was performed on mouse and human ovarian tissue, human granulosa cells and six human oocytes which were isolated from 2–3 mm follicles denuded of their granulosa cells by pipetting. The RNA was extracted as described previously (Chomczynski and Sacchi, 1987), DNase treated, (human oocyte RNA was left untreated to minimize the risk of losing rare transcripts), and reverse transcribed (Pharmacia First Strand cDNA Synthesis Kit; Pharmacia Biotech, St Albans, UK). RT–PCR (first-round, nested or semi-nested) was performed to amplify fragments of transferrin cDNA, using reagents obtained from Clontech (Palo Alto, USA). Amplification reaction mixes consisted of 1–1000 ng of template DNA, 0.5 IU of thermostable DNA polymerase, 0.2 mmol/l dNTPs, 1.5–3.0 mmol/l magnesium chloride, 10–30 pmol of primers and PCR reaction buffer (20 mmol/l Tris–HCl, pH 8.4 and 50 mM KCl), in a final volume of 20 µl. All samples were initially denatured at 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 50°C for 2 min and 72°C for 3 min. A final extension step of 72°C for 5 min was included. All PCR reactions were performed on a pre-programmed automated DNA thermal cycler (Perkin-Elmer, Warrington, UK). Using primers with redundancy values in the range 12 to 64 (Table I) designed from homologous regions of the amino acid alignment of all previously identified mammalian transferrins and chicken ovotransferrin (Figure 1A), a 219 bp fragment of the transferrin gene from mouse ovarian RNA was amplified, cloned and sequenced. A murine transferrin-specific primer B (+), based on the derived nucleotide sequence was used in conjunction with the vector primers M13F and SP6 to amplify the 3’ end of murine ovarian transferrin gene.
transcript from a cDNA library using a nested PCR strategy. The cDNA library was constructed from whole ovaries of 21–22 day old C57BL/6J strain mice into plasmid vector pSport1 (Jackson Laboratory, Bar Harbor, USA). First round amplification was performed using a redundant transferrin primer (TF2) and M13F, followed by second round amplification with the internal specific transferrin primer B(+) and SP6 (Figure 1B). Transferrin RT–PCR products were ligated into the plasmid vector pCRIL, transformed into One Shot competent cells (Invitrogen, Leek, The Netherlands) and subjected to automated solid phase sequencing by the dideoxynucleotide chain termination method (Dynal, Merseyside, UK). Nucleotide sequences of both strands were obtained from 12 independent sequencing reactions using three distinct clones in each case.

Table I. Primer sequences used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Redundancy</th>
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<tbody>
<tr>
<td>TF1(+)</td>
<td>GGIAA(AG)AA(AG)AG(CT)TG(CT)CA(CT)AC</td>
<td>32</td>
</tr>
<tr>
<td>TF2(+)</td>
<td>GGIAA(AG)AA(AG)TC(ACTG)TG(CT)A(CT)AC</td>
<td>64</td>
</tr>
<tr>
<td>TF3(+)</td>
<td>GGITGGAA(CT)AT(ACT)CC(ACTG)ATGGG</td>
<td>24</td>
</tr>
<tr>
<td>TF4(+)</td>
<td>GGITGGGTIAT(ACT)CC(ACTG)ATGGG</td>
<td>12</td>
</tr>
<tr>
<td>TF5(-)</td>
<td>A(AG)IGCICCIGT(GA)TA(ACTG)CC</td>
<td>16</td>
</tr>
<tr>
<td>TF6(-)</td>
<td>AAAGC(GC)CC(AT)GT(GA)TA(GA)CC</td>
<td>16</td>
</tr>
<tr>
<td>B(+)</td>
<td>CAGTCAAGGCTGCGCTCCCG</td>
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</tr>
<tr>
<td>M13F</td>
<td>GTTTTCCCAGTCACGACGTTG</td>
<td>1</td>
</tr>
<tr>
<td>SP6</td>
<td>ATTTAGGTGACACTATAG</td>
<td>1</td>
</tr>
</tbody>
</table>

