



**MATERNAL-EMBRYO INTERACTIONS AT THE TIME
OF IMPLANTATION IN EARLY PREGNANCY.**

BY

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SUMMARY.

As the fertilized mammalian egg travels down the reproductive tract it spends a short time as an unattached entity and the only connection between the dividing egg and the maternal tissues is the secreted fluid found in the lumen of the oviduct, and as it proceeds into the uterus, the secreted fluid within the uterine lumen. Development before blastulation is independent of the uterine environment, but once the egg becomes a blastocyst it becomes increasingly dependent on the uterine environment for further development. At this point during early development the interactions between the embryo and the maternal tissues are very important and determine whether the implantation of the embryo into the uterine wall occurs, pregnancy is established, and also if embryonic development is to proceed normally.

The aim of this study was to provide a better understanding of the embryo-maternal interactions during the peri-implantation period in early pregnancy. The work reported in this thesis, examined the *in vitro* culture of murine embryos either co-cultured with uterine cells, selected somatic cells or with the addition of growth factors.

In the first series of experiments, the effectiveness of co-culturing 8-cell embryos with a monolayer of mixed uterine cell types in the presence or absence of oestradiol and progesterone, alone and in combination was examined. Co-culture with a mixed uterine monolayer established from enzymatically dissociated uterine tissue increased the numbers of embryos developing in a minimum essential medium beyond the hatching blastocyst stage in the 5-day period of culture to 56.0% (343/613) compared with 30.2% (93/308) for embryos cultured in medium alone. The inclusion of progesterone (3.2×10^{-6} , 3.2×10^{-5} , or 3.2×10^{-4} M) or oestradiol (3.7×10^{-5} M) in the co-cultures increased the mean percentage of embryos developing to the advanced stages to 72.7-78.4%. The addition of progesterone (3.2×10^{-6} M) together with oestradiol (3.7×10^{-5} M) resulted in additional improvement to a mean of 86.5% (787/910, $P < 0.001$) but the combination was without significant effect on embryos cultured in media alone. Blastocyst viability was not impaired by co-culture as assessed by embryo survival following surgical transfer to pseudopregnant

recipients. In order to determine which cell type was responsible for the enhanced embryo survival and implantation in response to the presence of steroids in the culture system, further dissociation of the uterine tissue allowed co-culture of the embryos with epithelial and stromal monolayers. Co-culture of 8-cell embryos on epithelial monolayers in the presence of both steroids increased the numbers of embryos attaining post blastocyst stages of development as effectively as on mixed uterine monolayers. In addition, the culture of embryos on either epithelial or stromal monolayers allowed these embryos to hatch earlier and on stromal monolayers also to attach earlier. This initial series of experiments confirmed the feasibility of establishing a co-culture system to facilitate investigation of pre-implantation events *in vitro* and highlighted a role for steroid hormones in enhancing the capacity of uterine cells to support pre-implantation-stage embryos.

In the second series of experiments, 8-cell murine embryos were co-cultured on a number of selected somatic cell lines or with conditioned media from these monolayers in order to determine whether the enhanced embryo development and survival observed under co-culture conditions was uterine cell specific. The suitability of a number of epithelial (primary amnion and FL amnion) and fibroblastic (L cell and 3T3 cell) cell types as opposed to the uterine monolayer was examined. Co-culture of 8-cell embryos with primary human amnion cell monolayers and L-cell monolayers were equally effective as substrata for embryo development and survival *in vitro* (87.7%, 143/163; 85.8%, 91/106 respectively, $p < 0.01$) to post-hatching stages of development. Furthermore, conditioned media from these cells also enhanced embryo development to the post-hatching stages of development. In contrast the FL amnion cell line and the monolayer of 3T3 cells proved unsuitable substrata for embryo development as did conditioned media from these cell types. From this series of experiments it became apparent that particular cell monolayers such as primary amnion and L-cells act in an embryotrophic capacity to promote embryo growth and development *in vitro*; others such as FL amnion and 3T3 cells were ineffective as suitable substrata for embryo development *in vitro*. The determining factor as to whether a particular cell monolayer was either effective or ineffective as a substratum might reflect the type or the amounts of growth factors that were being produced *in vitro* under the

culture conditions specified, and whether these growth factors and their relative amounts were important to the embryo during its development from the 8-cell stage up to and including the implantation stage.

There is mounting evidence to suggest that a number of growth factors including cytokines and prostaglandins play an important role in embryogenesis particularly during the peri-implantation stage of development. In the third series of experiments, conditioned media from selected cell monolayers as well as from mixed uterine cells, epithelial cells or stromal cells from uterine tissues used for *in vitro* embryo co-culture, were assayed for the production of selected cytokines and prostaglandins. Interleukin-2 and Interleukin-3 could not be detected in any of the conditioned media, indicating very little production of these factors *in vitro*. Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) and Colony Stimulating Activity were evident in conditioned media from uterine cells with conditioned media from uterine epithelial cells showing the highest GM-CSF levels. There was no evidence for a marked increase in production following inclusion of either oestradiol or progesterone or both. The level of Interleukin-6 (IL-6) activity found in uterine epithelial cell conditioned media in the presence of oestradiol and progesterone in combination was 8 fold greater than the level of IL-6 activity seen in mixed uterine cell conditioned media both in the presence and absence of steroids, and the level of activity observed in stromal cell conditioned media was high in the absence of steroids and considerably reduced in the presence of steroids. Prostaglandin production by mixed uterine, uterine epithelial and stromal cell monolayers was examined. Both PGE-2 and PGF₂ α production was evident in conditioned media from mixed uterine cells cultured *in vitro* and that production was primarily by the stromal population and was not steroid dependent.

The fourth series of experiments was undertaken to examine whether the various somatic cell monolayers including mixed uterine monolayers used for co-culture of 8-cell embryos *in vitro* produce Insulin-like Growth Factor-I (IGF-I), Insulin-like Growth Factor-2 (IGF-2) and their associated binding proteins (IGF-BP). All conditioned media assayed showed substantial amounts of IGF-I, IGF-2 and associated IGF-BP's compared to control media. It became evident that the cell lines that provide an embryotrophic environment for

embryos to develop in, produced relatively less amounts of IGF's than FL amnion which was identified as an unsuitable substratum for embryo growth and development *in vitro*.

A number of recent studies have provided evidence that Epidermal Growth Factor (EGF) plays an important role throughout gestation, but there appear to be no reports on the role of EGF in very early events of embryogenesis. The fifth series of experiments was therefore undertaken to examine the possible role of EGF in early embryogenesis, in particular, in the culture/co-culture system established in this study. Following culture of 8-cell embryos in unsupplemented media or in media supplemented with increasing doses of EGF(ng/ml), no significant differences were observed in the rate of development or in the total number of embryos which attained post-hatching stages of development. In addition, no EGF was detected in conditioned media from any of the cell monolayers used for co-culture of 8-cell embryos *in vitro*. From this series of experiments it is concluded that EGF does not play a significant role in the development of 8-cell embryos cultured under the conditions described in this study.

In the sixth and final series of experiments, embryo culture media supplemented with 1000 U/ml E. coli derived recombinant human myeloid leukaemia inhibitory factor (LIF) increased the number of 8-cell murine embryos which developed beyond the *in vitro* hatched blastocyst stage from 64% to 80% ($p < 0.01$), which is comparable to the implantation rates seen in uterine co-culture in the presence of both progesterone and oestradiol. In the presence of LIF a significant number of embryos had commenced hatching by 96 hours post hCG treatment compared with controls (85.1% to 62.1% respectively, $p < 0.005$). Furthermore, 13.5% of LIF-treated embryos had begun to outgrow (implant) in culture as early as 120 hours post hCG treatment compared to none in the controls, and by 144 hours post hCG treatment all of the LIF-treated embryos that were going to implant (85.1%) had done so compared with only 47.0% in the unsupplemented group ($p < 0.001$). LIF-treated embryos also displayed a significantly greater outgrowth of trophoblast than controls as early as day 5 in culture ($p < 0.005$). As no effects were observed when 1-cell embryos were cultured in LIF supplemented media, the embryotrophic effect of LIF appears to be restricted to post 8-cell murine embryos.

Expression of LIF was evident in cultured stromal cells but not epithelial cells from day 3 pregnant uteri, and this expression was not steroid dependent. The data from this series of experiments identify an additional haemopoietic cytokine with embryotrophic activity.

The *in vitro* culture system established in this study clearly supports 8-cell embryo development when embryos are co-cultured with uterine cells in monolayer in the presence of oestradiol and progesterone. Other cells such as primary amnion and L cells in monolayer also support embryo development *in vitro*, whereas, FL amnion and 3T3 cells are unsuitable as monolayers in an embryo co-culture system. From this study it can be concluded that LIF plays an important communicative role between the embryo and the maternal tissues during the pre-implantation period in early embryogenesis.

DECLARATION.

The experimental work described in this thesis was conducted in the Department of Obstetrics and Gynaecology, University of Adelaide, South Australia, during 1986-1990. The data presented are from original studies by the author and any input from colleagues is duly acknowledged. This dissertation has not previously been submitted, wholly or in part, to any other University for any degree or diploma. I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

Tina Christine Lavranos.

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**PUBLICATIONS ARISING FROM INVESTIGATIONS
REPORTED IN THIS THESIS.**

1. Lavranos, T. C., and Seamark, R. F. (1987). Enhanced in vitro survival of mouse embryos co-cultured with uterine cells in the presence of steroids. Proc. Aust. Soc. Reprod. Biol. 19; 14 (Abst.).
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CHAPTER 1.

LITERATURE REVIEW.



1.1 INTRODUCTION.

The fertilized mouse egg travels down the oviduct and enters the uterine lumen; as it does this it also divides and prepares for implantation into the endometrium (Snell, 1941a). While the egg spends approximately 4½ days unattached in the reproductive tract, the only connection between the dividing egg and the maternal tissues is the secreted fluid from initially the epithelium of the oviduct and later the epithelium of the uterus. Early studies using such techniques as the transfer of embryos to ectopic sites (Fawcett, 1950; Kirby, 1962, 1969), *in vitro* microsurgery (Gardner, 1968) and *in vitro* culture (Brinster, 1969; Whitten, 1970) clearly indicated that development before blastulation of the mammalian egg is independent of the uterine environment. Once the egg becomes a blastocyst it becomes increasingly dependent on the uterine environment for further development. From early embryo transfer experiments in the rabbit (Chang, 1950), mice (McLaren and Michie, 1956) and sheep (Rowson and Moor, 1966), synchrony between the embryo and the uterine environment is very important if embryonic development is to proceed normally.

The work reported in this thesis examined the *in vitro* culture of murine embryos either co-cultured with uterine cells, selected somatic cells and their conditioned media, or with the addition of growth factors, in an attempt to expand our understanding of the maternal-embryo interactions during the peri-implantation period in early pregnancy. In this review the areas covered are: (1) biology of implantation, (2) maternal-embryo interactions during early pregnancy, and (3) *in vitro* culture of embryos. This literature review is not comprehensive but is intended as a summary of important developments in the areas mentioned.

1.2 BIOLOGY OF IMPLANTATION.

1.2.1 Introduction.

The establishment of pregnancy is dependent on the successful implantation of the embryo(s) in the wall of the uterus, and assumes that a number of changes have occurred in the embryo including the initial differentiation of the blastocyst into inner cell mass and trophoblast, and also in the uterus including chemical and cytological changes associated with the decidualization and differentiation of the endometrium (reviewed in Wimsatt, 1975; Finn, 1977; Hafez and Ludwig, 1977; Glasser and McCormack, 1981).

