Evaluation of the effect of interleukin-6 and human extracellullar matrix on embryonic development

N.Desai[1], M.Scarrow, J.Lawson, D.Kinzer and J.Goldfarb

Department of Reproductive Biology, Univ. MacDonald Women's Hospital, Case Western Reserve University, 11100 Euclid Av., Cleveland, OH 44106, USA

[1]To whom correspondence should be addressed

Extracellular matrices and their associated growth factors can modulate the in-vitro growth of cells. In this study, the effects of culture substrata and the cytokine interleukin-6 (IL-6) on embryonic development were investigated. In-vitro fertilized mouse oocytes were pooled and randomly distributed amongst treatment groups. The test treatments were: (i) IL-6, at either 500 or 1000 pg/ml; (ii) human extracellular matrix (HECM) applied to organ culture dishes at either 5.0 or 10.0 μg/ml; and (iii) HECM and IL-6 combined. A total of 1285 embryos was evaluated. The effect of IL-6 on embryos was dose dependent. Treated embryos exhibited higher blastulation and hatching rates than untreated control embryos. Culture of embryos on human matrix proteins versus standard culture surfaces significantly improved in-vitro hatching. The combination of both of these treatments was superior to the medium alone control, and the mean cell count per blastocyst was higher (131.7 ± 29.7 versus 82.5 ± 14.3 in control embryos; $P < 0.0001$). In a pilot study with human triploid embryos, the HECM/IL-6 culture system appeared to support embryonic compaction, blastulation and hatching. This work suggests that extracellular matrix components in combination with growth factors/cytokines may be another avenue for formulating more physiological culture systems.
Key words: blastocyst/co-culture/extracellular matrix/growth factor/Matrigel

Introduction

The retarded rates of embryo cleavage, blastulation and inadequate blastomere numbers observed in human blastocysts grown *in vitro* may be attributed to the absence of oviduct and uterine-derived growth factors. Culture of human embryos on somatic cell monolayers has been demonstrated to overcome some of these difficulties and to improve in-vitro development (Bongso *et al.*, 1990). Some of the beneficial effects may be attributed to the release of growth factors into the medium by the co-culture cells. The monkey Vero cell line has been widely used in clinical in-vitro fertilization (IVF) programmes for blastocyst cultivation (Ménézo *et al.*, 1992a,b) and its

secretions well characterized (Papaxanthos-Roche *et al.*, 1994; Kaufman *et al.*, 1995; Desai and Goldfarb, 1996, 1998).

The potential negatives of co-culture during human IVF are exposure of embryos to animal cells/proteins and the possibility of disease transmission. In addition, the co-culture technique is fairly labour intensive and requires tissue culture expertise, and the use of dividing cells can potentially introduce considerable variability into the embryo culture system (Desai and Goldfarb, 1998).

For the above reasons, an alternative to co-culture is highly desirable. Data from several investigations (Carnegie *et al.*, 1995; Piekos *et al.*, 1995) indicate that cultivation of embryos on basement membrane protein coated substrates can enhance aspects of early embryonic development. Culture supplementation with specific growth factors/cytokines may be another approach to further mimic the co-culture environment and influence in-vitro development (Dunglison *et al.*, 1996).

In this study, the potential use of human extracellular matrix (HECM) proteins isolated from human placenta was investigated as an alternative culture system for embryo development. The effect of exogenous interleukin-6 (IL-6) supplementation on embryo growth was also studied. This cytokine has been identified in embryo culture supernatants (Murray *et al.*, 1990; Austgulen *et al.*, 1995) and is also secreted at high concentrations by cell lines with embryotrophic properties (Desai and Goldfarb, 1996, 1998). Yet little is known about this cytokine and its interaction with the preimplantation embryo. Subsequent to the completion of the initial testing on mouse IVF embryos, a pilot study was conducted to determine if this culture system could be applied to human embryos.

Materials and methods

Mouse IVF assay

In order to increase the sensitivity of the bioassay, an IVF model was selected rather than the standard one or two-cell mouse embryo assay often used for evaluation of new culture parameters (Davidson *et al.*, 1988). A critical aspect of this study was the selection of a basal medium, which supported high blastocyst rates with both mouse and human embryos (Desai *et al.*, 1997).

