Development 121, 000-000 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

EGF, TGF-α and EGFR expression in human preimplantation embryos

Choy May Chia*, **Robert M. L. Winston and Alan H. Handyside**

Institute of Obstetrics and Gynaecology, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK

*Author for correspondence at Thomson Fertility Clinic, Thomson Medical Centre, 339 Thomson Road, Singapore 1130

SUMMARY

Epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α) through their common receptor, epidermal growth factor receptor (EGFR) are known to enhance mitogenesis, development and implantation in several species. In the mouse, co-culture of grouped embryos in microdrops increases the cell number and proportion developing to the blastocyst stage. A similar effect is observed with culture of single embryos in medium supplemented with EGF or TGF-α highlighting their embryotrophic effects.

To study the role of EGF, TGF-α and EGFR in early human development, two methods applicable for analysis of expression at the single embryo level have been employed. In the first method, reverse transcription-polymerase chain reaction has been used to examine the presence of transcripts. Following reverse transcription, strategically designed nested primers, optimised for specificity, were used for amplification from the cDNA equivalent of a single embryo. The products were then verified by restriction enzyme digestion and sequence analysis. In the second method, immunocytochemistry has been used to co-localise the expressed proteins. Individual embryos were paraffin embedded and serial sectioned, allowing adjacent sections to be examined with different antibodies and controls. Monoclonal TGF-α and polyclonal EGF and EGFR primary antibodies were used. Staining was performed by peroxidase-conjugated avidin-biotin immunocytochemistry with the appropriate controls. The combination of these two methods can potentially be used for simultaneous analysis of several growth factors and/or their receptors in the same human embryos.

Transcripts for EGF, TGF-α and EGFR were detected in unfertilized oocytes and embryos between 8-cell and blastocyst stages on day 3 to 6 post-insemination. Similarly, at the protein level, all three were detected in unfertilized oocytes and throughout preimplantation development to day 8. At the blastocyst stage, expression was observed in both the trophectoderm and inner cell mass but decreased in more advanced blastocysts except in the polar trophectoderm and inner cell mass. This pattern of expression contrasts with the murine and bovine species in which TGF-α and EGFR but not EGF are expressed at preimplantation stages. The concurrent co-expression of these growth factors and their receptor suggests a role for autocrine stimulation in preimplantation development. In the human, reduced protein levels of EGF, TGF-α and EGFR in advanced blastocysts may reflect a switch to dependence on paracrine stimulation. This may explain the inadequacy of simple culture media to maintain the development of human embryos at later stages and the benefits of co-culture with somatic cells. The continuous expression of EGF, TGF-α and EGFR in the polar trophectoderm may be significant for implantation.

Key words: human preimplantation embryos, growth factors, EGF, TGF-α, EGFR, RT-PCR, immunocytochemistry

INTRODUCTION

Despite over a decade of experience in human in vitro fertilisation (IVF), implantation rates have remained low, reaching only 22% per embryo transferred even in large clinics (Hardy, 1993). Causes of implantation failure have been attributed either to arrested development of embryos because of chromosomal defects(Papadalos et al., 1989; Jamieson et al., 1994), suboptimal culture conditions or alternatively transfer of embryos to a non-receptive endometrium. The success of oocyte donation programmes (Navot et al., 1991; Rozenwaks, 1987) has allowed the implantation window to be defined hormonally and cleavage-stage embryos are generally transferred to an endometrium that is appropriately primed at the time of implantation. Hence improvements in IVF success are more likely to result from better methods of selection of viable embryos and refinements in culture media through greater understanding of embryo requirements at different stages.

Growth factors such as epidermal growth factor (EGF), transforming growth factor-alpha (TGF-α), platelet derived growth factor (PDGF), insulin, insulin growth factor-1, (IGF-I) have been demonstrated to act as mitogens and stimulate differentiation in murine and bovine preimplantation embryonic development (Paria and Dey, 1990; Dardik and Schultz, 1991; Wood and Kaye, 1989; Larson et al., 1992; Harvey and Kaye, 1990, 1991). For this reason, the study of stage-specific expression of growth factors and their receptors in human preimplantation embryos forms a rational basis for

formulating improved culture media for in vitro development. This would facilitate the ultimate goal of replacing a single viable embryo to avoid the risk of multiple pregnancies resulting from replacing more than one embryo widely practiced in clinics to improve pregnancy rates.

The functional roles of EGFR and its ligands, EGF and TGF-α, in particular have been well demonstrated in rodent embryos. In the mouse, EGF or TGF-α increased protein synthesis, cell number, rate of cavitation and blastocyst expansion (Wood and Kaye, 1989; Paria and Dey, 1990; Dardik and Schultz, 1991). Moreover, the role of EGF and EGFR has been implicated in the initiation of implantation of both rat and mouse embryos respectively (Johnson and Chatterjee, 1993; Das et al., 1994b).

Except for reports of the presence of several growth factors and cytokines including PDGF (Zolti et al., 1991), interleukins (IL-1, IL-6), macrophage colony stimulating factor (CSF-1), tumour necrosis factor (TNF-α), IGF-II (Svalander et al., 1991) and TGF-α (Hemmings et al., 1992) in the medium in which IVF-derived embryos were cultured, nothing is known about the role of growth factors or their expression in human preimplantation embryos.

To study the role of EGF, TGF-α and EGFR in early human development, two methods applicable for analysis of expression at the single embryo level have been employed, reverse transcription-polymerase chain reaction (RT-PCR) and immunocytochemistry. The combination of these two methods can potentially be used for simultaneous analysis of other growth factors and their receptors in human embryos.

MATERIALS AND METHODS

Human eggs and embryos

The surplus human eggs and embryos used in this study were donated by patients undergoing IVF at the Wolfson Family Clinic, Hammersmith Hospital with written consent. Local ethical permission was granted by the research ethics committee of the Royal Postgraduate Medical School and the work was carried out under license from the Human Fertilization and Embryology Authority. Women were superovulated with human menopausal gonadotrophin (hMG; Pergonal, Serono, Welwyn Garden City) after pituitary-gonadal supression by a LHRH agonist (Buserelin, Hoechst, Hounslow) according to a stimulation regime by Rutherford et al. (1988). 34 hours before oocyte retrieval, human chorionic gonadotrophin (10,000 i.u., hCG, Profasi, Serono) was administered. The eggs were preincubated, inseminated and checked for pronuclei the following day as described by Hillier et al. (1984). Each embryo was cultured in 1 ml of Earle's Balanced Salt Solution supplemented with 10% maternal serum (collected before treatment cycle), 0.47 mM pyruvate (Sigma, Poole), 25 mM sodium bicarbonate (BDH, Lutterworth), 37.5 U/ml of streptomycin (Sigma, Poole) and 95.7 U/ml penicillin (Sigma, Poole) in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 37˚C. Eggs remaining unfertilized on day 2 (day 0= day of insemination) and normally fertilized embryos at 4-cell, 8-cell and blastocyst stages from day 2 to 8 were selected for study.