1.2.2 Cellular aspects of implantation.

The actual process of implantation begins with the attachment of the trophoblast of the blastocyst to the uterine wall, either invasively or not, depending on the species (reviewed by Finn, 1980) and ends with the formation of the placenta. The initial stage of implantation has been split into two sections, apposition (in which the blastocyst is positioned against the uterine luminal epithelium) and adhesion which involves actual attachment between the trophoblast and endometrium (Enders and Schlafke, 1967). Furthermore, localized stromal changes which result in increases in alkaline phosphatase activity (Finn and Hinchliffe, 1965) and permeability of the vasculature (McLaren, 1969) also occur followed by penetration of the epithelium, either in an intrusive manner (Enders and Schlafke, 1972) through displacement (Gulamhusein and Beck, 1973) or by fusion (Steer, 1971). Decidualization, which occurs during a limited period in pregnancy, and changes in cell proliferation and differentiation, are known to be in response to a large number of stimuli, none of which has as yet been found to be individually responsible for the initiation of this process, although evidence exists to suggest that proteinases are involved (Denker, 1980, 1981). Clearly there is communication between the embryo and

the maternal tissues; it is ambiguous whether the blastocyst triggers the changes that occur or vice versa (review by Renfree, 1980).

1.2.3 Hormonal requirements.

Implantation defines the action by which the early mammalian embryo constructs a fixed relationship with the endometrium. The nature of this process varies considerably between species and includes many events such as activation, positioning of the blastocyst, adhesion of the trophoblast of the blastocyst to the uterine luminal epithelium, invasion of the endometrium and development of the decidual response. Ovarian steroids play an important role in the complex series of synchronised changes in the blastocyst and uterus which result in the successful completion of these events (reviewed by Psychoyos, 1970; Nalbandov, 1971; McLaren, 1973).

There is increasing evidence for direct and indirect hormonal effects on embryonic development. Studies have identified steroidogenic activity by blastocysts in rabbits (Huff and Eik-Nes, 1966), pigs (early review by Heap *et al.*, 1982; Niemann and Elsaesser, 1984), mice (Kirkpatrick, 1971; Antila *et al.*, 1977; Sherman and Atienza, 1977; Wu, 1987), sheep, cows, roe deer, ferrets and cats (Gadsby *et al.*, 1980) and in post-implantation mouse embryos (Carson *et al.*, 1982). During early pregnancy the presence of steroid hormones in the uterine lumen can influence endometrial secretions (Aitken, 1979), assist the preimplantation embryo and can partake in the establishment of pregnancy (Heap *et al.*, 1977).

1.2.3 Role of the immune system.

The pregnant uterus appears to be a privileged immunologic site; in early pregnancy the maternal environment can either accept the embryo or reject it (reviews by Rocklin *et al.*, 1979; Clark *et al.*, 1987; Hill, 1990). Accumulated evidence suggests that hormones (Billingham, 1981) produced during pregnancy play a role in the down regulation of the

local immune response against paternal foreign transplantation antigens which are present on the fetus and also on the trophoblast, thereby allowing the fetus to escape graft rejection (reviews by Wegmann, 1988; Billington, 1989; Harbour and Blalock, 1989). Numerous other mechanisms have also been proposed to describe how this phenomenon occurs, and include synthesis of agents whose role is to maintain an immunosuppressive environment locally, production of immunosuppressive substances by the fetus which act within the maternal blood circulation, and maternal synthesis of agents which have a regulatory capacity within the fetoplacental unit (review by Billingham, 1981). Recently studies strongly suggest a role for lymphokines and cytokines in the establishment and maintenance of pregnancy (Pampfer *et al.*, 1991).

1.3. MATERNAL-EMBRYO INTERACTIONS DURING EARLY PREGNANCY.

1.3.1 Introduction.

In a number of species it has been well documented that embryonic mortality in the preimplantation or early implantation stages varies between 50% in humans (Roberts and Lowe, 1975), and around 30% in cattle (Ayalon, 1978), pigs (Flint *et al.*, 1982) and sheep (Kelly, 1984). With the establishment of *in vitro* fertilization (IVF) programmes, embryonic mortality has been found to be as high as 80% (Trounson, 1984). One way in which we can reduce embryonic mortality in commercially important domestic livestock and in human IVF programmes is to obtain a better understanding of the intricate and complex communication that occurs between the embryo and the maternal environment.

During embryogenesis a number of factors have been identified as mediators of cell-cell interaction and regulators of cell proliferation and differentiation and include, proteins (reviewed by Roberts and Bazer, 1988), haemopoietic growth factors (reviewed by Pollard, 1990; Pampfer *et al.*, 1991), prostaglandins (reviewed by Kennedy and Armstrong, 1981), insulin-like growth factors (reviewed by Bell, 1988, 1989; Daughaday and Rotwein, 1989), epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet derived growth

factor (PDGF), nerve growth factor (NGF), and transforming growth factor (TGF) (recent reviews by Hill et al., 1987; Rizzino, 1987; Brigstock et al., 1989; Pollard, 1990; Simmen and Simmen, 1991). A number of these will be covered in detail in the following sections.

1.3.2 Role of uterine proteins.

From numerous studies it has become evident that the uterus secretes a number of proteins throughout the preimplantation stages of development and beyond. Proteins generally can serve as enzymes, carrier molecules for hormones, growth factors, vitamins and minerals, and are possible regulators of genetic activity and may exhibit a number of functions throughout pregnancy (reviewed by Bazer, 1975). The composition of uterine proteins has been investigated in a number of species including the pig (reviewed by Roberts and Bazer, 1988), rabbit (early review by Beier, 1976; Bochsani *et al.*, 1988), rat (Surani, 1975, 1977a, 1977b; Kuivanen and DeSombre, 1985; Komm *et al.*, 1986), mice (Gore-Langton and Surani, 1976; Aitken, 1976, 1977a, 1977b; Fishel, 1980; Quarmby and Korach, 1984) human (Rutanen *et al.*, 1988; Kivalus *et al.*, 1987), cow (Bartol *et al.*, 1985), and sheep (Salamonsen *et al.*, 1985, 1986; Moffatt *et al.*, 1987). It appears that production of some of the proteins is regulated by ovarian hormones and involved in the control of embryo metabolism and implantation. Recent evidence also suggests that it is not only the uterine tissue at the time of implantation that is secreting proteins but that the embryo is also contributing to the pool of proteins in the local uterine lumen in early pregnancy. For example, in the sheep, one such protein is ovine trophoblast protein-1 (oTP-1) which is secreted by the embryo at the time in early pregnancy when maternal recognition of pregnancy takes place (Godkin *et al.*, 1982). A similar protein has been identified in the cow, bovine trophoblast protein-1, and it has been suggested that this protein has a similar role to oTP-1 in embryo-maternal communication at the time of implantation (Helmer *et al.*, 1987). Early reports on the patterns of protein synthesis by preimplantation mouse embryos suggest that between two-cell and the 8-16 cell stage of development is when most proteins are synthesised and only the rate of synthesis changes

once the embryo enters the uterus (Epstein and Smith, 1974; Van Blerkom and Brockway, 1975). Recent work has shown that mouse blastocysts cultured *in vitro* secrete proteins in to the medium which may be involved with blastocyst development and subsequent trophoblast attachment and outgrowth (Neider *et al.*, 1987; Neider, 1989, 1990; Paria and Dey, 1990). In addition there is evidence that the blastocyst (both cell populations, trophoblastic cells and inner cell mass) also secretes proteins into the blastocoel and these proteins may be involved in the development of the inner cell mass (Dardik and Schultz, 1991). Proteins therefore exhibit a number of functions during early stages of pregnancy and these in some cases are quite species specific but nevertheless essential to the network of maternal-embryo interactions which allow the recognition of pregnancy.

1.3.3 Haemopoietic growth factors.

It has become apparent in recent years that the reproductive system and the immune system work closely together to facilitate conception, prepare the uterus and the developing embryo for implantation and subsequent maintenance of pregnancy. Recent work by Wegmann (1988) has implicated lymphokines/cytokines, glycoproteins that control the production and function of blood-cells, in the regulation of embryonic growth and development. Recently Pollard *et al.* (1987) and Robertson *et al.* (1990) have suggested that granulocyte-macrophage colony stimulating factor (GM-CSF) present in the uterine environment, is embryotrophic. Other studies have shown that GM-CSF is synthesized by decidual cells (Wegmann *et al.*, 1989), and also stimulates ^3H -thymidine uptake by murine placental cells as does interleukin-3 (IL-3) (Athanasakis *et al.*, 1987). There is mounting evidence to implicate a number of other cytokines in the formation and maintenance of extraembryonic tissues during pregnancy (reviewed by Pollard, 1990). Work by Uzumaki *et al.* (1989) has identified receptors for granulocyte-colony stimulating factor (G-CSF) on human trophoblast cells and placental membranes. Furthermore, synthesis of colony stimulating factor-1 (CSF-1) by murine uterine glandular epithelium, regulated by the synergistic action of estradiol-17 β and progesterone, is elevated 10,000 fold during

pregnancy (Pollard *et al.*, 1987). In addition, uterine CSF-1 mRNA expression is seen at day 3, peaking at days 14-15 of pregnancy with CSF-1 mRNA expression evident in decidual cells and trophoblast at day 7.5 (Arceci *et al.*, 1989), suggesting that this growth factor has important functions both before and during implantation and also during the formation of the placenta (reviewed by Daiter and Pollard, 1992). Another member of the cytokine family of growth factors that has been identified as an autocrine/paracrine regulator in the establishment of pregnancy with similar effects to oTP-1 is interferon- α (Salamonsen *et al.*, 1988). Current work has identified the possible involvement of interleukin-6 (IL-6) during gestation. A primary source of IL-6 is uterine epithelial cells prior to and after mating and also later in pregnancy in the mouse (Robertson *et al.*, 1992). This work is supported by the findings of Nishino *et al.* (1990) where human placental trophoblast cells synthesize IL-6 as well as expressing receptors for IL-6. Myeloid leukaemia inhibitory factor (LIF) now recognised as having pleiotropic activities, has been studied extensively with respect to its actions and interactions on normal and leukaemic cells, and only recently been identified as important in the regulation of development (reviewed by Fry, 1992). Current studies have identified expression of LIF in uterine glandular epithelium at the time of implantation and this expression is under maternal control, such that the absence of this expression results in failure of the implantation of the embryos (Bhatt *et al.*, 1991; Shen and Leder, 1992; Stewart *et al.*, 1992). These studies certainly implicate the importance of certain members of the large family of cytokines in various roles throughout pregnancy and it appears that as research proceeds more of these factors may contribute to the maternal-embryo interactions during early pregnancy.

1.3.4 Role of prostaglandins.

Prostaglandin production by the uterus has been reported in a variety of species including the mouse (Rankin *et al.*, 1979), rat (Weems *et al.*, 1975), hamster (Saksena *et al.*, 1974), monkey (Demers *et al.*, 1974), rabbit (Scommegna *et al.*, 1977), ewe (Ellinwood *et al.*, 1979), cow, guinea pig, goat (reviewed by Flint and Hillier, 1975;

Horton and Poyser, 1976) and pig (Watson and Patek, 1979), and is known to play a role as a luteotrophin (eg. $\text{PGF2}\alpha$ in sheep, cattle and goats).