Figure 1. (A) Primer design for amplification of transferrin mRNA by reverse transcription–polymerase chain reaction (RT–PCR). Alignment of seven amino-acid sequences (RLTF = rat lactotransferrin; RabTF = rabbit serum transferrin; HuTF = human serum transferrin; HoTF = horse serum transferrin; MLTF = mouse lactotransferrin; COTF = chick ovotransferrin; XTF = Xenopus ovotransferrin). Highest homologies are shaded in black with lower and weaker homologies shaded in grey or unshaded respectively. Differences in the amino-acid lengths of intervening sequences in the alignment are indicated by numbers. (B) Cartoon of mouse ovarian library cDNA showing positional relationship between the expected RT–PCR products at 219–234 bp (shaded grey) amplified by redundant primers TF1 and TF6 from mouse follicles and sequences 5’ and 3’ to this (unshaded). Vector sequences are shaded in black. The 950 bp sequence represents 762 bp of the complete 3’ end of the murine transferrin cDNA plus 188 bp of vector, amplified from this library following sequencing of the original RT–PCR product and the use of vector primer Sp6 and sequence-specific primer B(+).

Quantification of transferrin in human follicular fluid
Samples of follicular fluid free of blood or media contamination were collected from a total of 20 patients aged 23–42 years (mean 34.4) undergoing oocyte retrieval for IVF. At least two samples from individual follicles per patient were collected into tubes containing heparin. A blood sample was also taken from each patient during the oocyte collection procedure for comparison, and all samples were centrifuged at 350 g for 10 min. Following end-point nephelometry (Nephelometer II; Behring, Marbuck, Germany), a correlation between follicular and serum transferrin levels was calculated using Pearson’s correlation coefficient of \((R^2)\). The coefficient of variation (CV) for these samples was 9.7%.

Results

Transferrin and transferrin receptor protein distribution
Transferrin distribution was investigated using sections of human ovarian tissue. In primordial follicles neither the oocyte nor the pregranulosa cells showed any evidence of transferrin labelling, although there was some weak labelling around the nuclear membrane of some oocytes (Figure 2A, arrow). Small growing follicles (1–2 layers) revealed some labelling for transferrin in the cytoplasm of their cuboidal granulosa cells (Figure 2B, arrowheads). The ooplasm in other follicles showed some labelling (Figure 2C, arrows), while a rim of weakly
positive reaction product associating with the oolemma could also be seen at this stage (Figure 2C, arrowheads and Figure 2B, arrows). The ooplasm and oolemmas of cells contained within antral follicles were strongly labelled, (Figure 2G) while expression in cumulus cells on either side of the unlabelled mural granulosa cell layer (between white lines) were more prominently labelled towards the antral cavity (Figure 2H; lower right of frame).

Transferrin was expressed in a subpopulation of human granulosa lutein cells isolated from follicular aspirates while other granulosa cells, red blood cells and negative controls were unlabelled (Figure 2J). Cytoplasmic labelling of these cells was particularly intense as was the labelling of the plasmalemma and the occasional nucleus. Some cells, however, displayed only labelling of the plasmalemma (arrowheads). The percentage of cells labelled for transferrin ranged from 40–50, with a similar range for expression of the transferrin receptor. However, in the absence of dual immunocytochemical
Follicular transferrin expression