Reverse transcription-polymerase chain reaction (RT-PCR)

(A) cDNA preparation

Human unfertilized oocytes and embryos were rinsed in phosphate-buffered saline (PBS) after removal of zonae with acid Tyrode and pooled eggs or embryos at the same stage (average of 5) lysed in 5 µl of lysis buffer (0.5% NP-40, 10 mM Tris (pH 8), 10 mM NaCl, 3 mM $MgCl_2$; Gilliland et al., 1990) in 0.5 ml Eppendorf tubes on ice. The tubes were microfuged for 2 minutes to remove the nuclei and cell debris. The supernatant was heated at 70˚C for 2 minutes and quickly chilled on ice. Reverse transcription was performed with 2 µl aliqouts of the supernatant in a 20 µl reaction volume containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DDT (Pharmacia), 300 pmol oligo$(dT)_{12-18}$ primer (Pharmacia) and 0.5 mM dNTP (Pharmacia) with and without 100 units of M-MLV reverse transcriptase (RT) (BRL) at 37˚C for 1 hour. The presence of hypoxanthine guanine phosphoribosyl transferase (HPRT) cDNA was used as control.

(B) Oligonucleotide primers for PCR

The sequences of oligonucleotide primers for EGF, TGF-α and EGFR and HPRT are given in Table 1. The outer primers for EGFR were selected based on the following constraints: minimum homology with other EGFR-related receptors eg c-erb B2 or tyrosine kinase consensus sites based on Hanks et al. (1988); for TGF-α, the outer primers were selected for minimal homology with EGF cDNA by avoiding the conserved cysteine rich domains (Blasband et al., 1990) and for EGF primers, the outer pair of primers spans exon 19 (EGF-like repeat no.8) which has <30% homology with EGF and exon 21. The coding region for mature EGF lies within exon 20 (Bell et al., 1986). Other criteria used for the primers were (a) inclusion of an intron in the amplicon for discrimination of products from genomic DNA and cDNA and (b) high annealing temperature of the amplicon with an appreciable temperature difference between primers and the amplicon. The inner primers for each gene selected from sequences identified above amplify the specific RT-PCR product and avoid non-specific priming associated with high numbers of PCR cycles. The best set of outer and inner primers fulfilling criterion (b) was chosen with the 'Oligo' primer analysis software (NBI).

(C) Co-amplification of EGFR and TGF-α cDNA

The cycling conditions used for PCR were: 94˚C, denaturation, 61˚C, annealing and 72˚C extension, each step at 1 minute with a final extension of 10 minutes. In the primary PCR for co-amplification of EGFR and TGF-α, 1-4 µl of cDNA sample were transferred into 25

Table 1. Oligonucleotide primers used for RT-PCR of EGF, EGFR, TGF-α and HPRT

Gene	Primer type	Forward primer	Reverse primer	Annealing temperature ˚C	Product length (bp)
EGF	Outer	5′-TGCCAACTGGGGGTGCACAG-3′	5′-CTGCCCGTGGCCAGCGTGGC-3′	58	339
	Nested	5′-TGCCAGCTGCACAAATACAGAGGG-3′	5′-CATCGTGGGACAGGGGACATTCA-3′	58	167
TGF-α	Outer	5′-CCTGCTGCCCGCCCGCCCGT-3′	5′-GCTGGCAGCCACCACGGCCA-3′	61	305
	Nested	5′-CTGCCCGCCCGCCCGTAAAA-3′	5′-CCGCATGCTCACAGCGTGCA-3′	61	275
EGFR	Outer	5′-GGACGACGTGGTGGATGCCG-3′	5′-GGCGCCTGTGGGGTCTGAGC-3′	61	208
	Nested	5′-CCTCATCCCACAGCAGGGCTTC-3′	5′GCTGTATCGCTGCAAGAAGCTGTC-3′	61	163
HPRT	Outer	5′-CTCCGCCTCCTCCTCTGCT-3′	5′-GCCTGACCAAGGAAAGCAAAG-3′	50	528
	Nested	5′-GCCGGCTCCGTTATGGCG-3′	5′-AGCCCCCCTTGAGCACACAGA-3′	55	226

μl of the PCR reaction mix containing 1.5 mm $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 8), 0.2 mM dNTP and 1.25 units of Amplitaq (Perkin Elmer). Amount of EGFR and TGF-α primers added was 2.2 nmol and 7.8 pmol of forward primers and 3.67 nmol and 8.7 pmol of reverse primers, respectively. Buffer conditions and reaction volume used in the secondary PCR were identical to the primary PCR except that 3.87 nmol and 4.5 nmol of EGFR nested forward primer and nested reverse primer respectively were used with 1 μl of the four-fold diluted primary co-PCR product. In a separate PCR assay for TGF-α, 2-4 μl of the undiluted primary co-PCR product were used with 4 and 4.7 pmol nested forward and reverse primers respectively.

(D) Amplification of EGF cDNA

PCR buffer conditions and reaction volume were as described for above primers. 1-4 μl of cDNA sample were used. Concentrations for outer forward and reverse primers were 3.3 pmol each. With inner forward and reverse primers, concentrations used were 2.4 and 2.3 pmol, respectively. Cycling conditions for PCR with EGF primers were: 94˚C, denaturation, 58˚C, annealing and 72˚C, extension, each step at 1 minute with a final extension of 10 minutes at 72˚C. 1-4 μl of cDNA sample were used for the primary PCR and 1 μl of the primary RT-PCR product for the secondary PCR. Amplification of HPRT cDNA was performed according to Ao et al. (1994).

All primers were synthesised either by Virology Department (Royal Postgraduate Medical School, London) or Oswel (Edinburgh). The HPRT primers were gift of Dr. A. Ao. In all PCR assays, 25 cycles of primary followed by 30 cycles of secondary PCR were performed respectively in a thermal cycler (Cetus, Perkin Elmer). All reactions were carried out in 0.5 μl Eppendorf tubes with an overlay of 30 μl silicon oil (BDH). 10 μl of the RT-PCR products were loaded per well for size analysis by 10 or 12% polyacrylamide gel electrophoresis in TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8) in an electrophoresis chamber (Mini-protean II, Bio-Rad) at 200 V constant voltage (Powerpac 3000, Bio-Rad). The ethidium bromide stained gel was visualized under u.v. Expected sizes of RT-PCR products for EGF, TGF-α, EGFR and HPRT are given in Table 1.

(E) Verification of RT-PCR products

The identities of RT-PCR products (EGF, TGF-α, EGFR) were verified by restriction enzyme analysis and sequencing (EGF and EGFR). Verification of RT-PCR products for EGF, TGF-α and EGFR was performed using 10 μl of the secondary PCR product with the appropriate buffer in a final reaction volume of 20 μl with and without the appropriate restriction enzymes at 37˚C for 1 hour. Digestion with DdeI (BRL) would yield fragments with expected sizes of 100 and 67 bp from RT-PCR product for EGF and three fragments with expected sizes of 9, 56 and 98 bp from RT-PCR product for EGFR. Similarly, digestion of the RT-PCR product for TGF-α with *Hin*fI (BRL) would yield two fragments with expected sizes of 164 and 111 bp. The size of digested products was analysed by electrophoresis as described above. For sequence analysis, the secondary PCR products for EGFR and EGF were purified using a kit (Magic PCR Preps DNA Purification System, Promega). The inner primers of EGFR and EGF were end-labelled and cycle sequencing was performed according to manufacturer's instructions (fmol DNA Sequencing System, Promega) modified to the annealing temperature profiles for each primer.