Minimum essential medium alpha (α-MEM; Gibco, Grand Island, NY, USA) supplemented with a plasma protein preparation (5%; Cutter Biological, Elkhart, IN, USA) was utilized for these experiments. Culture medium was supplemented with IL-6 (Biosource; Camarillo, CA, USA) and tested at two concentrations, 500 and 1000 pg/ml. Human extracellular matrix (HECM; Collaborative; Bedford, MA, USA) was coated onto dishes at a concentration of 5.0 or 10.0 μg/ml. Matrix was thawed on ice and pipetted into organ culture dishes. Dishes were allowed to set for 2 h at room temperature

before rinsing with culture medium. Control dishes contained protein-supplemented medium alone.

Six to eight week old female CB6F1 mice purchased from Charles River (Wilmington, MA, USA) were superovulated to obtain oocytes. Animals were injected with 7.5 IU (i.p.) of pregnant mare serum gonadotrophin (Sigma, St. Louis, MO, USA). A second injection, 10 IU of human chorionic gonadotrophin (HCG) (Sigma), was given 48 h later. Mice were killed 16–18 h post-HCG injection by cervical dislocation and their oviducts were excised. Cumuli containing unfertilized oocytes were isolated and placed in organ culture dishes containing pre-equilibrated medium. Dishes were incubated at 37°C in a humidified incubator with 5% CO_2. Spermatozoa isolated from the epididymis of a male CB6F1 mouse (8–10 weeks) were used for oocyte insemination.

Fertilization was determined the following morning by assessing cleavage to the 2-cell stage. The 2-cell embryos were pooled and randomly distributed amongst control and treatment dishes, and incubated at 37°C with 5% CO_2. At 96 h post-insemination, the percentage of 2-cell embryos undergoing blastocyst transformation and hatching was calculated for each treatment group.

In the last experiment, HECM and IL-6 treatment were combined. Each treatment was tested at its maximal concentration (10 μg/ml and 1000 pg/ml, respectively). At termination of the experiment, blastocysts from each treatment group were stained using the Hoechst nuclear staining protocol (Handyside and Hunter, 1984). Cell nuclei were counted with the aid of a computer based image analysis system. The total cell number per blastocyst was recorded.

Human embryo development

A pilot study of this culture system with human embryos was conducted using embryos arising from abnormally fertilized tripro-nucleate eggs from our IVF programme. The use of these triploid embryos for research was approved by our institutional review board. Dishes were coated with 1000 μg/ml of HECM, as described earlier. The basal culture medium was α-MEM with 5% plasma protein preparation. This was further supplemented with IL-6 at a concentration of 1000 pg/ml. Triploid embryos were placed in coated dishes containing the test medium on the day after fertilization. Dishes were incubated at 37°C with 5% CO_2. Development and morphology of embryos was monitored daily over a 5 day interval.

Statistical analysis

Differences in the rates of blastulation and hatching between treatment groups were analysed by the χ^2 test with the Yates correction to determine statistical significance. The Student's t-test was utilized to compare the mean cell numbers per blastocyst between treatment groups. Values of $P < 0.05$ were reported as being statistically significant.

Results

Table I illustrates the response of murine embryos to IL-6 supplementation. These data summarize the results of three separate experiments conducted with 441 murine oocytes fertilized *in vitro*. IL-6 effect on embryos appeared to be dose dependent. At 1000 pg/ml, IL-6 treated embryos exhibited higher blastulation rates than control embryos cultured in medium alone (92 versus 75%, respectively; $P < 0.001$). A two-fold increase in embryonic hatching was also observed (33 and 40% with IL-6 at 500 and 1000 pg/ml, respectively, versus 16% in untreated controls; $P < 0.001$).