It has been proposed that changes in endometrial vascular permeability are mediated by prostaglandins possibly in conjunction with other mediators, and an increase in endometrial vascular permeability precedes the decidual cell reaction (Reviews by Kennedy, 1980, 1986; Kennedy and Armstrong, 1981). Elevated levels of prostaglandin E (PGE) in rats and hamsters, prostaglandin I₂ (PGI₂) in rats and prostaglandin F (PGF) in rats but not hamsters, at the implantation sites but not in other areas of the uterus have been reported (reviewed by Kennedy and Armstrong, 1981). Prostaglandin F₂ α ($\text{PGF2}\alpha$) induced implantation in rats and mice have also been reported (review by Kennedy, 1980). There appears to be some doubt as to the exact role of each prostaglandin although $\text{PGF2}\alpha$ seems less effective than PGE₂ in inducing the decidual cell reaction. Considerable evidence demonstrates a role for PGE₂; some evidence also suggests a possible role for PGI₂, although there is evidence both for and against a role for $\text{PGF2}\alpha$. In addition there is some evidence to implicate both PGE₂ and PGI₂ in changes in vascular permeability during the inflammatory reaction (reviewed in Kennedy and Armstrong, 1981). Interestingly, more recently work by Low and Hansen (1988) has found that PGE₂ inhibited IL-2 dependent proliferation of lymphocytes and in a concentration-dependent manner and also suppressed PHA- and mixed lymphocyte reaction in ovine and bovine lymphocytes from pregnant animals. They implicate PGE₂ as a local immunoregulatory substance whose role may be to protect the allogenic fetus from immunological attack by the mother.

Early studies have suggested that stimulation of prostaglandin production at the time of implantation is in response to the presence of the blastocyst in close contact with the uterine epithelium (Psychoyos, 1973). More recently others have looked at the possibility that the embryo is another source of prostaglandin production. Shemesh *et al.* (1979) found that bovine blastocysts collected at days 13, 15 and 16, and cultured for 48 hours then frozen prior to assay, produced significantly greater amounts of PGF and PGE₂ than embryos that were not cultured and frozen at the time of collection. Similar results were also obtained in the sheep; a study by Hyland *et al.* (1982) found that ovine embryos

collected at day 14 and cultured for 8.5 hours produced a total (PGF and PGE) of 28 times more prostaglandin than embryos that had not been cultured. In addition they reported that co-culture of embryos with uterine tissue resulted in similar levels of prostaglandins as those produced by embryos cultured alone. Further support is seen from a number of subsequent studies; Davis *et al.* (1983) postulate that prostaglandins are of embryonic origin and may be associated with elongation and migration as well as recognition of early pregnancy in the pig. 8-cell and morula stage murine embryos contain PGE₂ as detected by indirect immunofluorescence and fluorescence diminished when these embryos were cultured in the presence of indomethacin (an inhibitor of prostaglandin production) *in vitro*, suggesting that murine embryos also produce prostaglandins (Niimura and Ishida, 1987). Prostaglandin production by either the embryo or the uterine tissues certainly plays a role in the maternal-fetal interactions in early development and as evidence accumulates it appears likely they may play a more definitive role than just as a luteotrophin.

1.3.5 IGFs and their role in early development.

Insulin-like growth factors (IGFs) and their binding proteins (IGF-BPs) have been found in saliva, lymph, breast milk, amniotic fluid (Reviewed by Sara and Hall, 1990) and IGFs in urine and cerebral spinal fluid (reviewed by Daughaday and Rotwein, 1989). IGFs are like a number of other growth factors, in that the target cells will respond to their presence in a number of ways, either by stimulation of cell proliferation, or stimulation of DNA, RNA or protein synthesis, and the presence of IGF-BPs can modulate the cells' response to the IGFs (reviewed by Baxter, 1988; Sara and Hall, 1990). DNA synthesis and cell proliferation have been stimulated *in vitro* in primary cell lines, established cell lines and organ explants in the presence of IGF-I and IGF-II (reviewed by Sara and Hall, 1990).

The function of IGFs in embryogenesis is yet to be defined. IGF-II has been identified as the major insulin-like growth factor in humans and rats, with both IGF-I and IGF-II receptors present in fetal tissues (Sara and Hall, 1984), and low levels IGF-II mRNA have been found in early organogenesis (Pratten *et al.*, 1988). In addition, explants

of a number of mouse fetal tissues that were cultured in serum-free medium were found to release IGF-I between the 11th and 16th day of gestation; similarly explants of human placenta also released into the conditioned media both IGF-I and IGF-II (reviewed by Daughaday and Rotwein, 1989). A study by Rotwein *et al.* (1987) also found IGF-I mRNA and IGF-II mRNA between days 11 to 14 of gestation in the rat. During earlier development only insulin has been shown to stimulate RNA, DNA and protein synthesis as early as the morula stage of development in the mouse, with both IGF-I and IGF-II having no effect on the embryo at pre-implantation stages of development (Rao *et al.*, 1990). IGF-BPs on the other hand, particularly of the molecular weight 29-35 kilodaltons, have been reported to be the major soluble secretory proteins produced by decidual cells of the endometrium, and it has been postulated that since IGF-BP inhibits IGF-I action, then local elevated levels of decidual IGF-BP could play a role in preventing the trophoblast, which have receptors for IGF-I, from proliferating when in contact with decidual tissue (reviewed by Bell., 1988, 1989). The full function of IGFs and IGF-BPs in early embryogenesis has not been defined as yet, although a number of recent studies have provided evidence that insulin and IGFs play an important role in the preimplantation stages of development (Harvey and Kaye, 1988, 1990, 1991a,b,c).

1.4 IN VITRO CULTURE OF EMBRYOS.

1.4.1 Introduction.

The capacity to culture early embryos *in vitro* in complete and simple media has been significant to the development of a large number of reproductive technologies in current use. However, these complete and simple media are unable to duplicate the oviductal or uterine environments necessary for normal embryo development, and this fact might be reflected in the developmental blocks seen following *in vitro* culture of early stage embryos in numerous species including the cow (Thibault, 1966), pig (Polge, 1982), sheep (Gandolfi and Moor, 1987) and also in some strains of mice (Whittingham and Biggers,

1967). Support for the embryo *in vivo* may be given by either the oviduct cells which may secrete nonspecific compounds (Heyman and Ménézo, 1984) or following stimulation by the embryo may release specific substances (Villalon *et al.*, 1982) allowing normal development to proceed. Once the embryo enters the uterine lumen, additional support to the embryo may be given by the uterine cells through production of specific growth and developmental factors which may act either on the cells in an autocrine fashion to prime or prepare the uterus for implantation or act directly on the embryo. The oviductal and uterine environments are also responsible for the maintenance of the correct pH, ion concentration, amino acids, bicarbonate, and metabolites at different times of the reproductive cycle and conditions of the reproductive tract (reviewed by Beier, 1976).

1.4.2 Culture of embryos in simple media.

The initial report by Whitten (1957) that 2-cell murine embryos could be cultured to blastocysts *in vitro* in a simple medium has since generated extensive investigations into the optimal conditions and nutritional requirements of preimplantation embryos during early development. Early studies were conducted in a number of species including the hamster (Bavister *et al.*, 1983), mouse (Brinster, 1965a, 1965b; Chen and Hsu, 1982), rabbit (Maurer *et al.*, 1969; Kane and Foote, 1971; Ogawa *et al.*, 1971; Kane 1972), human (Edwards *et al.*, 1969; Edwards *et al.*, 1980) and sheep (Walker *et al.*, 1988) to determine suitable culture conditions. A number of culture methods have been employed to study later stages of embryo post-implantation development *in vitro* including explantation of embryos, culture in a watch glass, rotating tubes and bottles and a system in which the embryo(s) is stationary with medium circulating around it (reviewed by New, 1978).

1.4.2.1 Media.

Embryo culture for a short time is crucial to many embryo manipulation and also transfer experiments; the culture media and culture conditions must be optimal to ensure

that the embryo has the best possible chance of survival. The majority of studies have concentrated on the development of media for early stages of development (reviews by Brinster, 1969; Biggers 1987). More recently Quinn *et al.* (1985a,b) developed a culture medium based on human tubal fluid (HTF) which provided optimal conditions for murine embryo development *in vitro*. Bovine embryos have been cultured in simple media such as Dulbecco's phosphate buffered saline (Smith *et al.*, 1986) and complete media such as modified Eagles media, TCM-199, Ham's F-10, Synthetic oviduct fluid, Brinster's mouse ova culture medium, and Whitten's medium, with Ham's F-10 providing the optimal support to embryo development past the blastocyst stage compared with all other media tried (Wright *et al.*, 1976). Others have tried culture of embryos in human amniotic fluid and Whittingham's T6 medium (Gianaroli *et al.*, 1986) for human embryo development *in vitro* prior to IVF with successful results.

Embryo development over the implantation period requires complete media such as Waymouth's media and Eagle's media as support to the attaching blastocyst and for outgrowth of the trophoblast (reviewed by McLaren and Hensleigh, 1975). Development of culture systems to support post-implantation stages of development are less advanced than for early stages and media found to support these later stages of development include McCoy's 5A and Waymouth's media (Steele, 1975).

1.4.2.2 Media supplementation.

The composition of simple media has a profound effect on the success of embryo development while *in vitro* (Whitten, 1970). Media has been supplemented with a protein source such as serum or its albumin fraction (serum albumin). The source of serum varies; commonly used protein supplements include fetal calf serum (Wright *et al.*, 1976; Robl and Davis, 1981; Gandolfi and Moor, 1987; Heyman *et al.*, 1987), bovine serum albumin (Wright, 1977; Quinn *et al.*, 1985a,b), bovine serum (Menino *et al.*, 1985; Rajamadran *et al.*, 1985; Canfield *et al.*, 1986; Blum *et al.*, 1987), lamb serum (Herr and Wright, 1986), human cord serum (Bates *et al.*, 1985), sheep serum (Moore, 1970; Moor and Cragle,

1971; Moore and Spry, 1972; Robl and Davis, 1981) and rabbit serum (Herrmann *et al.*, 1981). While the concentration of sera in these studies varies from 10-20%, bovine serum albumin concentration varied between 0.05 to 64mg/ml. Others have also tried supplementation of media with uterine fluid/flushings from human (Aitken and Maathuis, 1978), rabbit and rat (Lambert *et al.*, 1981) and bovine uterine fluid (Menino *et al.*, 1985), although a number of reports have implied that uterine fluids may contain factors which could be toxic to the embryos (Lambert *et al.*, 1981; Psychoyos and Casimiri, 1981).

1.4.2.3 Atmospheric conditions.

Early work by Whitten (1971) established the optimal oxygen concentration for one-cell and two-cell mouse embryos cultured in simple media *in vitro* to be 5%O₂, 5%CO₂ and 90%N₂. Work by Quinn and Harlow (1978) subsequently demonstrated that blastocysts developing in a gas atmosphere greater than 5%O₂ had fewer blastomeres, and the rate of development declined considerably. In addition others have shown that 5%O₂ is also optimal for the culture of cow embryos (Wright *et al.*, 1976) and sheep embryos (Tervit *et al.*, 1972), although a recent report seems to be contradictory and suggests that increasing oxygen concentration to 20% does not effect ovine embryo development (Betterbed and Wright, 1985). Atmospheric conditions may have an indirect role in the microenvironment in which the embryo develops *in vitro*, and possibly influence embryonic responsiveness to developmental cues.

1.4.2.4 Energy substrates.

The major energy source for developmental stages beyond the 8-cell stage has not been extensively studied although for mouse *in vitro* culture, pyruvate has been found to be the major energy substrate (Brinster, 1969). Early studies have found that utilization of energy substrates changes during development *in vitro* (Brinster, 1965b,c). Pyruvate, lactate and glucose are constituents of most simple media (reviewed by Kaye, 1986). Variations on

the concentrations of these substrates seem to vary according to the culture media used, for example, Pomp *et al.* (1988) found a lower concentration of sodium lactate in Whitten's medium was beneficial in supporting early murine embryonic development.