Immunocytochemistry of paraffin embedded sections of eggs/embryos

Eggs and embryos at the same stage were fixed in 4% paraformaldehyde in PBS for 1 hour, serially dehyrated in alcohol and paraffin embedded in gelatin capsules. Serial sections (5 μm) were retrieved individually on silane-coated slides prepared by method of Senior et al. (1988) taking care not to miss the first section. Immunocytochemistry was performed using rabbit polyclonal anti-human EGF (Ab-3) and anti-human EGFR (Ab-4) and mouse monoclonal anti-human TGF-α (Ab-2) antibodies on individual dewaxed sections of eggs and embryos, which had earlier been located under bright-field microscopy. All the antibodies were obtained from Oncogene Science (Cambridge Bioscience) and used at a dilution of 2.5 to 5 μg/ml. All incubations were carried out in humidified chambers at ambient temperature. After incubation with blocking serum (normal horse serum) and the primary antibody for 20 minutes and 1 hour respectively, amplification (1/2 hour incubation) with the relevant biotinylated secondary antibody (5 μg/ml), peroxidase-conjugated avidin-biotin complex (Oncogene Science) was applied to the sections ($\frac{1}{2}$ hour). For visualisation, diaminobenzidine tetrahydrochloride/H_2O_2 (DAB at 1 mg/ml, 0.3% H_2O_2) and counterstain Mayer's Haematoxylin were used. Finally, the sections were subsequently dehydrated and mounted in DPX (BDH). Each change of reagent was followed by careful washings (3×) of the sections with PBS. Negative controls included substituting (1) PBS for primary antibody and (2) substituting anti-TGF-α with anti-TrpE (mouse monoclonal to a *E. coli* protein). Controls for specificity of antibodies included competitive binding of the primary antibody with the relevant ligands i.e. human recombinant EGF (E3264, Sigma), EGFR peptide-1 (PP28, Oncogene Science) and human TGF-α peptide (PF008-20, Oncogene Science). Primary antibodies were serially diluted for optimal neutralisation using antigen concentrations varying from 500 to 1200 ng.

RESULTS

Detection of EGF, TGF-α and EGFR transcripts

Fig. 1 shows a typical gel electrophoresis of RT-PCR products for EGF, TGF-α and EGFR from pooled eggs, 8-cell embryos and blastocysts. In Fig. 1 (top panel)**,** the major RT-PCR product for EGF corresponds to the expected fragment size of 167 bp (topmost arrowhead, lanes 2, 4 and 6). No products were detected in lanes loaded with corresponding RT-PCR products where RT has been omitted (lanes 1, 3 and 5). Digestion of RT-PCR product with Dde I yields two fragments of the expected sizes of 100 and 67 bp (lower two arrowheads, lane 8).

In Fig. 1 (middle panel), the major RT-PCR product for TGF-α corresponds to the expected fragment size of 275 bp (topmost arrowhead, lanes 2, 4 and 6). However, a larger non-specific band above 300 bp could be observed occassionally. No products were detected in lanes loaded with corresponding RT-PCR products where RT has been omitted (lanes 1, 3 and 5). Digestion of RT-PCR product with *Hin*fI yields two fragments of the expected sizes of 164 and 111 bp (lower two arrowheads, lane 8).

Transcripts of EGFR (Fig. 1, bottom panel) of the predicted size (163 bp) were seen at all stages analysed (topmost arrowhead, lanes 2, 4 and 6). No products were detected in lanes loaded with corresponding RT-PCR samples where RT has been omitted (lanes 1, 3 and 5). Bands below 50 bp formed by primer dimers could be seen in these lanes (1, 3 and 5). These were not observed with primers for EGF and TGF-α, presumably because of differences in susceptibility to self annealing with these primers. The identity of the 163 bp fragment was verified by Dde I digestion and two bands of predicted sizes at 98 and 56 bp respectively were detected (lower two arrowheads, bottom panel, lane 8). The smallest fragment of 9 bp migrated from the gel during electrophoresis and therefore was not detectable. The experiments for RT-PCR of EGF, TGF-α and EGFR were repeated for a minimum of three times with eggs and embryos at each stage with different lysates. Transcripts were consistently detected at cDNA equiv-

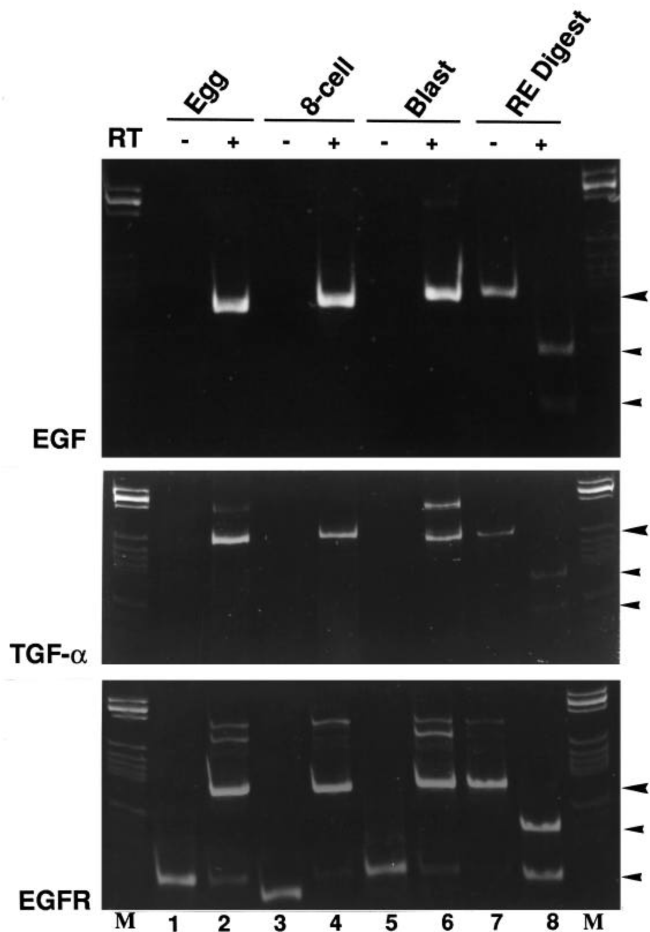

Fig. 1. Gel electrophoresis of RT-PCR products for EGF (upper panel), TGF-α (middle panel) and EGFR (lower panel). Lanes 1 and 2: Unfertilized eggs without and with reverse transcriptase, respectively (–/+RT); 3 and 4: 8-cell embryos (–/+RT); 5 and 6: blastocysts (–/+RT); and, 7 and 8: without and with restriction enzyme digest (–/+ RE digest). M: Marker, pBR322, *Hae*III digest. Large arrowheads show major RT-PCR products; small arrowheads show digested fragments (see text for details). Eggs and embryos were pooled for cDNA synthesis. Aliquots of cDNA, equivalent to 2/5-1/2 of an individual egg or embryo were used for amplification.

alent of 2/5 to 1/2 an egg or embryo with lysates of pooled eggs or embryos using HPRT cDNA as a control.