Figure 3. Composite image of four gels showing reverse transcription–polymerase chain reaction (RT–PCR) products for murine transferrin (lanes 2, 4, 5, 7) human transferrin (lanes 11, 12) and human β-actin (lanes 9 and 10). Lanes 1, 3, 6 and 8 contain a 100 bp ladder from each of the original gel sections shown in lane 2, lane 4, lanes 5 and 7 and lanes 9–13 respectively. For clarity, some stretching of these images was undertaken to ensure that the markers aligned as closely as possible. The 219 bp product amplified by primers TF1/TF6 is indicated in lanes 2 and 4; the expected product amplified by the M13 and B/H11001 primers is not clear in lane 5 but is the sole product amplified following nesting with SP6 and B/H11001 (lane 7). A 234 bp product was generated by the same redundant primers (TF1/TF6) from human granulosa–lutein cells (lane 11) but not from human oocytes (lane 12). The 320 bp product for β-actin was amplified from both of these cell types (lanes 9 and 10). Lane 13 is a control using the redundant primers on oocyte RNA but without adding reverse transcriptase to the first strand mix.

labelling, we cannot be certain that the same cells were labelled in each case. In comparison to its ligand, labelling of the transferrin receptor was not clearly restricted to the plasmalemma in any of the observed cells (Figure 2K).

**Isolation and sequencing of mammalian ovarian transferrin gene transcripts**

Figure 3 is a composite image showing the results of nested RT–PCR using the redundant primers listed in Table I. Only the first round product is shown unless otherwise indicated. A 219 bp product was amplified from both mouse ovarian and liver RNAs using primers TF1 and TF6 (lanes 2 and 4). The ovarian product was 98% homologous to a fragment of hepatic transferrin mRNA, representing part of the 3′ end of the murine ovarian transferrin gene transcript coding for an open reading frame of 73 amino acids (see Figure 4B). Using nested primers specific to the cloned mouse ovarian transferrin transcript, the full 3′ sequence was amplified from the mouse ovarian library (pSport; Figure 1B). The product (lane 7) was 950 bp in length (762 bp of transcript plus 188 bp of vector) and coded for an open reading frame of 195 amino acids with 174 bp of 3′ UTR (Figure 1B). Repeated attempts to amplify the 5′ end of the gene were unsuccessful. The same redundant primers amplified a 234 bp product from human granulosa lutein cells (lane 11) that could not be amplified from isolated human oocytes (lane 12), despite verification of template viability (lanes 9 and 10). We were unable to clone this product and resorted to PCR with the TF3/TF6 primers in order to generate a hemi-nested 182 bp product (not shown). This product was successfully sequenced and shown to encode a transcript for a 60 amino acid fragment with 76% (32/42) identity to human (Yang et al., 1984) and 98% identity (59/60) to murine hepatic transferrin respectively, at the amino acid level. The human and murine ovarian sequences also share a 98% (59/60) identity at this level (Figure 4).

**Quantification of transferrin in human follicular fluid**

Transferrin was measured in all follicular fluid and serum samples and ranged from 0.93–2.79 g/l (2.00 0.39) and 1.54–3.25 g/l (2.33 0.43) respectively. There was a strong correlation between transferrin levels in the paired samples ($R^2$ of 0.75; Figure 5).