1.4.2.5 Addition of growth factors.

It is widely accepted that the uterus provides a complete but complex environment for embryo development. A large number of studies (reviewed by Hill *et al.*, 1987 and Brigstock *et al.*, 1989) have indicated that many growth factors, for example, platelet-derived growth factor and fibroblast growth factor are present in the uterine environment and appear to be important to the attachment and implantation of the embryo and the growth and development of the embryo and foetus. Recent studies have investigated the effects of media supplementation with growth factors. For example, work by Menino *et al.* (1989), who examined the effects of supplementing culture media with various growth factors (T-cell growth factor TCGF, marrow cell stimulator MCS, fibroblast growth factor FGF, endothelial cell growth factor ECGF, nerve growth factor 2.5S and 7S NGF2.5S NGF7S, multiplication stimulating activity MSA, and liver cell growth factor LCGF) on *in vitro* 2-cell mouse embryo development indicated that some factors (MCS > NGF7S > ECGF > MSA) enhanced embryo hatching better than others. Menino and O'Claray (1986) cultured 2-cell mouse embryos *in vitro* in Whitten's medium supplemented with either plasmin or plasminogen and found that attachment and trophoblastic outgrowth were enhanced in these groups compared to unsupplemented media.

Other groups have also studied the effects of adding growth factors to embryo culture, for example addition of insulin to *in vitro* culture resulted in increased rates of development of preimplantation embryos, (Gardner and Kaye, 1986), rates of compaction and blastocyst formation (Gardner and Kaye, 1991) and stimulation of protein synthesis in morulae, blastocysts and expanded blastocysts (Harvey and Kaye, 1988). Addition of transferrin and selenite with insulin increased DNA synthesis in hatching blastocysts (DeLuca and Lopata,

1988). The supplementation of culture media has been restricted to studies on very early embryo development; little has been done on post 8-cell stages of development.

1.4.3 Co-culture of embryos *in vitro*.

1.4.3.1 Cells from the reproductive tract.

The use of co-culture was first reported by Biggers *et al.* (1962) in which mouse embryos were cultured with oviduct tissue. Later studies also examined the benefits of culturing embryos with a selection of cells from the reproductive tract including oviduct epithelium (Eyestone *et al.*, 1987; Gandolfi and Moor, 1987; Rexroad and Powell, 1986, 1988a,b; Sakkas *et al.*, 1988, 1989; Eyestone and First, 1989; Sakkas and Trounson, 1990; McCaffrey *et al.*, 1991), oviducts in organ culture (Whittingham, 1968; Northey and Leibfried-Rutledge, 1985; Kirher *et al.*, 1989), uterine cells (Salomon and Sherman, 1975; Van Blerkom and Chavez, 1981; Allen and Wright, 1984; Voelkel *et al.*, 1985; Khurana and Wales, 1987; Lindenberg *et al.*, 1988) and trophoblastic vesicles (Camous *et al.*, 1984; Heyman and Ménézo, 1984; Heyman *et al.*, 1987; Rexroad and Powell, 1988a). Most of these studies have shown that co-culture with cells from the reproductive tract has enhanced embryo development *in vitro*.

1.4.3.2 Other cells.

One of the earliest reports on the use of established cell lines as a suitable matrix for embryo development *in vitro* was by Cole and Paul (1965). They showed that mouse embryos could develop to post-implantation stages of development and more closely resembled *in vivo* embryos at similar developmental stages than embryos cultured *in vitro* in simple media. More recently work by Ouhibi *et al* (1990) examined the effect of co-culturing murine embryos with MDBK (bovine kidney) cells and observed high rates of blastocyst formation. Similar results were seen when Vero (Green monkey kidney) cells

were co-cultured with human embryos and significant improvement in embryo development was seen (Ménézo *et al.*, 1990), and also when mouse embryos were co-cultured with either hamster hepatocyte cells, hamster liver fibroblast cells or transformed mouse fibroblast cells (L cells) (Overskei and Cincotta, 1987), embryo development was enhanced compared to simple media. It is possible that these different cell types secrete non-specific compounds or are stimulated by the presence of the embryo to release specific peptides or factors which act in a beneficial manner to the embryo. Co-culture may also help in the identification of factors which may be present in the uterine environment but do not contribute to the early stages of embryonic development. For example, a study by Schneider *et al.* (1989) showed that co-culture of 4-cell murine embryos with activated human peritoneal macrophages (produce IL-1) did not effect embryo development. A culture system which uses established cell lines is convenient and repeatable in comparison to the use of primary cell lines, and in addition provides the investigator with the opportunity to identify unique growth factors or substances which could play a significant role in embryo development at different stages of gestation.

1.4.3.3 Conditioned media from various cells.

There is mounting evidence to suggest that the oviduct and uterus produce proteins and growth factors such as cytokines (reviewed by Pampfer *et al.*, 1991), or improve the environmental matrix possibly by removal of limiting metabolic products or by modification of inhibitory substances, for example hypoxanthine which is known to induce a 2-cell block in mice (Loutradis *et al.*, 1987). Production of these various substances might be stimulated by the presence of the embryo or in response to steroids. Culture and co-culture of cells from the reproductive tract *in vitro* has provided further evidence as to the possible nature of these substances. Consequently a number of studies have extended these initial observations by using conditioned media, (ie media cultured with cells for a specific period of time after which the cells are removed) to gain insight into the exact nature of the soluble secreted products of the cells. One study by Sakkas *et al.* (1987) investigated the use of

conditioned culture media from mouse and sheep oviduct explants and from sheep epithelial monolayers to culture 2-cell murine embryos to the blastocyst stage. They found that a significantly higher proportion of embryos reached the 8-cell stage of development when cultured in conditioned media than in non-conditioned media, and subsequently identified the consistent presence of three secretory proteins in all three conditioned media examined. Furthermore they concluded that conditioned media used in their culture system clearly enhanced embryo development. Other workers have confirmed these findings. More recently another study analysed the effect of culturing early bovine embryos with oviduct-tissue conditioned media, and their results demonstrated that conditioned media supported embryo development to post-blastocyst stages of development, and have also shown that a single freeze thaw of their conditioned media did not affect the stability and ability of the conditioned media to support embryo development (Eyestone and First, 1989; Eyestone *et al.*, 1990, 1991).

1.4.4 Manipulation of cultured embryos.

1.4.4.1 Introduction.

The use of various culture systems aids the reproductive biologist, interested in a better understanding of normal/abnormal reproductive physiology, the agricultural scientist, interested in improving health and reproductive performance of animals exhibiting economically viable traits, and clinicians, involved in treating infertility, in accomplishing their aims.

Complex technologies have been developed which allow extensive manipulation of early embryos. One area of research has been involved with the extensive and successful use of chimeras to investigate a number of questions involved with development (LeDouarin and McLaren, 1984). In addition, genetic engineering has been utilized to produce transgenic animals which will most certainly revolutionize livestock breeding (reviewed by Seamark, 1989). Furthermore, routine use of *in vitro* fertilization programmes

and related procedures aid infertile couples to conceive and establish pregnancy (Steptoe, 1980). All of these technologies rely on short-term *in vitro* culture of early embryos, which allows the evaluation of manipulated embryos before they are committed to the host.

1.4.4.2 Chimeras.

The fate of certain cells and the potential of cells to commit to certain cell lineages has been comprehensively studied with the use of chimeras. The mouse predominantly has been used as a model (reviewed by Rossant, 1984) although work with a number of other species such as the rat, sheep, rabbit (reviewed by Papaioannou and Dieterlen-Lievre, 1984), cows (Summers *et al.*, 1983) and goats (Fehilly *et al.*, 1984; Meinecke-Tillman and Meinecke, 1984) have verified and expanded early work on the mouse. For example, studies have investigated regulation of cell numbers in early embryo development in the rat (Mayer and Fritz, 1974), rabbit (Gardner and Munro, 1974), and mouse (Buehr and McLaren, 1974; Rand 1985), and cell determination of the trophectoderm and inner cell mass in the mouse (Rossant *et al.*, 1982), and in interspecific chimeras of sheep and goat (Polzin *et al.*, 1986).

1.4.4.3 Transgenics.

Experimental manipulation of the embryo prior to short term culture followed by transfer to the host, can include single gene modifications, microinjection prior to first cell division and integration of the gene prior to cell differentiation and can improve the genetic makeup of animals especially domestic livestock. The mouse, as a model, has been utilized for the development of these techniques in the production of transgenic animals (reviewed by Allen *et al.*, 1987). Economically viable traits in domestic species that have been improved using transgenic technology include wool growth (Ward, 1982), milk quality (Lathe *et al.*, 1986; Simons *et al.*, 1987) litter size (Piper and Bindon, 1985; Jonmundsson and Adelsteinsson, 1985) and growth hormone production (Woychik *et al.*, 1982). Progress

in this field of research appears to be endless and is only limited by the constraints of the time taken to continue the development of transgenic technology.

1.4.4.4 IVF and related programmes.

Treatment of human infertility has changed enormously since the development and routine use of *in vitro* fertilization (IVF) programmes and related fertility programmes. Essential to IVF and some of the other programmes is the *in vitro* culture of early stage embryos (Stephoe, 1980; Kerin *et al.*, 1984a). Constant improvement of culture conditions (Quinn *et al.*, 1984b, 1985a, 1985b) aims at consistency in pregnancy rates. Human tubal fluid (HTF) medium was developed and is optimal for support of preimplantation embryos (Quinn *et al.*, 1985a, 1985b). Constant research in this area examines other suitable media for embryo culture. Examples of this is the use of human amniotic fluid (Gianaroli *et al.*, 1986) and other culture techniques such as tube culture and organ culture (Marrs *et al.*, 1984) for increased success rates in established *in vitro* fertilization and embryo transfer programmes.

1.5 DISCUSSION.

As yet the ability to reduce the high embryonic mortality seen in the preimplantation or early implantation stages of development of manipulated embryos has not been attained following culture *in vitro*. This implies that the culture systems that have been developed are possibly limiting, especially in the peri-implantation stages of development, and further indicates that researchers do not have a complete understanding of the complex communication between the embryo and the maternal tissues.

The aim of this study was to provide a better understanding of the embryo-maternal interactions during the peri-implantation period in early pregnancy. In order to do this it was necessary firstly to establish a culture system for 8-cell embryos which would mimic the *in vivo* environment, and the study described in chapter 2 examines the effectiveness of

co-culturing murine 8-cell embryos with a monolayer of mixed uterine cell types from day 3 pregnant mice. In view of the importance of steroid hormones in stimulating the production of factors by the uterine cells (reviewed here) the addition of oestradiol and progesterone alone and in combination was also examined. Further dissociation of the uterine tissue enabled the investigation of cell specific support by the different uterine cell types for pre-implantation embryos *in vitro*.

Since others have used somatic cell types for culture of embryos (reviewed here), it was of interest to co-culture 8-cell murine embryos on a number of selected somatic cell lines in order to determine whether the enhanced embryo development and survival observed under co-culture conditions was uterine cell specific, (described in chapter 3). This was done either with primary cell culture or with the use of established cell lines. Conditioned media from all cell monolayers used for embryo co-culture were also examined for their ability to support embryo development. The ability to support embryo development might give some indication as to whether the cells were producing a soluble factor in response to the steroid hormones as in the case of the uterine cells or some other factor that was intrinsic to the particular cell type.