In a separate series of murine IVF experiments ($n = 7$),

Table I. Development of murine in-vitro fertilized (IVF) embryos following interleukin-6 (IL-6) treatment

	Control	IL-6 (500 pg/ml)	IL-6 (1000 pg/ml)
Two-cell	126	166	149
Blastocysts	95	137	137
	(75)	(83)	(92)[a]
Hatching	20	45	59
	(16)	(33)[a]	(40)[a]

Blastocyst and hatching rates expressed as percentage of total fertilized (2-cell) embryos cultured.
Values in parentheses are percentages.
[a]Significantly different from control: $P < 0.001$.

Table II. Development of murine in-vitro fertilized (IVF) embryos on human extracellular matrix (HCEM) versus plastic

	Control (uncoated)	HECM (5.0 μg/ml)	HECM (10.0 μg/ml)
n	230	246	243
Blastocysts	133	177	159
	(58)	(72)[a]	(65)
Hatching	87	137	129
	(38)	(56)[b]	(53)[a]

Blastocyst and hatching rates expressed as percentage of total fertilized (2-cell) embryos cultured.
Values in parentheses are percentages.
[a,b]Significantly different from control: [a]$P < 0.01$; [b]$P < 0.001$.

Table III. The combined effect of human extracellular matrix (HCEM) proteins and interleukin-6 (IL-6) on blastulation hatching and total cell number

Treatment	n	Blastocysts (%)	Hatching (%)	Cell number
Control	62	75.8	37.1	82.5 ± 14.3
HECM + IL-6	63	90.5	63.5[a]	131.7 ± 29.7[b]

Dishes coated with HECM (10 μg/ml) and media supplemented with IL-6 (1000 pg/ml). Blastocyst and hatching rates expressed as percentage of total fertilized (2-cell) murine embryos.
[a,b]Significantly different from control: [a]$P < 0.01$; [b]$P < 0.001$.

embryonic development on plastic dishes was contrasted to that achieved on matrix coated dishes. A total of 719 embryos were divided amongst three treatment regimens as shown in Table II. HECM coating was effective at both 5 and 10 μg/ml. A 15% increase in overall hatching rate was observed ($P < 0.01$) after culture of embryos on human matrix proteins (56 and 53% respectively) versus standard culture surfaces (38%). A statistical difference in the blastocyst rate was observed at the lower HECM (5.0 μg/ml) concentration but not at 10 μg/ml HECM.

HECM coating and IL-6 supplementation were combined in the last experiment, and results shown in Table III. The blastulation and hatching rates (90.5 and 63.5% respectively) were higher than those observed in the untreated control dish. Total cell counts performed at termination of the experiment indicated a positive effect of this treatment on blastocyst cell number. The mean cell number in the control group was 82.5 ± 14.3. This was significantly lower ($P < 0.0001$) than

the mean cell count of 131.7 ± 29.7, determined for embryos cultured on extracellular matrix in the presence of IL-6.

The final phase of these experiments was to conduct a pilot study to determine if this culture system could be applied to human embryos. The HECM/IL-6 culture system was tested on tripronucleate human embryos arising from our clinical IVF programme. The number of tripronucleate embryos available for use in testing was limited and for this reason parallel controls were not run in standard culture vessels and medium, and we instead relied on historical controls. In our in-vitro programme, triploid embryos have generally exhibited cleavage rates of greater than 80% with 15–20% blastocyst formation and a 40–50% hatching rate. In this initial trial ($n = 18$), no evidence was found of a direct adverse effect of HECM/IL-6 on human embryo development. With the exception of a single embryo, all cultured triploids continued to cleave. Approximately 39% (7/18) underwent compaction, 22% (4/18) blastulated and 75% of the blastocysts went on to hatch *in vitro*.

Discussion

The modulation of cellular function and development both *in vivo* and *in vitro* is a function of soluble signals such as those derived from growth factors, cytokines and hormones, and insoluble signals arising from the extracellular matrix (ECM) or scaffolding upon which the cells develop. Extracellular matrices consist of collagen matrices to which cells are bound through adhesion proteins such as laminin or fibronectin. Also associated with matrix components are low concentrations of growth factors. The nature and type of extracellular matrix has been shown to contribute to cell differentiation *in vitro* (Martin and Kleinman, 1981; Reid and Jefferson, 1984). The application of matrix proteins to culture systems for growth of embryos is therefore a logical extension in the quest to better simulate the in-vivo culture environment.