Sequence analysis

Confirmation by sequence analysis showed that the 163 bp and 167 bp RT-PCR product for EGFR (from nucleotides no.3207 to 3414, GenBank accession no.X04571) and EGF (from nucleotides no. 3276 to no. 3342, GenBank accession no. X00588) respectively matched published sequences in GenBank database (results not shown).

Immunocytochemistry

Micrographs of sectioned eggs and embryos illustrating the observed pattern of staining with antibodies to EGF, TGF-α and EGFR are presented in Figs 2-5. In most of the eggs and embryos, zonae dissolved during the 90% dehydration step, so almost all the sections had no zonae. In general, cytoplasmic staining, more intense peripherally was observed in sections of eggs (Fig. 2A,D,G (less obvious)), 4-cell embryos (Fig. 2B,E,H) and early blastocysts (Fig. 2C,F,I, less obvious). In some, staining for TGF-α was less intense than with the other two antibodies in adjacent sections of the same egg, eg. Fig. 2A,D,G. Similarly with the other two antibodies, variable intensities of staining were observed within the same embryos even though same concentration of antibodies were used. These could reflect the variable amount of growth factors and receptors within the same embryo and between different embryos. Controls for specificity of antibodies and reagents all demonstrated ablation of staining in serial or adjacent sections (Figs 3A-D, 4B).

At later blastocyst stages, staining with all three antibodies was observed in both the inner cell mass and trophectoderm with preferential staining at the polar end and patchy staining of varying intensity in the mural trophectoderm. Fig. 4A shows an example of a day 6 blastocyst with patchy staining for EGFR especially in the mural trophectoderm (te) and stronger staining in the inner cell mass (icm) and overlying polar trophectoderm. Advanced blastocysts (day 7 and 8) showed markedly reduced staining throughout. Fig. 5 shows an example of the weaker staining in the trophectoderm and inner cell mass compared to Fig. 4A. However, some retention of staining is still observed in the inner cell mass and polar trophectoderm at this late stage compared to the mural trophectoderm (Fig. 5, te). Complete absence of staining for EGF was observed in two of the 10 expanded blastocysts.

A summary of the intensity of staining with antibody to each of the growth factors and receptor observed in sections of 7-14 embryos for each developmental stage is given in Table 2. Owing to the multiple washing steps, some sections were lost. Therefore, most of these results were compiled from serial but not necessarily adjacent sections of the same embryo. On a scale of 1+ to 4+ (most intense), the majority of sections of each developmental stage stained 3+ to 4+ for antibodies to EGF and TGF-α and 4+ for EGFR. Generally, the periphery of these sections stained 4+. Diminished staining, occurring in patches begins in the trophectoderm (te) at the expanded blastocyst stage on day 6 and 7 (Figs 4A and 5).

DISCUSSION

We have demonstrated the concurrent expression of the ligands EGF, TGF-α and their common receptor EGFR throughout human preimplantation development by RT-PCR and immunocytochemistry. Transcripts of EGF, TGF-α and EGFR were detected in eggs, 8-cell embryos and blastocysts sensitive to the single embryo level (Fig. 1). Co-expression at the protein level in serial sections of individual embryos were observed from day 2 to 8 (Figs 2-5). The combination of these two techniques is a powerful approach to the study of growth factors in human preimplantation embryos. For example, it was possible to co-amplify TGF-α and EGFR consistently from cDNA aliquots, equivalent to 2/5 to 1/2 of an egg or embryo. Also, 5 (eggs/cleavage stages) to 7-10 (blastocysts) serial sections from individual eggs or embryos were available for analysis with different antibodies.

The presence of transcripts in unfertilized eggs, at cleavage and blastocyst stages suggests that maternal transcription

Table 2. Immunocytochemical localisation of EGF, TGF-α and EGFR in human eggs and embryos

Stage	Intensity of staining		
	anti-EGF	anti-TGF-α	anti-EGFR
Egg to morula	3+ to 4+	3+ to 4+	4+
Early blastocyst (days 4-5) (TE and ICM)	3+ to 4+	3+ to 4+	3+ to 4+
Blastocyst (day 6)			
Mural TE*	2+/–	2+/–	2+/–
Polar TE	3+ to 4+	3+ to 4+	3+ to 4+
ICM	3+ to 4+	3+ to 4+	3+ to 4+
Late blastocyst (days 7-8)			
Mural TE*	+/–	+/–	+/–
Polar TE	1+	1+	1+
ICM	1+	1+	1+

7 to 14 embryos from each developmental stage were examined. Scale of intensity : 1+ to 4+ (most intense).

*Mural trophectoderm (TE) from days 6-8 blastocysts showed differential staining with large patches unstained; ICM, inner cell mass.

occurs in the egg and that these transcripts persist at cleavage stages before activation of the embryonic genome. Evidence that zygotic transcription begins at the 4-8 cells in human embryos is based on inhibition of the synthesis of polypeptides using the transcriptional inhibitor, α-amanitin (Braude et al., 1988) and absence of uridine labelling of nascent RNA transcripts in the nucleoli prior to the 6 to 8-cell stage in electron microscopic autoradiographic studies (Tesarik et al., 1986). Recently, however, Y-chromosome-specific transcripts have been detected as early as the pronucleate stage (Ao et al., 1994). Also, in the mouse, there is evidence of persistence of maternal mRNA and proteins which are functional throughout preimplantation development eg. gap junction protein connexion 32 (Barron et al., 1989) and glucose phosphate isomerase (Kidder, 1993). In human embryos, the presence of zonula-adherens-type junctions and gap junctions seen in the plasma membrane were not dependent on embryonic transcription and is present from 2-cell stage onwards indicating that oocyte encoded messages could be functional up to blastocyst stage (Tesarik, 1989). Therefore, without quantitative PCR and/or use of transcriptional inhibitors, or detection of polymorphisms, it is not possible to distinguish whether the transcripts we detect are maternal or zygotic.

The concurrent expression of EGF, TGF-α and EGFR in human eggs and embryos contrasts with the pattern of expression in other species. In the mouse, cow, pig and sheep, EGF is absent throughout preimplantation development (Rappolee et al., 1988; Vaughan et al., 1992; Watson et al., 1992, 1994). TGF-α is present in eggs and later from the 8-cell stage indicating both maternal and zygotic transcription in the mouse (Rappolee et al., 1988) whereas EGFR transcripts detected by RT-PCR and Southern blotting are present from 1-cell to the early blastocyst stages (Wiley et al., 1992). In the cow, transcripts for TGF-α were detected from 1-cell to the blastocyst stage (Watson et al., 1992).