The review indicates that prostaglandins, a number of cytokines and insulin-like growth factors and their binding proteins contribute to the preparation of both the embryo and the uterine environment and also to early developmental events leading up to and including implantation and also recognition of early pregnancy. The studies described in chapters 4 and 5 examine the identity of the factor(s) made available to the embryo in the co-culture system.

Finally since culture conditions for embryos from the 8-cell stage onwards are still relatively undefined with respect to the role of a number of growth factors and in view of the vast studies conducted on earlier embryos as discussed in this review, it was necessary to include a number of growth factors. Those tried were EGF (Chapter 6) and LIF (Chapter 7), each growth factor was added to the culture system in use, without the support from a cellular substrata, in order to investigate whether the support seen from steroid hormone stimulated uterine cells or other supportive cells could be mimicked by one factor, and

subsequently or concurrently identify the production of these growth factors *in vitro* by the different cell types used for co-culture.

The network of signals that occur between mother and embryo at the peri-implantation stage of pregnancy is so extensive that one study could not cover them all. Thus within the study presented here, a small number of growth factors was selected and their importance in an *in vitro* culture system addressed.

CHAPTER 2.

**CO-CULTURE OF MURINE EMBRYOS: ADDITION OF OESTRADIOL AND
PROGESTERONE TO EMBRYO-UTERINE MONOLAYER CO-CULTURE
ENHANCES EMBRYO SURVIVAL AND IMPLANTATION *IN VITRO*.**

2.1 INTRODUCTION.

The ability to culture early mammalian embryos *in vitro* in simple defined media has been centrally important to the development of the wide range of advanced reproductive technologies in present use. However, maintenance of embryo viability in these simple media, when culture periods are extended beyond a few days, is still uncertain even in familiar laboratory species such as the mouse (Brinster, 1972; Hsu, 1978,1979).

Recently there have been several reports indicating that embryo development *in vitro* can be improved if embryos are co-cultured with selected somatic cells; these include studies from the rat (Glasser and McCormack, 1980), pig (Kuzan and Wright, 1982a; Allen and Wright, 1984), cow (Voelkel *et al.*, 1985; Kuzan and Wright, 1982b) and sheep (Rexroad and Powell, 1986; Gandolfi and Moor, 1987). The nature of the support given by somatic cells to the embryo is unknown but may incorporate provision of an improved environmental matrix, through making available specific growth and developmental factors or by removal of limiting metabolic products.

As a basis for *in vitro* studies on the pre-implantation embryo I have examined the utility of a simple mixed uterine cell monolayer as a support for the development of eight-cell murine embryos *in vitro*. A significant role for progesterone and oestradiol supplementation of the media to enhance survival in the co-culture system was identified.

2.2 MATERIALS AND METHODS.

2.2.1 Animals.

Balb-C x C57 3-4 week old F1 female mice were primed with 7.5 i.u. PMSG (Folligon; Intervet, Australia), followed 48 hours later with 7.5 i.u. hCG (Chorulon; Intervet, Australia) to achieve superovulation. Immediately following the hCG injection, treated females were placed with fertile males (CBA x C57 strain, one female plus one male per cage). The next morning each female was checked for the presence of a vaginal plug as evidence of mating (Snell, 1941b). This was then considered as day 1 of pregnancy. In

embryo transplantation experiments, recipients were prepared by placing females with vasectomized males (CBA x C57 strain, two females plus one male per cage). The following morning each female was checked for the presence of a vaginal plug. This was then considered as day 1 of pseudo pregnancy.

2.2.2 Media.

The culture medium was prepared from powdered Minimal Essential Medium (MEM; Eagle, with Earle's salts, with L-glutamine without sodium bicarbonate; Flow Laboratories, U.K.) dissolved in Milli-Q water and supplemented with 25 mM sodium bicarbonate and 10% (v/v) heat-inactivated fetal calf serum (FCS; CSL, Australia). An antibiotic-antimycotic solution was also added to provide per 100mL of solution:

10 000 units penicillin, 10 000 μg streptomycin and 25 μg fungizone (CSL, Australia). The pH and osmolarity of the media were adjusted to 7.40 and 280 mOsm respectively. At this point the medium was sterilized by filtration (Acrodisc 0.2 μm filter; Gelman Sciences Inc., U.S.A.).

In some experiments the medium was supplemented by addition of either or both of the following steroids: (1) oestradiol (1,3,5(10)-oestratrien-3 β ,17 β -diol, Steraloids Inc., U.S.A.); (2) progesterone (4-pregnen-3,20-dione, Steraloids Inc., U.S.A.); the concentration of each varied according to the experiment.

2.2.3 Embryos.

On Day 3 of pregnancy females were killed between 1300 and 1500 hours, i.e. 71-73 hours post-hCG injection, by cervical dislocation. The whole reproductive tract was dissected out and placed in Earle's balanced salt solution without calcium and magnesium (EBSS) at 37°C. Subsequently, eight-cell embryos were teased or flushed out of the oviduct-uterus junction (Pratt, 1987) and after being washed once in culture medium were

placed into the control or the experimental group (see below) and maintained in a humidified gas environment of 5% CO₂ in air, at 37°C.

2.2.4 Uterine Monolayers.

The procedure for preparation of mixed monolayers from whole uteri, described previously by Salomon and Sherman (1975), was modified as follows: after collection of the embryos from day 3 pregnant females, uteri were dissected free of remaining oviductal and fatty tissue and washed twice in EBSS at 37°C under sterile conditions; they were then cut into smaller pieces (approximately 3mm long) and placed in a petri dish containing 0.5% trypsin and 0.02% EDTA in EBSS (EBSS-T-E), for 15 minutes at 37°C. The enzyme solution was then replaced with fresh enzyme solution, the uterine tissue dissociated by mincing with sterile scissors for about 1 minute and the mixture incubated at 37°C for 30 minutes. After the second enzyme treatment, heat-inactivated FCS (5%) was added and the cell suspension centrifuged at 500g for 20 minutes, the supernatant discarded and the pellet resuspended in culture medium supplemented with steroid hormone, where indicated. Cells were plated out into four-well multidishes (Nunc, W. Germany) at 1.3×10^6 cells per well and the dishes placed in an incubator at 37°C, 5% CO₂ in air for 24 hours prior to the addition of the embryos collected from other mice. The growth of the mixed uterine cells at this concentration was sufficient to achieve confluence during this initial period.

2.2.5 Epithelial and stromal monolayers.

Whole uteri from day 3 pregnant female mice were used to prepare epithelial and stromal cell monolayers. The procedure used was a modification of the protocol detailed by Sherman (1978), and can be described as follows: individual uteri were trimmed free of any oviduct, fat, mesentery and blood vessels, slit lengthwise to expose the luminal surface and washed twice in cold phosphate buffered saline (PBS) pH 7.2, within 10-15 minutes of collection, under sterile conditions. Uteri were then placed in a tube containing 0.5%

trypsin, 2.5% pancreatin (bovine pancreatic, type III, Sigma) in PBS (1 uterine horn per ml) for 1 hour on ice followed by 1 hour at room temperature. Approximately 10% MEM containing 10% FCS was added to the final volume of enzymatic solution. The mixture was then agitated by passage up and down through a sterile plastic pipette for a minute. The supernatant which contained epithelial (approximately 95% of cells) and other detached cells was centrifuged at 200g for 10 minutes, the cell pellet was washed once in MEM. The stromal population was obtained from the remaining tissue by placing it into a petri dish containing EBSS-T-E (1 uterine horn per ml) for 45 minutes at 37°C. As for the epithelial fraction, MEM+FCS was added, the mixture was similarly agitated and the supernatant containing the stromal cells was pelleted at 200g for 10 minutes, and subsequently washed once in MEM. Both the epithelial and stromal cells were plated out in MEM supplemented with oestradiol ($3.7 \times 10^{-5}\text{M}$) and progesterone ($3.2 \times 10^{-6}\text{M}$) at a concentration of 1.0×10^6 cells per well in four well multidishes, and incubated for 24 hours at 37°C in 5% CO₂ in air, prior to the addition of embryos.

2.2.6 Culture of embryos.

For experimentation, eight-cell embryos were randomly assigned to a control or experimental group, with each group consisting of three-eight replicates, with embryos from four to six mice per replicate. Embryos were added 10-20 per well, 2-10 wells per replicate, approximately 15-20 minutes after being recovered from the oviduct-uterus junction and were maintained *in vitro* for a period of 5 days in wells containing the culture medium alone (1mL per well) or the culture medium and the mixed uterine, epithelial or stromal cell monolayer with or without steroid supplementation as indicated.

2.2.7 Assessment of morphological development.

Observations on embryo development were made daily using an inverted microscope, and the numbers of embryos achieving morula, blastocyst or hatching

blastocyst stage were recorded (Hsu, 1979). On days 4-5 of culture many embryos underwent developmental changes associated with implantation (Sherman, 1978). For this study post-hatching embryos were recorded as achieving stage 1 when they displayed proliferating trophoctoderm cells and stage 2 when they showed outgrowth of trophoctoderm cells on the bottom of the culture vessel when cultured in medium alone or invasive outgrowth when cultured with the uterine monolayer (examples of blastocysts and implanting embryos in co-culture can be seen in Figure 2.1).

2.2.8 Uterine transfers.

Once collected, 8-cell embryos were cultured in either media alone supplemented with both progesterone and oestradiol (controls) or co-cultured with mixed uterine monolayers supplemented with progesterone ($3.2 \times 10^{-6}\text{M}$) and oestradiol ($3.7 \times 10^{-5}\text{M}$) until the expanded blastocyst stage (3 days in culture). The embryos were then surgically transferred to 6-12 Day 2 pseudopregnant recipient females with 3-4 embryos each uterine horn. Uterine horns were scored on Day 15 for the number of implantation sites and the number of foetuses.

2.2.9 Statistics.

Experimental conditions for replicate experiments were kept as identical as possible, with variability between wells being less than 10%, and between replicates less than 15%. All results presented here were analysed using Chi-square tests, with $p < 0.05$ as significant.

2.3 RESULTS.

2.3.1 Control of embryo culture.

Of a total of 308 eight-cell embryos cultured in control wells in medium alone, 214 (69.5%) developed normally to the blastocyst stage following 2 days in culture, with 178 (57.8%) hatching by 3 days in culture. Of these, 93 (30.2%) continued the development to stage 2 following 5 days in culture.

2.3.2 Embryo co-culture with mixed uterine cell monolayer.

Embryos cultured in the medium but on a mixed uterine cell monolayer showed increased developmental capacity, with 465 of the 613 embryos established in co-culture (75.9%) forming blastocysts and 343 (56.0%) implanting (stage 2) within the uterine monolayer. Survival and development of embryos in co-culture was further enhanced by the inclusion of steroids in the medium. Progesterone at all dosages tested (3.2×10^{-6} , 3.2×10^{-5} and 3.2×10^{-4} M)-markedly enhanced survival to all stages of development (Table 1.). Oestradiol at a concentration of 3.7×10^{-5} M also had an enhancing effect, whereas oestradiol at dosages of 3.7×10^{-6} and 3.7×10^{-4} M was ineffective, indicating that the oestradiol effect may be dose dependent (Table 2.). A preliminary experiment to investigate the combination of progesterone (3.2×10^{-6} M) with oestradiol (3.7×10^{-5} M) indicated that the steroids may act synergistically to enhance embryo survival, as 809 of the 910 embryos co-cultured with the combined steroid developed to the hatched blastocyst stage with 787 (86.5%) implanting (stage 2) in the uterine monolayer; no significant effect on the survival or development of the embryos cultured in steroid supplemented media (no cells) was evident (Table 3). This was confirmed in a second experiment investigating the effects on embryo development of adding varying amounts of progesterone (3.2×10^{-6} , 3.2×10^{-7} and 3.2×10^{-8} M) in conjunction with oestradiol (3.7×10^{-5} M) to culture medium (Table 4.). The response to steroid hormones was only evident in co-cultures as the inclusion of

steroid hormones (progesterone 3.2×10^{-6} to 3.2×10^{-8} M and oestradiol 3.7×10^{-5} M) had no significant effect on the survival or development of the embryos cultured in medium alone (Table 4.).