Embryo development on fibronectin coated dishes has been compared to that in an oviduct co-culture system (Piekos *et al.*, 1995). Blastocyst development was similar in both treatments and significantly greater than in the control group. Yet hatching and the total cell number per embryo was still greater in the co-cultured blastocysts, suggesting that fibronectin coating alone was not an adequate substitute for co-culture. Matrigel, a commercially available extracellular matrix product, containing collagen IV, laminin, entactin and proteoglycans isolated from mouse tumour cells has also been tested (Carnegie *et al.*, 1995). Development of preimplantation embryos cultivated in conventional culture was contrasted to that of embryos grown on either Matrigel or on Vero cell monolayers. With both matrix and co-culture, in-vitro blastocyst formation and hatching were significantly enhanced over that observed in the medium-alone control. These investigators concluded that Matrigel might be an acceptable alternative to co-culture. Contradictory findings have been reported (Dawson *et al.*, 1997). Their data in fact suggest that the extracellular matrix product might be inhibitory. The variable nature of commercial ECM products may in part account for these findings.

In the present study, an extracellular matrix product isolated from human placenta was chosen. To our knowledge, embryo growth on placenta-derived matrix proteins has never before been tested. It was felt that an extracellular matrix similar in composition to Matrigel but of non-tumour origin might be more desirable should this product later be considered for introduction into the clinical laboratory for human embryo culture. Extracellular matrix was not found to be inhibitory to either mouse or human embryos. The observed positive effect of HECM on mouse blastocyst formation may be related in part to the low concentrations of growth factors associated with the matrix components. While the specific growth factor profile of the placenta-derived HECM has not yet been determined, Matrigel has been shown to contain variable amounts of epidermal growth factor (EGF) (0.5–1.3 ng/ml), platelet-derived growth factor (PDGF) (5–48 pg/ml), insulin-like growth factor-I (IGF-I) (11–24 ng/ml) and transforming growth factor-β (TGFβ) (1.7–4.7 ng/ml) (Mannuzza, 1994). All of these identified factors can influence specific aspects of embryonic development. Cell metabolism and cell proliferation, especially in the inner cell mass of the developing blastocyst, have been shown to be stimulated by IGF-I (Smith *et al.*, 1993). Addition of EGF or TGFβ to mouse embryo culture media enhances in-vitro blastulation (Paria and Dey, 1990).

The most pronounced effect of the HECM was on the hatching rate. One explanation is that the zona hardening phenomenon often associated with in-vitro fertilized embryos is attenuated by culture on a more physiological substratum. Co-culture systems likely provide a similar advantage. Studies with both in-vivo and in-vitro fertilized mouse zygotes have shown that cultivation of embryos on cell monolayers enhances blastocyst hatching (Lai *et al.*, 1992; Desai *et al.*, 1994; Piekos *et al.*, 1995). Human embryos cultivated on bovine oviductal cell monolayers exhibit more zona thickness variation than embryos grown directly on conventional tissue culture dishes (Morgan *et al.*, 1995). This non-uniformity in zona thickness could potentially facilitate embryonic hatching.

Protease activity in the early embryo can also be affected by culture substrata and extracellular matrix components. Embryonic secretion of basement membrane degrading proteases, such as urokinase plasminogen activator and type IV collagenase, is believed to be crucial in the implantation process, allowing penetration of the embryo through the decidua (Turpeenniemi-Hujanen *et al.*, 1995). The effect of extracellular matrix components, fibronectin, collagen type IV and laminin on trophectodermal and inner cell mass cells isolated from sheep blastocysts has been examined (Bartlett and Menino, 1995). The type of ECM was shown directly to influence total cellular outgrowth and secretion of urokinase plasminogen activator. Higher collagenase type IV activity when trophoblasts were cultured with laminin versus on plastic substrata was measured (Emonard *et al.*, 1990). In human embryo cultures, type IV collagenase activity was shown to peak, just prior to implantation and to decrease thereafter (Turpeenniemi-Hujanen *et al.*, 1992). Elevated type IV collagenase activity could be sustained by addition of laminin to the culture system. Laminin also promoted in-vitro hatching of cultured human embryos (Turpeenniemi-Hujanen *et al.*, 1995). All of these data clearly suggest that incorporation of

ECM components into an embryo culture system may serve to augment the implantation process, allowing the embryo to continue with its 'differentiated functions' (e.g. protease secretion) in the in-vitro environment and later upon transfer.