The role of EGF, TGF-α and EGFR in the human preimplantation embryos is not yet established. EGF and TGF-α have different functions at different stages of preimplantation development in the mouse. Single mouse embryos supplemented with 4 ng/ml EGF cultured from the 2-cell stage have increased cell numbers which are comparable to those obtained in vivo at the blastocyst stage and to embryos cultured in groups (Paria and Dey, 1990). TGF-α also have similar mitogenic effects on embryo development although less potent. A markedly improved rate of zona hatching of blastocysts is observed with individual embryos cultured in medium supplemented with EGF (Paria and Dey, 1990). In addition, the increase in blastocoel expansion of embryos requires continuous exposure to picomolar concentrations of EGF or TGF-α suggesting a differentiative function for these growth factors (Dardik and Schultz, 1991). Data supporting a role for EGF and EGFR in implantation include: (1) upregulation of EGFR mRNA in delayed blastocysts prior to implantation (Paria et al., 1993), (2) EGF binding increases during periimplantation period (Chakraborty et al., 1988), (3) 6-7 hours prior to attachment, uterine heparin-binding EGF, one of the ligands for EGFR is expressed exclusively in the luminal epithelium surrounding the blastocyst (Das et al., 1994b), (4) administration of EGF by intrauterine and intravenous routes could initiate implantation of delayed blastocysts (Johnson and Chatterjee, 1993) and (5) blastocysts from transgenic mice homozygous for mutated EGFR genes die at time of implantation suggesting that EGFR is essential for implantation (unpublished work from Terry Magnuson's laboratory, cited in Das et al. (1994b)).

In the mouse, high levels of EGFR mRNA were observed with day 4 blastocysts in vivo at the periimplantation stage (Paria et al., 1993). In ovarietomised mice, an eight-fold induction of EGFR mRNA from basal levels, occurs in dormant blastocysts 8 hours after administration of estradiol (Paria et al., 1993). This upregulation of EGFR in dormant blastocysts could not be induced in vitro by estrogen alone, suggesting the mediation of an uterine factor (Paria et al., 1993). EGF may be one of the uterine mediators as it has been shown to replace estrogen in initiating implantation in ovarietomised rodents implying that synthesis of EGF is acting downstream to hormonal stimulation (Huet-Hudson et al., 1990; Johnson and Chatterjee, 1993). Also, estrogen stimulates the synthesis of EGFR in vivo in the rodent endometrium (Mukku and Stancel, 1985; Das et al., 1994a) and EGF stimulates EGFR in mouse blastocysts in vitro (Wiley et al., 1992).

Based on morphology and increasing blastocoel expansion, the hatched and expanded blastocysts used for immunocytochemistry did not appear to be degenerating. Given the fact that EGF and TGF-α are remarkably stable even under extremes of pH and are resistant to enzymes such as pepsin and trypsin (Gregory, 1985), it is doubtful that they would be degraded so rapidly as to be undetectable at this stage. The diminished staining with all three antibodies observed for expanded and hatched blastocysts (e.g. Fig. 5) may therefore reflect depletion of growth factors from the maternal serum, inadequacy of the endogeneous pool of growth factors and a dependency on external stimulation for continued synthesis of these growth factors at this stage.

Interestingly, embryos cultured under conditions identical to ours, transferred at the blastocyst stage did not result in improved pregnancy rates despite having been preselected for a potential for development (Bolton et al., 1991). This may be

Fig. 2. Immunocytochemistry of serial sections of a human egg, 4-cell embryos and an early blastocyst stained with antibodies to TGF-α (A–C), EGF (D-F) and EGFR (G-I). (A,D,G) Adjacent sections of an unfertilized egg with first polar body (arrowhead). Metaphase II stage nucleus is stained blue with Mayer's Haematoxylin (A). Cytoplasmic staining, more intense peripherally is observed with TGF-α (A) and EGF (D) but comparatively less pronounced with EGFR (G). (B,E,H) Typical sections of different 4-cell embryos. B shows variable cytoplasmic staining with anti-TGF-α in individual blastomeres, but all show more intense staining peripherally. The nucleus is clearly visible in the uppermost blastomere. E and H also show more intense peripheral staining in all the blastomeres. The nuclei of the blastomeres (uppermost and lower right in E, upper and lower in H) stained blue with Mayer's Haematoxylin. (C,F,I) Serial sections of the same early human blastocyst (late day 4). Strong cytoplasmic staining is observed throughout the nascent trophectoderm and inner cell mass. Nuclei in the cells counterstained blue but appeared purplish due to masking by the brown stain. Magnification 40×. Bar scale represents 20 μm.

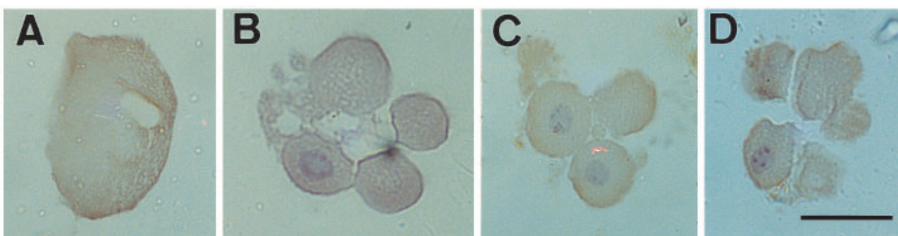

Fig. 3. (A-D) Controls for specificity of the antibodies to TGF-α, EGF and EGFR. (A) Section of an egg stained with anti-EGFR (3.3 μg/ ml) neutralised with 500 ng EGFR peptide-1; (B) section of an 8-cell embryo stained with anti-TGF-α (62.5 ng/ml) neutralised with 1.2 μg TGF-α peptide; (C) section of another 8-cell embryo stained with anti-Trp E (5 μg/ml), a mouse monoclonal to a bacterial protein; (D) section of another 8-cell embryo (3.3 μg/ml) stained with anti-EGF neutralised with 100 ng EGF. Magnification 20×. Bar scale represents 40 μm.