Table 1. Development of embryos on uterine monolayers with increasing concentrations of progesterone in the media.

| Developmental Stage | Progesterone (M) | | | |
|---------------------|------------------|----------------------|----------------------|----------------------|
| | 0 | 3.2×10^{-6} | 3.2×10^{-5} | 3.2×10^{-4} |
| 8-cell* | 613 | 422 | 412 | 395 |
| Morula | 89.4 | 97.9 | 94.7 | 95.2 |
| Blastocyst | 75.9 | 90.3 | 89.6 | 88.4 |
| Hatched Blastocyst | 68.0 | 86.0 | 84.0 | 83.3 |
| Post-hatch Stage 1 | 62.2 | 78.9 | 78.2 | 72.7 |
| Post-hatch Stage 2 | 56.0 | 78.4** | 77.7** | 72.7** |

* Number of 8-cell embryos. All other values expressed as a percentage of initial numbers of 8-cell embryos cultured which developed to the stage cited.

** Significantly different to control group, but not significantly different between treatment groups. $p < 0.01$, Chi-square.

Figure 2.1. *In vitro* co-culture of murine embryos on mixed uterine monolayers.

- (a) Seven murine blastocysts in co-culture with a uterine monolayer, one blastocyst has begun to hatch (arrow).
- (b) One murine embryo implanting (designated stage 2) within the uterine monolayer, approximately day 5 in culture, * indicating ICM and ▲ indicating trophoblast which has displaced the uterine cells.

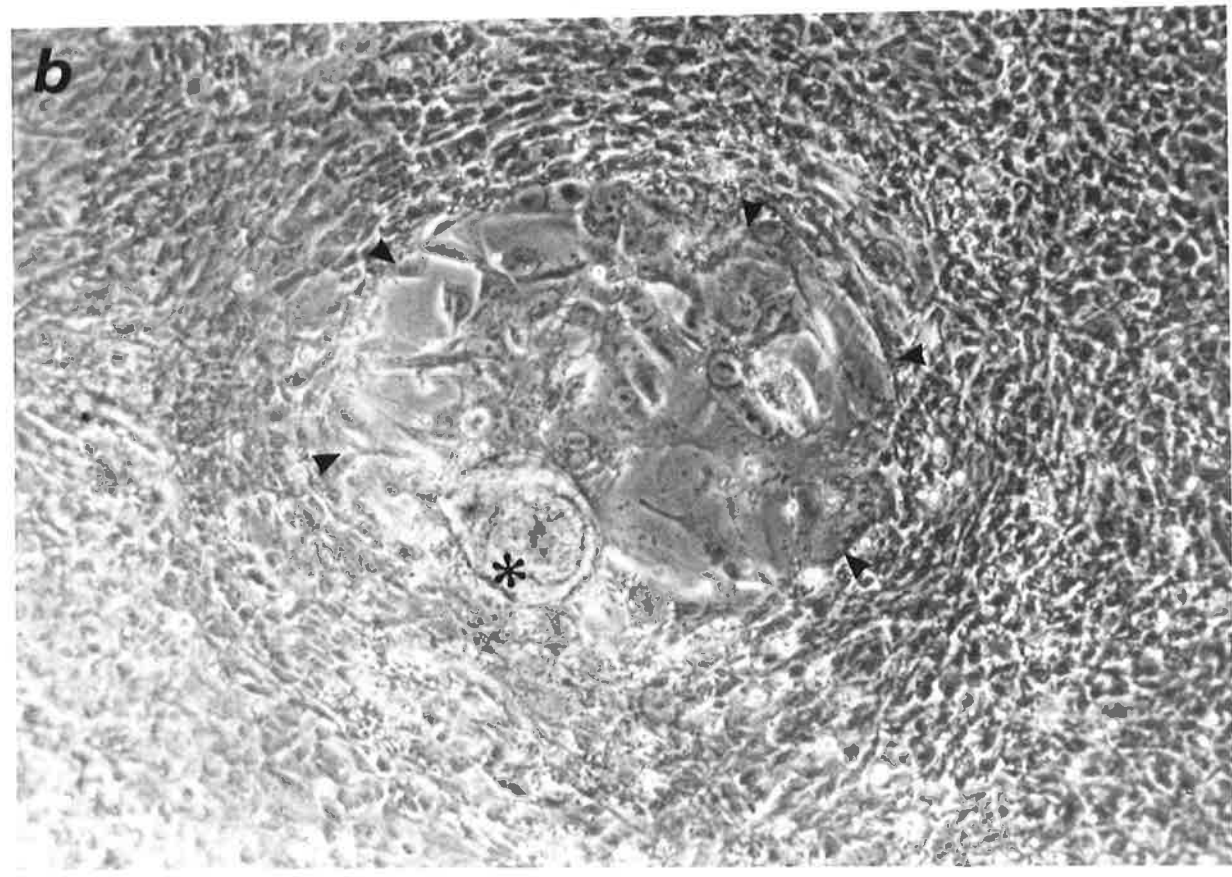
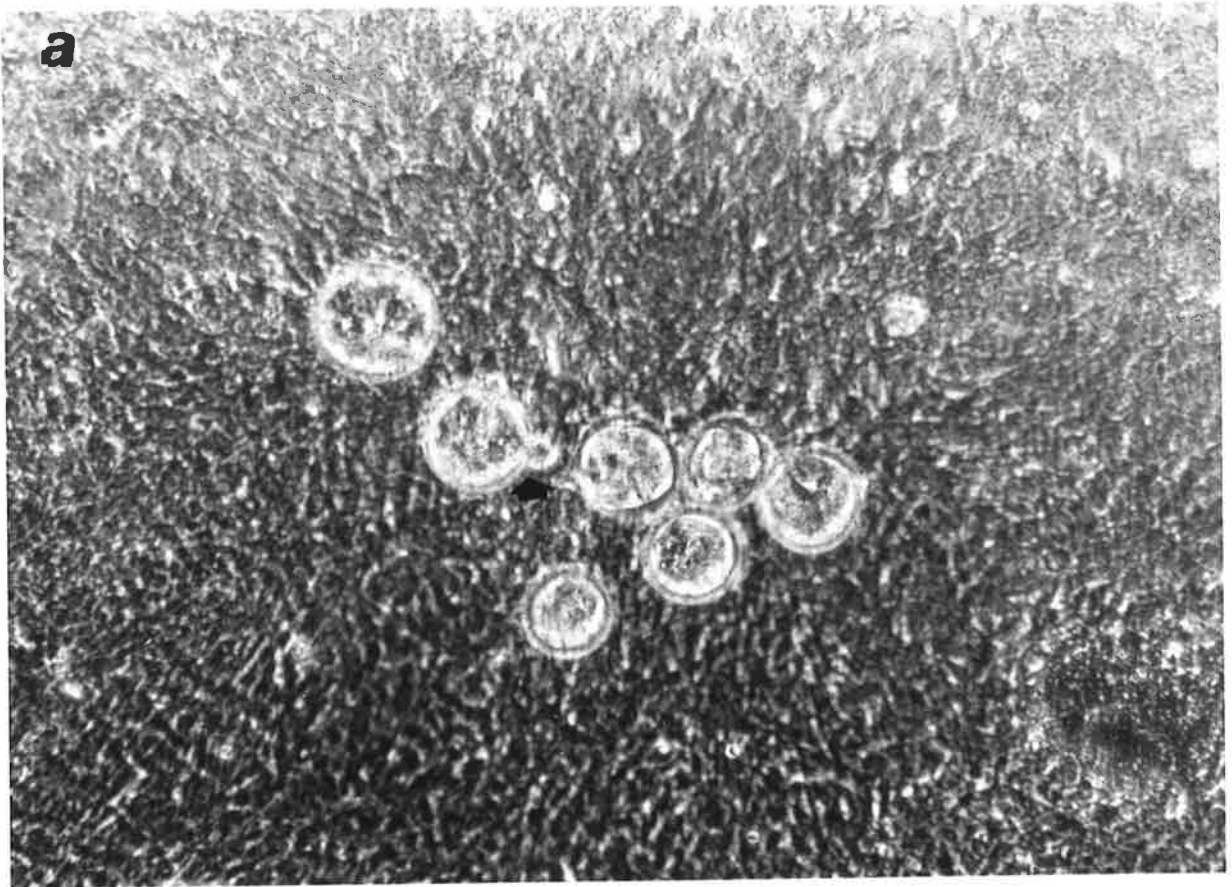


Table 2. Development of embryos on uterine monolayers with increasing concentrations of oestradiol in media.

| Developmental Stage | Oestradiol (M) | | | |
|---------------------|----------------|----------------------|----------------------|----------------------|
| | 0 | 3.7×10^{-6} | 3.7×10^{-5} | 3.7×10^{-4} |
| 8-cell* | 613 | 563 | 377 | 421 |
| Morula | 89.4 | 91.8 | 96.3 | 96.2 |
| Blastocyst | 75.9 | 75.1 | 89.9 | 86.5 |
| Hatched Blastocyst | 68.0 | 65.7 | 82.5 | 74.6 |
| Post-hatch Stage 1 | 62.2 | 56.3 | 76.7 | 67.0 |
| Post-hatch Stage 2 | 56.0 | 51.9 | 74.8+ | 62.7 |

* Number of 8-cell embryos. All other values expressed as a percentage of initial numbers of 8-cell embryos cultured which developed to the stage cited.

+ Significantly different when compared to other groups at the same stage of development. $p < 0.05$, Chi-square.

Table 3. Development of embryos in the presence of steroids (Progesterone, 3.2×10^{-6} ; oestradiol, 3.7×10^{-3}) with or without a uterine monolayer.

| Developmental Stage | Media alone | Uterine monolayer |
|---------------------|-------------|-------------------|
| 8-cell* | 221 | 910 |
| Morula | 87.8 | 94.9 |
| Blastocyst** | 72.4 | 92.6 |
| Hatched Blastocyst | 57.9 | 89.3** |
| Post-hatch Stage 1 | 46.6 | 86.9** |
| Post-hatch Stage 2 | 43.4 | 86.5** |

* Number of 8-cell embryos. All other values expressed as a percentage of initial number of 8-cell embryos cultured which developed to the stage cited.