**Discussion**

Immunocytochemistry confirmed that transferrin and its receptor are expressed in the ovary of both human and mouse. In addition, to our knowledge, this is the first report demonstrating that cumulus granulosa cells, but not oocytes, have the potential to synthesize their own transferrin from an endogenous supply of mRNA coding for this protein. More specifically, we were able to detect transferrin mRNA in the granulosa cells of follicles by RT–PCR, but not in the oocytes, while antral follicles exhibited high levels of the protein in both oocytes and granulosa cells. Relying on a subjective examination of these sections, we observed a gradual increase in transferrin labelling within granulosa cells and oocytes at progressively more advanced stages of follicle development with the most intense labelling observed in the cumulus cells of antral follicles and after luteinization. Furthermore, we found that labelling in the cumulus granulosa cells appeared to increase gradually towards the antral cavity and there was little or no expression apparent in the mural granulosa cells. These results differ from those of other authors (Aleshire et al., 1989) where
relatively constant cytoplasmic labelling of granulosa cells at all developmental stages except primordial follicles was reported. A sub-population of granulosa/granulosa–lutein cells isolated from the cumulus mass of mature follicles contained high levels of transferrin and transferrin receptor with the former localized to the plasmalemma as well as the cytoplasm. The transferrin receptor has been observed in oocytes by others (Balboni et al., 1987). Taken together, the immunocytochemical and molecular data suggest that some granulosa cells can synthesize their own transferrin which is translocated to and across the membrane for endocytosis by the oocyte. This idea bears close comparison with the biochemical situation in the testis where transferrin production by the Sertoli cells occurs to compensate for poor iron transport across the blood/testis barrier. The developing spermatocytes are thought to take up the iron-bound transferrin from the Sertoli cells by an endocytotic process (Skinner et al., 1984; Vannelli et al., 1986). While no equivalent blood/follicle barrier exists in the ovary (Gosden et al., 1988), indeed, there are near identical levels of transferrin in follicular fluid and serum, it is possible that the de-novo expression of transferrin in the follicle may still be necessary to compensate for impoverished microenvironments isolated from the near equilibrium in extracellular transferrin values. Microenvironments are a poorly understood aspect of repro-

![Figure 4](image-url)
productive physiology and their importance cannot be underestimated (Hunter, 1994). Alternative explanations are; that locally expressed transferrin is part of the distinct signalling pathway (de Jong, 1990) or that ovarian transferrin and its receptor are redundant to the ovary. The presence of similarly variable patterns of expression for a number of gene products known to have key roles in follicle development, e.g. luteinizing hormone (LH) receptor (Amsterdam et al., 1975; Eppig, 1991) weighs against the latter interpretation.

To help resolve conflicting explanations for the likely significance of localized transferrin expression, we have also provided partial sequence data for both the mouse and human ovarian transcripts. Alignment of these sequences with each other gave a 98% match at the amino acid level of identity. Interestingly, human ovarian transferrin is also 98% homologous at this level to murine hepatic transferrin, a closer relationship than with other human transferrins (Yang et al., 1984). Other alignments from the transferrin family [Multalin multiple alignment programme, Institute of Biology and Chemistry of Proteins (IBCP), France] demonstrated an appreciable homology between them. The highest level of homology was with rat lactotransferrin (Escriva et al., 1995), with 70% identity at the amino acid level. High levels of homology were also revealed with rabbit serum transferrin (Banfield et al., 1991), horse serum transferrin (Carpenter and Broad, 1993), mouse lactotransferrin (Pentecost and Teng, 1987), chicken ovotransferrin (Jeltsch and Chambron, 1982), and Xenopus transferrin (Moskaitis et al., 1990). The extent of homology shared with other transferrins ranged from 67% to rabbit serum transferrin down to 58% to Xenopus transferrin. Together, these data support the conclusion that mammalian granulosa cells can synthesize a functional transferrin protein de novo that may be a distinctive isovariant. To explore this possibility further, the full ovarian sequences are needed but have proved difficult to obtain especially at the 5’ ends, as is the case in other species (Denovan-Wright et al., 1996). A combination of in-situ hybridization and/or immunocytochemistry with probes/antibodies specific to the serum and ovarian transferrin RNAs/proteins could then be used to considerable effect. Indeed, the possibility that the antiserum used in this study is a poor discriminator of ovarian versus serum transferrin could be eliminated by the availability of more specific reagents.

The information which these techniques can generate will help to determine the precise role that ovarian transferrin plays in the follicle. In-vitro culture of follicles has traditionally included exogenously added serum transferrin in the media. If, as we suspect, a hitherto overlooked dynamic relationship exists between serum and ovarian transferrin expression in situ, then this may have to be taken into account if the in-vitro maturation technique is to be optimized for efficient production of mature follicles.

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References


Figure 5. Regression analysis of mean follicular and serum transferrin concentrations in samples obtained from 20 in-vitro fertilization patients.
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