We initially became interested in understanding the role of IL-6 during embryo development because of work in our laboratory characterizing growth factor/cytokine secretion by the Vero cell line used for human embryo co-culture (Desai and Goldfarb *et al.*, 1998). Of the factors secreted, IL-6 was measured in patient co-culture supernatants at relatively high concentrations (300–1000 pg/ml). It was felt important to clarify further the interaction of this factor with the developing embryo. Studies demonstrating the presence of this cytokine in human and mouse embryo culture fluids, along with the detection of mRNA transcripts for IL-6 in mouse blastocysts, all point to an autocrine role for this factor (Murray *et al.*, 1990; Austgulen *et al.*, 1995). In addition, IL-6 shares a common signal transducer molecule with leukaemia inhibitory factor (LIF), a molecule identified to play an important role in the implantation process in mouse (Gearing *et al.*, 1992). Both factors are present in the uterus at elevated concentrations just prior to implantation (Charnock-Jones *et al.*, 1994; Tabibzadeh *et al.*, 1995). IL-6 secretion by endometrial cells is apparently stimulated by interferon-γ (IFN-γ), another immunomodulator found to be critical for pregnancy (Nasu *et al.*, 1998).

Results from this study indicate that the development of in-vitro fertilized oocytes to the hatched blastocyst stage can be modulated by addition of IL-6 to the culture medium. This finding is the first direct evidence of IL-6 influence on preimplantation embryonic development and will require further study. The combination of IL-6 supplementation with extracellular matrix coating of dishes resulted in a culture system that distinctly enhanced at least three parameters of embryonic quality, namely blastocyst formation, hatching, and total cell number per blastocyst. The latter parameter is of especial importance since it is often compromised in embryos developing to blastocysts under suboptimal conditions. Deficiencies in overall blastomere number may hinder proper allocation of cells to the inner cell mass, thereby impairing subsequent development.

Compared to the murine model, human embryo cultivation to the blastocyst stage and production of healthy offspring has been much more of a challenge. Until recently, the best method for obtaining high quality human blastocysts was through the utilization of co-culture techniques. The availability of newer media formulations that better support human blastocyst development may, however, completely eliminate the necessity of co-culture (Forsdahl *et al.*, 1994; Gardner *et al.*, 1998). While initial trials in these new serum-free blastocyst culture media look promising, it still remains to be seen if these media alone can sufficiently support the embryos of all categories of patients. The combination of blastocyst culture media with growth factor and ECM components may prove to be a new avenue for formulating more physiological culture systems.

In summary, the data presented in this study suggest that the design of a culture system for in-vitro embryo development needs not only to examine the basal medium and nutrient requirement of embryos at various cell stages but should also consider the choice of culture substrata and the effect of growth promoting factors. As IVF clinics consider shifting to day 5 transfers with blastocysts, the in-vitro environment will be likely to impact even more on successful pregnancy outcomes. Implantation failure after human IVF may be related to zona hardening and the subsequent inability of transferred embryos to hatch appropriately. This study has identified two culture components that have a striking effect on embryonic hatching, namely IL-6 and human extracellular matrix proteins. In combination, these factors also promoted cell proliferation in the developing embryo.