** Significantly different between groups. $p < 0.05$, Chi-square.

Table 4. Development of embryos *in vitro*: the effect of addition of progesterone (P, 0, 3.2×10^{-8} , 3.2×10^{-7} , 3.2×10^{-6} M) on the development of embryos cultured in a minimum essential medium with oestradiol (E, 3.7×10^{-5} M) in the absence or presence of uterine cell monolayer.

| Developmental Stage | Progesterone (3.2x M) | | | | | | | |
|---------------------|-----------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| | 0 | Medium alone | | | Co-culture | | | |
| | 0 | 10^{-8} | 10^{-7} | 10^{-6} | 0 | 10^{-8} | 10^{-7} | 10^{-6} |
| 8-cell* | 97 | 99 | 100 | 102 | 95 | 98 | 101 | 95 |
| M | 92.8 ^a | 94.9 ^a | 95.0 ^a | 91.2 ^a | 96.8 ^a | 94.9 ^a | 100 ^a | 98.9 ^a |
| B | 82.5 ^a | 82.8 ^a | 84.4 ^a | 81.4 ^a | 89.5 ^a | 89.8 ^a | 92.1 ^a | 93.7 ^a |
| HB | 60.8 ^a | 63.6 ^a | 65.0 ^a | 66.6 ^a | 71.6 ^b | 75.5 ^b | 87.1 ^c | 81.1 ^c |
| P-h S1 | 50.5 ^a | 53.5 ^a | 59.0 ^a | 56.9 ^a | 61.1 ^a | 70.4 ^b | 82.2 ^c | 76.8 ^c |
| P-h S2 | 46.4 ^a | 45.5 ^a | 53.0 ^a | 53.9 ^a | 61.1 ^a | 64.3 ^a | 81.2 ^c | 76.8 ^c |

Values for any developmental stage not having a common superscript differ (^{b-c}, $p < 0.01$; ^{a-c}, $p < 0.001$)

* Number of 8-cell embryos. All other values expressed as a percentage of initial numbers of 8-cell embryos cultured that developed to the stage cited.

KEY: M morula, B blastocyst, HB hatched blastocyst, P-h S1 post-hatch stage 1, P-h S2 post-hatch stage 2.

2.3.3 Embryo viability as assessed by uterine transfers.

To assess embryo viability *in vitro*, 91 embryos at the expanded blastocyst stage after 3 days of co-culture in the presence of progesterone (3.2×10^{-6} M) and oestradiol (3.7×10^{-5} M) were surgically transferred to a total of 12 recipients (day 2). The recipients were sacrificed and evidence of 60 implantation sites (65.9%) and 34 fetuses (37.4%) identified. This compared with the 15 (31.3%) fetuses and 27 implantation sites (56.3%) obtained following transfer of 48 morphologically normal embryos at the expanded blastocyst stage after 3 days in culture in the simple culture medium supplemented with steroid hormones (Table 5.).

Table 5. The effect of embryo-uterine monolayer co-culture supplemented with steroids on embryo viability following *in vitro* culture and subsequent uterine transfer to pseudopregnant females.

| | Embryos transferred at day 3 of co-culture |
|--------------------------|---|
| Controls (media alone) | |
| No. transferred | 48 |
| % implanted | 56.25 |
| % reabsorbed | 25.0 |
| % fetuses | 31.25 |
| Uterine monolayer + P+ E | |
| No. transferred | 91 |
| % implanted | 65.9* |
| % reabsorbed | 23.08 |
| % fetuses | 37.4 |

* Significantly different from control group, $p < 0.05$ Chi-square.
KEY: P progesterone 3.2×10^{-6} M; E oestradiol 3.7×10^{-5} M

2.3.4 Effect of steroids on the uterine monolayer.

In the initial 24-hour period following plating out of the uterine cells, the major cell type present that attached to the culture vessel, had the morphological characteristics of stromal cells, with the remainder equally divided between epithelial cells and fibroblastic myometrial cells. Over the 5-day period of culture the cells proliferated and underwent compaction so that the epithelial cells and the fibroblastic cells became arranged in separate clusters throughout a monolayer of stromal cells. Decidualization was evident from day 4 in culture in approximately 20-30% of the stromal population.

The addition of steroid hormones to the culture medium, progesterone and oestradiol, either alone or in combination, increased the number of stromal cells that

underwent decidualization to polygonal, bi-, tri- and multinucleate forms (Bell and Searle, 1981) to approximately 70%.

2.3.5 Effect of co-culture on post-blastocyst development.

As described earlier when embryos were co-cultured with mixed uterine cells in the presence of progesterone ($3.2 \times 10^{-6} \text{M}$) and oestradiol ($3.7 \times 10^{-5} \text{M}$) an increase in the number of embryos (82/101, 81.2%) which developed to stage 2 compared to controls (45/97, 46.4%) was observed. In order to determine the developmental stage of sensitivity to the steroid supplemented mixed uterine monolayer co-culture system, embryos were cultured in media alone from 8-cell to the blastocyst stage then transferred to a steroid supplemented mixed uterine monolayer and allowed to develop to stage 2; of 98 embryos only 56 (57.1%) attained post-hatch stage 2 and this was not significantly different from the control group (media alone; 53/98, 54.1%), but significantly less than embryos co-cultured throughout (70/82, 85.4%; $p < 0.05$). Conversely, development to post-hatch stage 2 of embryos co-cultured from 8-cell to blastocyst and then transferred to media alone, a significant increase was seen (54/71, 76.1%) compared to embryos cultured in media alone throughout (54.1%, $p < 0.05$), but was not significantly different from embryos co-cultured throughout (85.4%) (Table 6.).

Table 6. Development of embryos *in vitro*: determination of when uterine cell contact is important, before or after the blastocyst stage of development.

| Developmental Stage | Controls | | Experimental | |
|---------------------|------------------------|------------------------------|----------------------------------|----------------------------------|
| | media alone throughout | uterine monolayer throughout | media alone to uterine monolayer | uterine monolayer to media alone |
| 8-cell* | 98 | 82 | 98 | 71 |
| Morula | 90.8 | 100 | 99.0 | 100 |
| Blastocyst | 82.7 | 100 | 90.8 | 100 |
| Hatched Blastocyst | 72.4 | 95.1 | 77.6 | 87.3 |
| Post-hatch Stage 1 | 63.3 | 85.4 | 58.2 | 79.9 |
| Post-hatch Stage 2 | 54.1 | 85.4 | 57.1** | 76.1+ |

* Numbers of 8-cell embryos. All other values expressed as a percentage of initial numbers of 8-cell embryos achieving developmental stage cited.

** Significantly different from control group (uterine monolayer throughout) and uterine monolayer to media alone group $p < 0.05$, Chi-square.

+ Significantly different to control (media alone throughout) $p < 0.05$, Chi-square.

2.3.6 Embryo co-culture with epithelial and stromal monolayers.

As shown earlier when 8-cell embryos were co-cultured on uterine monolayers in the presence of both oestradiol and progesterone, the number of embryos developing to post-hatching stages of development significantly increased. It was necessary to determine whether one particular cell type was responding to the steroid supplementation and therefore providing a more suitable environment for the embryos to develop in. In order to investigate this further, 8-cell embryos were co-cultured with either epithelial or stromal cell monolayers in the presence of both oestradiol and progesterone at a concentration of $3.7 \times 10^{-5}M$ and $3.2 \times 10^{-6}M$ respectively. When 8-cell embryos were cultured in media alone or co-cultured with either epithelial or stromal monolayers in the presence of steroids no significant difference was seen in the numbers of embryos developing to the hatching blastocyst stage as can be seen in Table 7. Significant differences between these groups

became evident at the hatched blastocyst stage of development. Of the 158 embryos in the media alone group only 58.9% (93/158) hatched which was significantly different to both those co-cultured with steroid supplemented epithelial monolayers (77/98, 78.6%; $p < 0.05$) and steroid supplemented stromal monolayers (94/118, 79.7%; $p < 0.01$). By post-hatch stage 2 of development only 55.7% (88/158) of the embryos cultured in media alone attained this stage compared to 68.6% (81/118) on stromal monolayers and both these groups were significantly different to those co-cultured with epithelial monolayers (77/98, 78.6%), $p < 0.01$. (Table 7).

Table 7. The effect of co-culture of 8-cell embryos on epithelial and stromal cell monolayers in the presence of oestradiol (3.7×10^{-5} M) and progesterone (3.2×10^{-6} M) on embryo development and survival *in vitro*.

| Developmental Stage | Media alone | Epithelial | Stromal |
|---------------------|-------------|------------|---------|
| 8-cell* | 158 | 98 | 118 |
| Morula | 100 | 100 | 100 |
| Blastocyst | 94.3 | 100 | 99.2 |
| Hatching Blastocyst | 86.7 | 100 | 97.5 |
| Hatched Blastocyst | 58.9 | 78.6+ | 79.7++ |
| Post-hatch stage 1 | 56.3 | 78.6 | 69.5 |
| Post-hatch stage 2 | 55.7 | 78.6++ | 68.6 |

* Number of embryos. All other values expressed as percentage of initial numbers of 8-cell embryos cultured that developed to stage cited.

+ Significantly different to media alone, $p < 0.05$.

++ Significantly different to media alone, $p < 0.01$.

2.3.7 Effect of co-culture with epithelial and stromal monolayers on the rate of hatching and attachment.

During the co-culture of 8-cell embryos with steroid supplemented epithelial and stromal monolayers it became evident that there were differences in the numbers of embryos attaining the hatching and attachment stages of development. On closer observation it became clear that a larger proportion of embryos co-cultured with either of the monolayers were hatching earlier, that is, only 0.6% (1/158) of embryos cultured in media alone were hatching at 96 hours post-hCG compared to 5.1% (5/98, $p < 0.01$) on epithelial monolayers and 2.5% (3/118, $p < 0.05$) on stromal monolayers. By 100 hours post-hCG these differences were still evident as 7.6% (12/158) of embryos in media alone were hatching which was significantly different to those on epithelial monolayers (13.3%, 13/98 $p < 0.05$), although the number hatching on stromal monolayers was equivalent to those hatching in media alone (5.9%, 7/118). No differences were observed between groups at 120 and 124 hours post-hCG whereas the total numbers of embryos completely hatching in both the epithelial and stromal groups (78.6%, 77/98; and 79.7%, 94/118 respectively) were significantly different to the media alone group (58.9%, 93/158). (Table 8).

Table 8. The effect of 8-cell embryo co-culture with epithelial and stromal monolayers in the presence of oestradiol (3.7×10^{-5} M) and progesterone (3.2×10^{-6} M) on the rate of hatching, *in vitro*.

| Culture Conditions | % 8-cell embryos hatching (hrs post-hCG) | | | | Total % hatched |
|--------------------------------|--|-------|------|------|-----------------|
| | 96 | 100 | 120 | 124 | |
| media alone (n= 158)+ | 0.6 | 7.6 | 47.5 | 49.4 | 58.9 |
| epithelial monolayers (n= 98)+ | 5.1** | 13.3* | 60.2 | 43.9 | 78.6* |
| stromal monolayers (n= 118)+ | 2.5* | 5.9* | 53.4 | 43.2 | 79.7** |

+ Number of embryos. All other values expressed as percentage of initial numbers of 8-cell embryos cultured that developed to stage cited.

* Significantly different to media alone, in addition at 100 hours epithelial vs stromal monolayers significantly different, $p < 0.05$.

** Significantly different to media alone, $P < 0.01$.

In addition not only were embryos hatching earlier in co-culture but they were also attaching to the monolayers earlier. As early as 120 hours post-hCG 0.8% (1/118) of embryos on stromal monolayers had begun to attach to the monolayer compared to 0% in both of the other two groups. By 124 hours post-hCG the number of embryos attaching in the stromal group had increased to 2.5% (3/118) which was significantly different to the media alone group (0%, 0/158) $p < 0.01$, but not significantly different to that seen in the epithelial monolayer group (1.0%, 1/98). At 168 hours post-hCG the total number of embryos that implanted did not increase and only 55.7% (88/158) of those in media alone attained post-hatch stage 2 which was significantly less than the embryos reaching this stage when co-cultured with epithelial monolayers (78.6%, 77/98) $p < 0.01$, but not significantly different to those co-cultured with stromal monolayers (68.6%, 81/118) as can be seen in Table 9.