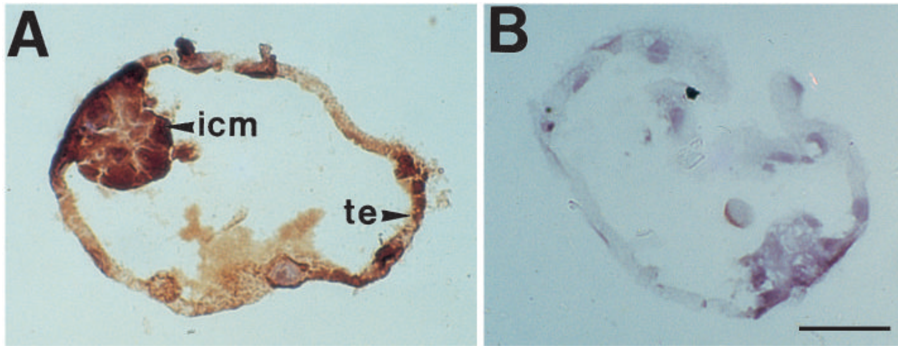

Fig. 4. (A) Staining with anti-EGFR on section of an expanded blastocyst (day 6). Non-uniform staining of the trophectoderm (te) is observed with intense staining in the inner cell mass (icm) and overlying polar trophectoderm. (B) Negative control for anti-EGFR with no staining observed when PBS was substituted for anti-EGFR on a section of a day 6 blastocyst. Magnification 20×. Bar scale represents 40 μm.

explained by down regulation of growth factors synthesis reflected in reduced staining of EGF, TGF-α and EGFR, detectable in the trophectoderm on day 6, under inadequate culture conditions. Moreover, reports of improved rates of blastocyst formation and pregnancies of human embryos co-cultured with monolayer feeder cells may be explained by presence of growth factors provided by the feeder cells stimulating later stages of preimplantation development in vitro (Menezo et al., 1992; Bongso et al., 1992; Yeung et al., 1992). It would be interesting to test this hypothesis by performing immunocytochemistry on day 6 blastocysts co-cultured with somatic cells to see if staining for EGF, TGF-α and EGFR is maintained.

Human embryos are cultured in the presence of maternal serum, which may contain low levels of EGF (Westergaard and Andersen, 1989) and other factors. The possibility that the staining observed with anti-EGF and anti-TGF-α was derived solely from EGF and TGF-α present in maternal serum and taken up via EGF receptors in the human preimplantation embryos was unlikely as embryos cultured overnight, from day 4-5 in serum-free medium showed similar staining intensities (data not shown). Staining for EGF, TGF-α and EGFR of the human blastocyst shows a patchy appearance in the mural trophectoderm (eg Fig. 4A) with strong staining in the inner cell mass and polar trophectoderm. The binding of gold-labelled EGF on mouse blastocysts has been shown to be mediated by EGFR localised on the apical and subsequently to the basolateral surface of the trophectoderm. Expression of TGF-α was concentrated in the inner cell mass. An autocrine role has been postulated for TGF-α in promoting blastocoel expansion through the EGFR receptors located on the basolateral surface (Dardik et al., 1992). In the absence of proteases required to cleave the mature peptide from its membrane spanning precursors, proEGF and proTGF-α have only restricted effect through neighbouring receptors on adjacent blastomeres thus assuming an juxtacrine role and also localising its influence. This juxtacrine mechanism could operate between trophectoderm cells and cells from the inner cell mass as well as cell-cell interaction within the inner cell mass, regulating their growth. Hence the preferential staining seen in inner cell mass and the polar trophectoderm (Fig. 4A) probably highlights the importance of regulated growth by these growth factors and their receptor for normal development.

The prevalence of cytoplasmic staining for EGF, TGF-α and EGFR may represent the traffic of growth factors and their receptor from the cytoplasm to the plasma membrane or vice-

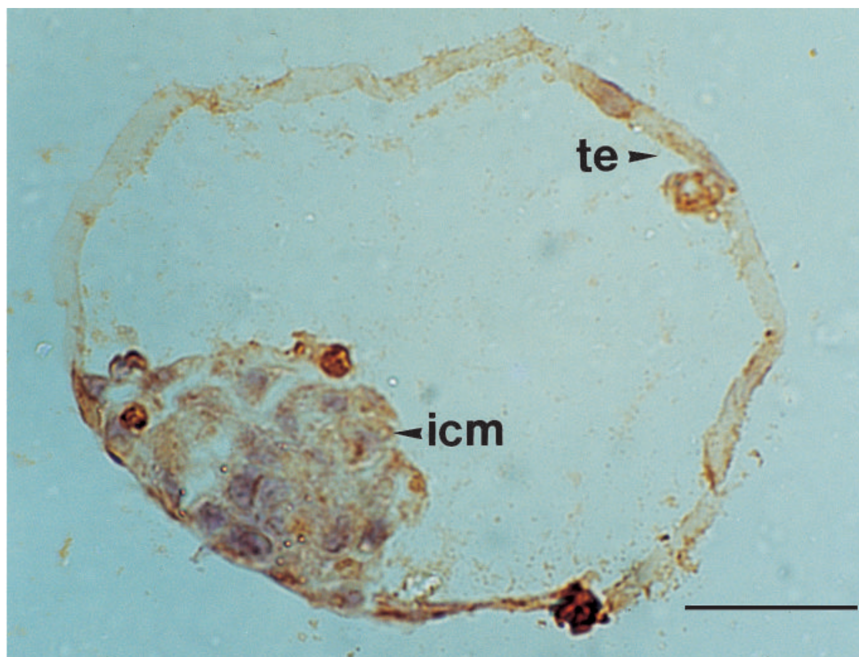

Fig. 5. Markedly reduced staining of section of an advanced blastocyst (day 7) with anti-EGFR (compare with Fig.4A). Most of the mural trophectoderm (te) shows very weak staining whereas staining is stronger in the inner cell mass (icm) and polar trophectoderm. Magnification 40×. Bar scale represents 40 μm.

versa. In fact, functional ligand/receptor complexes have been detected intracellularly in A431 cells, which highly expresses EGF receptors (Cohen and Fava, 1985). More intense staining at the periphery was observed at the plasma membrane with all three antibodies in most sections suggesting the interaction of the processed or membrane-bound growth factors EGF and TGF-α with their common receptor (Massague and Pandiella, 1993). The staining for the ligands, EGF and TGF-α may both be derived from autocrine synthesis and maternal serum.

The presence of the secreted form of TGF-α (mature TGF-α) has been reported in culture medium from 6 human embryos from cleavage to blastocyst stages (Hemmings et al., 1992). It is possible that autocrine regulation by secreted mature forms of EGF and TGF-α produced by the embryo interacting with EGF receptors sited further away but on the same embryo could also be operative simultaneously with juxtacrine stimulation between neighbouring blastomeres. Although the specificity of the processing enzyme for release of the mature forms of EGF and TGF-α is unknown, increased secretion of soluble growth factors may upregulate enzymic activity such as metalloproteinases and collagenases implicated in degrading the matrix of the basement membrane of the endometrial cells near the peri-implantation period (Brenner et al., 1989; Turpeenniemi-Hujanen et al., 1992). This would potentiate embryo signalling as well as paracrine interaction with EGF receptors present on the endometrium. Conversely, at the same time, secreted EGF and TGF-α or their integral membrane precursors from the endometrium could interact with EGFR on the embryo in corresponding paracrine or juxtacrine stimulation. Endogeneous production of EGF, TGF-α and EGFR has been confirmed in human endometrium by RT-PCR and immunocytochemistry recently (Haining et al., 1991; Chegini et al., 1992).