Despite the positive results presented in this study, it is recognized that contaminating factors associated with the commercial ECM products may ultimately limit their clinical application. Growth factors associated with matrix products can act as either activators or inhibitors, thereby having contradictory effects on differentiation of the blastocyst inner cell mass and trophectodermal compartments. Additional testing of individual matrix components in combination with a variety of growth factors and cytokines should therefore be conducted with the goal of optimizing culture strategies and media formulations. Future studies should include analysis of cell distribution between the trophectoderm and inner cell mass with each treatment. This may ultimately aid in understanding the nature of the interaction between the embryo, the extracellular matrix and associated growth factors.

References

Austgulen, R., Arntzen, K., Vatten, L. *et al.* (1995) Detection of cytokines (interleukin-1, interleukin-6, transforming growth factor-beta) and soluble tumour necrosis factor receptors in embryo culture fluids during in-vitro fertilization. *Hum. Reprod.*, **10**, 171–176.

Bartlett, S. and Menino, A. (1995) Evaluation of extracellular matrices and the plasminogen activator system in sheep inner cell mass and trophectodermal outgrowth *in vitro. Biol. Reprod.*, **52**, 1436–1445.

Bongso, A., Ng, S. and Ratnam, S. (1990) Co-cultures: their relevance to assisted reproduction. *Hum. Reprod.*, **5**, 893–900.

Carnegie, J., Claman, P., Lawrence, C. and Cabaca, O. (1995) Can matrigel substitute for Vero cells in promoting the in-vitro development of mouse embryos? *Hum. Reprod.*, **10**, 636–641.

Charnock-Jones, D., Sharkey, A., Fenwick, P. and Smith, S. (1994) Leukemia inhibitory factor mRNA concentration peaks in human endometrium at the time of implantation and the blastocyst containing mRNA for the receptor at this time. *J. Reprod. Fertil.*, **101**, 421–426.

Davidson, A.V.M., Lobo, R.A. and Paulson, R.J. (1988) Mouse embryo culture as quality control for human *in vitro* fertilization: the one-cell versus the two-cell model. *Fertil. Steril.*, **49**, 516–521.

Dawson, K., Baltz, J. and Claman, P. (1997) Culture with matrigel inhibits development of mouse zygotes. *J. Assist. Reprod. Gen.*, **14**, 543–548.

Desai, N. and Goldfarb, J. (1996) Growth factor/cytokine secretion by a permanent human endometrial cell line with embryotrophic properties. *J. Assist. Reprod. Gen.*, **13**, 546–550.

Desai, N. and Goldfarb, N. (1998) Co-cultured human embryos may be subjected to widely different microenvironments: pattern of growth factor/ cytokine release by Vero cells during the co-culture interval. *Hum. Reprod.*, **13**, 1600–1605.

Desai, N., Kennard, E., Kniss, D. and Friedman, C. (1994) Novel human endometrial cell line promotes blastocyst development. *Fertil. Steril.,* **61,** 760–766.

Desai, N., Kinzer, D., Loeb, A. and Goldfarb, J. (1997) Use of synthetic serum substitute and alpha-minimum essential medium for the extended culture of human embryos to the blastocyst stage. *Hum. Reprod.,* **12,** 328–335.

Dunglison, G., Barlow, D. and Sargent, L. (1996) Leukemia inhibitory factor significantly enhances the blastocyst formation rates of human embryos cultured in serum-free medium. *Hum. Reprod.,* **11,** 191–196.

Emonard, H., Christiane, Y., Smet, M. *et al*. (1990) Type IV and interstitial collagenolytic activities in normal and malignant trophoblast cells are specifically regulated by extracellular matrix. *Invasion Metastasis*, **10**, 170–177.

Forsdahl, F., Bertheussen, K., Bungum, L.J. *et al*. (1994) A study on extended culture time with embryo replacement at the morula and blastocyst stage. *Hum. Reprod.*, **9**, 142–143.

Gardner, D., Vella, P., Lane, M. *et al*. (1998) Culture and transfer of human blastocysts increases implantation rates and reduces the need for multiple embryo transfers. *Fertil. Steril.*, **69**, 84–88.

Gearing, D., Comeau, M., Frerd, D. *et al*. (1992) The IL-6 signal transducer, gp 130: an oncostatin M receptor and affinity converter for the LIF receptor. *Science*, **255**, 1434–1437.