CONCLUSION

In summary, the simultaneous expression of EGF, TGF-α and EGFR from egg to blastocyst stage suggests that their sustained expression may be important in human preimplantation development. Analysis of transcripts and expression of protein for EGF, TGF-α and EGFR were possible, sensitive to the single embryo level. The presence of EGF in human preimplantation embryos is probably unique to mammalian preimplantation development as it is absent in a number of species. Reduced protein levels of EGF, TGF-α and EGFR at later stages of development suggest inadequacy of existing culture conditions. Further work is necessary to study the functional role of EGF, TGF-α and EGFR in human preimplantation embryos. The establishment of quantitative PCR would help to distinguish a fall from a rise (indicating the transition from maternal to embryonic transcription) in the level of growth factor transcripts. The use of EGFR-specific tyrphostins (Paria et al., 1991) or antisense oligonucleotides (Brice et al., 1993) may be useful in studying the effects of inactivating the EGF receptor in human preimplantation development.

The authors would like to thank Karin Dawson and the IVF team at the Wolfson Family Clinic, Hammersmith Hospital for their support and encouragement and the assistance of Peter Moore in sectioning. Finally, C. M. C. gratefully acknowledges the sponsorship of Thomson Medical Centre, Singapore.

REFERENCES

Ao, A., Erickson, R. P., Winston, R. M. L. and Handyside, A. H. (1994). Transcription of paternal Y-linked genes in the human zygote as early as the pronucleate stage. *Zygote* **2**, 281-288.

Barron, D. J., Valdimarsson, G., Paul, D. L. and Kidder, G. M. (1989). Connexin 32, a gap junction protein, is a persistent oogenetic product through preimplantation development of the mouse. *Dev. Genet.* **10**, 318-323.

Bell, G. I., Fong, N. M., Stempien, M. M., Wormsted, M. A., Caput, D., Ku, L. L., Urdea, M. S., Rall, L. B. and Sanchez Pescador, R. (1986). Human epidermal growth factor precursor: cDNA sequence, expression in vitro and gene organization. *Nucleic Acids Res.* **14**, 8427-8446.

Blasband, A. J., Rogers, K. T., Chen, X. R., Azizkhan, J. C. and Lee, D. (1990). Characterization of the rat transforming growth factor alpha gene and identification of promoter sequences. *Mol. Cell Biol.* **10**, 2111-2121.

Bolton, V. N., Wren, M. E. and Parsons, J. H. (1991). Pregnancies after in vitro fertilization and transfer of human blastocysts. *Fertil. Steril.* **55**, 830-832.

Bongso, A., Ng, S. C., Fong, C. Y., Anandakumar, C., Marshall, B., Edirisinghe, R. and Ratnam, S. S. (1992). Improved pregnancy rate after transfer of embryos grown in human fallopian tubal cell coculture. *Fertil. Steril.* **58**, 569-574.

Braude, P., Bolton, V. and Moore, S. (1988). Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* **332**, 459-461.

Brenner, C. A., Adler, R. R., Rappolee, D. A., Pedersen, R. A. and Werb, Z. (1989). Genes for extracellular-matrix-degrading metalloproteinases and their inhibitor, TIMP, are expressed during early mammalian development. *Genes Dev.* **3**, 848-859.

Brice, E. C., Wu, J. X., Muraro, R., Adamson, E. D. and Wiley, L. M. (1993). Modulation of mouse preimplantation development by epidermal growth factor receptor antibodies, antisense RNA, and deoxyoligonucleotides. *Dev. Genet.* **14**, 174-184.

Chakraborty, C., Tawfik, O. W. and Dey, S. K. (1988). Epidermal growth factor binding in rat uterus during the peri-implantation period. *Biochem. Biophys. Res. Commun.* **153**, 564-569.

Chegini, N., Michael, J. R. and Masterson, B. J. (1992). Platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and PDGF β-receptors in human endometrial tissue: localization and in vitro action. *Endocrinology* **130**, 2373-2385.

Cohen, S. and Fava, R. A. (1985). Internalization of functional epidermal growth factor:receptor/kinase complexes in A-431 cells. *J. Biol. Chem.* **260**, 12351-12358.

Dardik, A. and Schultz, R. M. (1991). Blastocoel expansion in the preimplantation mouse embryo: stimulatory effect of TGF-alpha and EGF. *Development* **113**, 919-930.

Dardik, A., Smith, R. M. and Schultz, R. M. (1992). Colocalization of transforming growth factor-alpha and a functional epidermal growth factor receptor (EGFR) to the inner cell mass and preferential localization of the EGFR on the basolateral surface of the trophectoderm in the mouse blastocyst. *Dev. Biol.* **154**, 396-409.

Das, S. K., Tsukamura, H., Paria, B. C., Andrews, G. K. and Dey, S. K. (1994a). Differential expression of epidermal growth factor receptor (EGF-R) gene and regulation of EGF-R bioactivity by progesterone and estrogen in the adult mouse uterus. *Endocrinology* **134**, 971-981.

Das, S. K., Wang, X. N., Paria, B. C., Damm, D., Abraham, J. A., Klagsbrun, M., Andrews, G. K. and Dey, S. K. (1994b). Heparin-binding EGF-like growth factor gene is involved in the mouse uterus temporally by the blastocyst solely at the sites of its apposition: a possible ligand for interaction with blastocyst EGF receptor in implantation. *Development* **120**, 1071-1063.

Gilliland, G., Perrin, S. and Bunn, H. F. (1990). Competitive PCR for quantitation of mRNA. In *PCR Protocols. A Guide to Methods and Applications.* (A. I. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White, eds). pp. 60-69. San Diego: Academic Press.

Gregory, H. (1985). In vivo aspects of urogastrone-epidermal growth factor. *J. Cell Sci. Suppl.* **3**, 11-17.

Haining, R. E. B., Schofield, J. P., Jones, D. S. C., Rajput-Williams, J. and Smith, S. K. (1991). Identification of mRNA for epidermal growth factor and TGF-α present in low copy number in human endometrium and decidua using reverse transcription-polymerase chain reaction. *J. Mol. Endocrin.* **6**, 207-214.

Hanks, S. K., Quinn, A. M. and Hunter, T. (1988). The protein kinase family:

conserved features and deduced phylogeny of the catalytic domains. *Science* **241**, 42-52.
Hardy, K. (1993). Development of human blastocysts in vitro. In *Preimplantation Embryo Development.* (ed. B. Bavister). pp. 184-199. New York: Springer-Verlag.
Harvey, M. B. and Kaye, P. L. (1990). Insulin increases the cell number of the inner cell mass and stimulates morphological development of mouse blastocysts in vitro. *Development* **110**, 963-967.
Harvey, M. B. and Kaye, P. L. (1991). Mouse blastocysts respond metabolically to short-term stimulation by insulin and IGF-1 through the insulin receptor. *Mol. Reprod. Dev.* **29**, 253-258.
Hemmings, R., Langlais, J., Falcone, T., Granger, L., Miron, P. and Guyda, H. (1992). Human embryos produce transforming growth factors alpha activity and insulin-like growth factors II. *Fertil. Steril.* **58**, 101-104.
Hillier, S. G., Dawson, K. J., Afnan, M., Margara, R. A., Ryder, T. A., Wickings, E. J. and Winston, R. M. L. (1984). Embryo culture: quality control in in vitro fertilization In *Proceedings of the Twelth Study Group of the Royal College of Obstetricians and Gynaecologists.* (ed. W. Thompson, D. N. Joyce, and J. R. Newton). pp. 125-140.
Huet-Hudson, Y. M., Chakraborty, C., De, S. K., Suzuki, Y., Andrews, G. K. and Dey, S. K. (1990). Estrogen regulates the synthesis of epidermal growth factor in mouse uterine epithelial cells. *Mol. Endocrin.* **4**, 510-523.
Jamieson, M. E., Coutts, J. R. T. and Connor, J. M. (1994). The chromosome constituition of human preimplantation embryos fertilized in vitro. *Human Reprod.* **9**, 709-715.
Johnson, D. C. and Chatterjee, S. (1993). Epidermal growth factor (EGF) replaces estradiol for the initiation of embryo implantation in the hypophysectomized rat. *Placenta* **14**, 429-438.
Kidder, G. M. (1993). Genes Involved in Cleavage, Compaction, and Blastocyst Formation. In *Genes in Mammalian Development.* (ed. R. B. L. Gwatkin). pp. 45-71. New York: Wiley-Liss.
Larson, R. C., Ignotz, G. G. and Currie, W. B. (1992). Platelet derived growth factor (PDGF) stimulates development of bovine embryos during the fourth cell cycle. *Development* **115**, 821-826.
Massague, J. and Pandiella, A. (1993). Membrane-anchored growth factors. *Annu. Rev. Biochem.* **62**, 515-541.
Menezo, Y., Hazout, A., Dumont, M., Herbaut, N. and Nicollet, B. (1992). Coculture of embryos on Vero cells and transfer of blastocysts in humans. *Human Reprod.* **7**, 101-106.
Mukku, V. R. and Stancel, G. M. (1985). Regulation of epidermal growth factor receptor by estrogen. *J. Biol. Chem.* **260**, 9820-9824.
Navot, D., Scott, R. T., Droesch, K., Veeck, L. L., Liu, H. C. and Rosenwaks, Z. (1991). The window of embryo transfer and the efficiency of human conception in vitro. *Fertil. Steril.* **55**, 114-118.
Papadalos, G., Templeton, A., Fisk, N. and Randall, J. (1989). The frequency of chromosome anomalies in human preimplantation embryos after IVF. *Human Reprod.* **4**, 91-98.
Paria, B. C. and Dey, S. K. (1990). Preimplantation embryo development in vitro: cooperative interactions among embryos and role of growth factors. *Proc. Natl. Acad. Sci. USA* **87**, 4756-4760.
Paria, B. C., Tsukamura, H. and Dey, S. K. (1991). Epidermal growth factor-specific protein tyrosine phosphorylation in preimplantation embryo development. *Biol. Reprod.* **45**, 711-718.
Paria, B. C., Das, S. K., Andrews, G. K. and Dey, S. K. (1993). Expression of the epidermal growth factor receptor gene is regulated in mouse blastocysts during delayed implantation. *Proc. Natl. Acad. Sci. USA* **90**, 55-59.
Rappolee, D. A., Brenner, C. A., Schultz, R., Mark, D. and Werb, Z. (1988). Developmental expression of PDGF, TGF-alpha, and TGF-beta genes in preimplantation mouse embryos. *Science* **241**, 1823-1825.
Rozenwaks, Z. (1987). Donor eggs: their application in modern reproductive technologies. *Fertil. Steril.* **47**, 895-909.
Rutherford, A. J., Subak Sharpe, R. J., Dawson, K. J., Margara, R. A., Franks, S. and Winston, R. M. (1988). Improvement of in vitro fertilisation after treatment with buserelin, an agonist of luteinising hormone releasing hormone. *Br. Med. J.* **296**, 1765-1768.
Senior, P. V., Critchley, D. R., Beck, F., Walker, R. A. and Varley, J. M. (1988). The localisation of laminin mRNA and protein in the postimplantation embryo and placenta of the mouse: an in situ hybridization and immunocytochemical study. *Development* **104**, 431-116.
Svalander, P. C., Holmes, P. V., Olovsson, M., Wikland, M., Gemzell Danielsson, K. and Bygdeman, M. (1991). Platelet-derived growth factor is detected in human blastocyst culture medium but not in human follicular fluid--a preliminary report. *Fertil. Steril.* **56**, 367-369.
Tesarik, J., Kopecny, V., Plachot, M. and Mandelbaum, J. (1986). Activation of nucleolar and extranucleolar RNA synthesis and changes in the ribosomal content of human embryos developing in vitro. *J. Reprod. Fertil.* **78**, 463-470.
Tesarik, J. (1989). Involvement of oocyte-coded message in cell differentiation control of early human embryos. *Development* **105**, 317-322.
Turpeenniemi-Hujanen, T., Ronnberg, L., Kauppila, A. and Puistola, U. (1992). Laminin in the human embryo implantation: analogy to the invasion by malignant cells. *Fertil. Steril.* **58**, 105-113.
Vaughan, T., James, P. S., Pascall, J. C, and Brown, K. D. (1992). Expression of the genes for TGF-α, EGF and the EGF receptor during early pig development. *Development* **116**, 663-669.
Watson, A. J., Hogan, A., Hahnel, A., Wiemer, K. E. and Schultz, G. A. (1992). Expression of growth factor ligand and receptor genes in the preimplantation bovine embryo. *Mol. Reprod. Dev.* **31**, 87-95.
Watson, A. J., Watson, P. H., Arcellana Panlilio, M., Warnes, D., Walker, S. K., Schultz, G. A., Armstrong, D. T. and Seamark, R. F. (1994). A growth factor phenotype map for ovine preimplantation development. *Biol. Reprod.* **50**, 725-733.
Westergaard, L. G. and Andersen, C. Y. (1989). Epidermal growth factor (EGF) in human preovulatory follicles. *Hum. Reprod.* **4**, 257-260.
Wiley, L. M., Wu, J. X., Harari, I. and Adamson, E. D. (1992). Epidermal growth factor receptor mRNA and protein increase after the four-cell preimplantation stage in murine development. *Dev. Biol.* **149**, 247-260.
Wood, S. A. and Kaye, P. L. (1989). Effects of epidermal growth factor on preimplantation mouse embryos. *J. Reprod. Fertil.* **85**, 575-582.
Yeung, W. S., Ho, P. C., Lau, E. Y. and Chan, S. T. (1992). Improved development of human embryos in vitro by a human oviductal cell co-culture system. *Hum. Reprod.* **7**, 1144-1149.
Zolti, M., Ben Rafael, Z., Meirom, R., Shemesh, M., Bider, D., Mashiach, S. and Apte, R. N. (1991). Cytokine involvement in oocytes and early embryos. *Fertil. Steril.* **56**, 265-272.

(Accepted October 26 1994)