Handyside, A. and Hunter, S. (1984) A rapid procedure for visualizing the inner cell mass and trophectoderm nuclei of mouse blastocysts *in situ* using polynucleotide-specific fluorochromes. *J. Exp. Zool.*, **231**, 429–434.

Kaufman, R., Ménézo, Y., Hazout, A. *et al*. (1995) Cocultured blastocyst cryopreservation: experience of more than 500 transfer cycles. *Fertil. Steril.*, **64**, 1125–1129.

Lai, Y., Stein, D., Soong, Y. *et al*. (1992) Evaluation of Vero cell co-culture system for mouse embryos in various media. *Hum. Reprod.*, **7,** 276–280.

Mannuzza, F. (1994) Growth factor reduced Matrigel basement membrane matrix: a matrix preparation with greatly reduced concentrations of growth factors and proteinases. *Cell*, *Line*, **4**, 1–2.

Martin, G. and Kleinman, H. (1981) Extracellular matrix proteins give new life to cell culture. *Hepatology.*, **1**, 264–266.

Ménézo, Y., Nicollet, B., Herbaut, N. and Andre, D. (1992a) Freezing co-cultured human blastocysts. *Fertil. Steril.*, **58**, 977–980.

Ménézo, Y., Hazout, A., Dumont, M. *et al*. (1992b) Co-culture of embryos on Vero cells and transfer of blastocysts in humans. *Hum. Reprod.*, **7**, 101–106.

Morgan, K., Wiemer, K., Steuerwald, N. *et al*. (1995) Use of videocinematography to assess morphological qualities of conventionally cultured and co-cultured embryos. *Hum. Reprod.*, **10**, 2371–2376.

Murray, R., Lee, F. and Chiu, C. (1990) The genes for leukemia inhibitory factor and interleukin-6 are expressed in mouse blastocyst prior to the onset of hemopoiesis. *Mol. Cell Biol.*, **10**, 4953–4956.

Nasu, K., Nohiko, M., Narahara, H. *et al*. (1998) Effects of interferon-γ on cytokine production by endometrial stromal cells. *Hum. Reprod.*, **13**, 2598–2601.

Papaxanthos-Roche, A., Taupin, J., Mayer, G. *et al*. (1994) Human interleukin for DA cells or leukemia inhibitory factor is released by Vero cells in human embryo co-culture. *Fertil Steril.*, **63**, 648–650.

Paria, B. and Dey, S. (1990) Preimplantation embryo development *in vitro*: cooperative interactions among embryos and role of growth factors. *PNAS*, **87**, 4756–4760.

Piekos, M., Frasor, J., Mack, S. *et al*. (1995) Evaluation of co-culture and alternative culture systems for promoting in-vitro development of mouse embryos. *Hum. Reprod.*, **10**, 1486–1491.

Reid, L.M. and Jefferson, D. (1984) Cell culture studies using extracts of extracellular matrix to study growth and differentiation in mammalian cells. In Mather, J. (ed.), *Mammalian Cell Culture.* Plenum, New York, pp. 239–280.

Smith, R., Garside, W., Aghayan, M. *et al.* (1993) Mouse preimplantation embryos exhibit receptor-mediated binding and transcytosis of maternal insulin-like growth factor I. *Biol. Reprod.*, **49**, 1–12.

Tabibzadeh, S., Kong, Q., Babaknia, A. and May, L. (1995) Progressive rise in the expression of interleukin-6 in human endometrium during menstrual cycle is initiated during the implantation window. *Molec. Hum. Reprod.*, **10**, 2793–2799.

Turpeenniemi-Hujanen, T., Feinberg, R., Kauppila, A. and Puistola, U. (1995) Extracellular matrix interactions in early human embryos: implications for normal implantation events. *Fertil. Steril.*, **64**, 132–138.

Turpeenniemi-Hujanen, T., Ronnberg, L., Kauppila, A. and Puistola, U. (1992) Laminin in the human embryo implantation: analogy to the invasion by tumor cells. *Fertil. Steril.*, **58**, 105–113.