Table 9. The effect of 8-cell embryos co-cultured with epithelial and stromal monolayers in the presence of oestradiol (3.7×10^{-5} M) and progesterone (3.2×10^{-6} M) on the rate of attachment, *in vitro*.

| Culture Conditions | n* | % 8-cell embryos attaching (hours post-hCG) | | | |
|-----------------------|-----|---|-------|------|--------|
| | | 120 | 124 | 148 | 168 |
| media alone | 158 | 0 | 0 | 51.9 | 55.7 |
| epithelial monolayers | 98 | 0 | 1 | 64.3 | 78.6** |
| stromal monolayers | 118 | 0.8 | 2.5** | 66.1 | 68.6 |

* Number of embryos. All other values expressed as percentage of initial numbers of 8-cell embryos cultured that developed to stage cited.

** Significantly different to media alone, $p < 0.01$.

2.4 DISCUSSION.

Successful *in vitro* culture of mouse embryos, first achieved over 40 years ago by Hammond (1949) is now routine in many laboratories. Development of eight-cell embryos to the blastocyst stage can be readily sustained *in vitro* in simple defined medium comprising a balanced salt solution containing glucose and a protein source (Whitten, 1956). However, as demonstrated by Harlow and Quinn (1982), embryo viability after culture in simple medium is compromised.

There are simple culture media available, the composition of which has been optimized to support the development of pre-implantation murine embryos, and HTF was developed for this purpose (Quinn *et al.*, 1985a, 1985b). However, HTF proved inferior to MEM as a nutrient medium for uterine cells and in view of the objectives of this series of experiments and all subsequent experiments, MEM was selected.

Co-culture of embryos with uterine or other somatic cells has been employed previously as a means of investigating implantation *in vitro* (Salomon and Sherman, 1975; Sherman, 1978; Enders *et al.*, 1981). Recently the possibilities of the use of such systems to enhance pre-implantation embryo survival and development has been identified (Kuzan

and Wright, 1982a, 1982b; Allen and Wright 1984; Khurana and Wales, 1987; Gandolfi and Moor, 1987). This series of experiments confirms that co-culture techniques can be used to improve embryo survival *in vitro*, dramatically so if steroid hormones are included in the co-culture medium.

Although it is known that pre-implantation mouse embryos are sensitive to steroid hormones (Kirkpatrick, 1971; Whitten, 1957), the viability of surviving embryos exposed to steroid hormones was unimpaired whether they had been subject to co-culture or not. The positive effects obtained in this series of experiments were shown to be mediated through interaction of the steroids with the uterine cells. Both progesterone and oestrogen are known to play an active role in the maintenance of the uterine environment during early stages of embryo development and are obligatory for the initiation of blastocyst implantation *in vivo* (Nalbandov, 1971; Glasser and Clark, 1975). However, critical studies on the effect of these steroids on mouse blastocyst implantation in co-culture with uterine cells indicate that progesterone and oestrogen are not obligatory for analogous events *in vitro* (Salomon and Sherman, 1975).

There appear to be no previous reports of the positive effects of steroid hormones on survival of mouse embryos in co-culture. Presumably, in enhancing embryo survival in the simple culture system employed, the steroid hormones act to promote interaction between embryonic and uterine tissues (Fishel and Surani, 1980; Neider *et al.*, 1987). It is well known that the uterine tissues respond to steroid hormones and stage-specific proteins have been isolated from uterine fluids before implantation (Aitken, 1977a). In the present series of experiments there was a marked increase in the number of stromal cells that underwent decidualization in response to the presence of the steroid hormones with the uterine cell monolayer responding to both progesterone and oestradiol, singly and in combination as judged morphologically.

However, relating the morphological changes occurring in the mixed cell culture to the chemical events recently described as occurring in tissues in response to steroid hormones is complex (Neider *et al.*, 1987). It may be anticipated that each cell type will

respond differently to a given steroid hormone and it is known that there is considerable interaction between steroids. Progesterone, for example, has no discernible effect on protein synthesis in rat tissue but can inhibit the synthesis and secretion of specific proteins induced by oestrogen (Wheeler *et al.*, 1987). Further differentiation of these responses will probably be best achieved using studies based on separated uterine cell types (Glasser and McCormack, 1980).

Techniques for the isolation of relatively pure cell lines of epithelial and stromal cells from the mouse uterus are available (Sherman, 1978). Encouragement for this approach is obtained from a recent study by Gandolfi and Moor (1987), who demonstrated increased viability of sheep embryos following co-culture with tubular epithelial cells but not with somatic (fibroblasts) cells. Other workers have shown that co-culture of mouse embryos on relatively pure cell lines of uterine epithelial cells provides a superior environment of development of embryos as assessed by studies of embryo carbohydrate metabolism (Khurana and Wales, 1987). These findings support the studies done by these other groups; it is evident that co-culture of 8-cell embryos on epithelial monolayers in the presence of steroids increases the number of embryos attaining post blastocyst stages of development as effectively as on mixed uterine monolayers. In addition, the percentage of embryos co-cultured on either epithelial or stromal monolayers allows these embryos to hatch earlier, and on stromal monolayers, to also attach earlier, which suggests that these co-culture systems provide a better culture system *in vitro*, and allow development to proceed closer to *in vivo* development.

In summary, co-culture of eight-cell embryos with uterine cells in MEM is improved when steroids are added. Further investigation of the changes induced by the steroid hormones, which led to the enhanced embryo survival, is warranted.

CHAPTER 3.

**CO-CULTURE OF MURINE EMBRYOS: THE EFFECT OF EMBRYO
CO-CULTURE ON SELECTED SOMATIC CELL MONOLAYERS COMPARED
WITH UTERINE MONOLAYERS ON EMBRYO SURVIVAL AND
IMPLANTATION *IN VITRO*.**

3.1 INTRODUCTION.

In recent years a number of studies have suggested that embryo development *in vitro* can be improved if embryos are co-cultured with selected somatic cells. Most investigations have centred around the use of primary cultures of cells from the reproductive tract although there are a few reports which have examined the use of established cell lines.

As described in chapter 2 we have found that co-culture of 8-cell murine embryos with steroid supplemented mixed uterine cell monolayers results in a significant increase in embryos developing beyond the hatching blastocyst stage. Other studies have identified several other cell types also with similar beneficial effects; these include studies with bovine embryos co-cultured with uterine monolayers (Voelkel *et al.*, 1985), with trophoblastic vesicles (Camous *et al.*, 1984; Heyman and Ménézo 1984) or with oviduct epithelium (Eyestone *et al.*, 1987; Pollard *et al.*, 1989), porcine embryos co-cultured with endometrial cell monolayers (Allen and Wright 1984) or with oviducts in organ culture (Krisher *et al.*, 1989), murine embryos co-cultured with oviduct cells (Sakkas *et al.*, 1989, Sakkas and Trounson 1990), also sheep embryos co-cultured with oviduct epithelial cells (Gandolfi and Moor 1987) or with trophoblastic vesicles (Rexroad and Powell 1988a).

Recently a couple of studies have investigated the use of established cell lines as a suitable matrix for embryo development *in vitro*. One group examined the effect of murine embryos co-cultured with MDBK cells (bovine kidney) and observed high rates of blastocyst formation (Ouhibi *et al.*, 1990), others found co-culture of human embryos with Vero cells (Green monkey kidney) significantly improved embryo development (Ménézo *et al.*, 1990). The use of established cell lines allows the investigator to use a system which is not only convenient and repeatable but also allows us to identify unique growth factors which could play a significant role in embryo development.

To expand our initial observation on the suitability of utilising uterine cell monolayers as a support for the development of eight-cell murine embryos *in vitro*, we examined the suitability of a number of primary and established cell lines and their conditioned media to support embryo development and survival *in vitro*.

3.2 MATERIALS AND METHODS.

3.2.1 Animals.

Balb-C x C57 3-4 week old F1 female mice were primed with 7.5 i.u. PMSG followed 48 hours later with 7.5 i.u. hCG to achieve superovulation. Immediately following the hCG injection, treated females were placed with fertile males (CBA x C57 strain, one female with one male per cage). The next morning each female was checked for the presence of a vaginal plug as evidence of mating. This was then considered as day 1 of pregnancy.

3.2.2 Media.

The culture media were prepared from powdered Minimal Essential Medium, dissolved in Milli-Q water and supplemented with 25mM sodium bicarbonate and 10% (v/v) heat-inactivated FCS. An antibiotic-antimycotic solution was also added to provide per 100mL of solution: 10 000 units penicillin, 10 000 μ g streptomycin and 25 μ g fungizone. The pH and osmolarity of the media were adjusted to 7.4 and 280 mOsm respectively. At this point the medium was sterilized by filtration through a 0.2 μ m filter.

3.2.3 Embryos.

On day 3 of pregnancy females were killed 71-73 hours post-hCG injection, by cervical dislocation. The whole reproductive tract was dissected out and placed in EBSS at 37°C. Subsequently eight-cell embryos were teased and/or flushed out of the oviduct-uterus junction and after being washed once in culture medium were placed into the control group(s) or the experimental group (selected cell monolayer) as specified and maintained in a humidified gas environment of 5% CO₂ in air, at 37°C.

3.2.4 Epithelial Cell Monolayers.

3.2.4.1 Primary Amnion Monolayers.

Amnion tissue was collected from human placentas obtained at elective caesarian section deliveries. It was collected by stripping the amnion from the chorion under sterile conditions, and placed into sterile containers containing HEPES-buffered Earle's balanced salt solution without calcium and magnesium (HEPES-EBSS) at 37°C and transported immediately back to the laboratory. The amnion tissue was then cut into small pieces (approximately 3mm square) and washed twice in EBSS at 37°C under sterile conditions. Following this step approximately 30 pieces were placed into a petri dish containing EBSS-T-E, and incubated for 30 minutes at 37°C. The amnion pieces were then dissociated further by mincing with sterile scissors for about 1 minute and the mixture returned to the incubator for a further 45 minutes at 37°C. Following the enzyme treatment, heat-inactivated FCS (5%) was added and the cell suspension centrifuged at 750g for 20 minutes, the supernatant discarded and the pellet resuspended in culture medium. Cells were plated out into four-well multidishes at 1.0×10^6 cells per well, and the dishes placed in a humidified incubator at 37°C, 5% CO₂ in air for 24-48 hours prior to the addition of 8-cell embryos. The growth of the primary amnion cells at that concentration was sufficient to achieve confluence over this initial period.

3.2.4.2 FL Amnion.

FL amnion is an established cell line derived from human amnion epithelial cells (ATCC CCL62, 1988) maintained in MEM. FL amnion cells were cultured in 75 cubic cm culture flasks (Costar, Cambridge) in a humidified incubator, 5% CO₂ in air at 37°C, and were prepared as follows prior to the addition of embryos. The monolayer of cells was washed twice in EBSS at 37°C for 5 minutes each, then EBSS was replaced with EBSS-T-E and incubated for 5-10 minutes at 37°C, or until the cell monolayer had detached from the

bottom of the flask. This was followed by the addition of approximately 2 mL of heat-inactivated FCS. The cell suspension was transferred to a 10 mL tube and centrifuged at 500g for 10 minutes. The supernatant was discarded and the pellet resuspended in 2 mL of MEM at 37°C. FL amnion cells were plated out into four-well multidishes at 5×10^5 cells per well, and the dishes placed in a humidified incubator at 37°C, 5% CO₂ in air for 24 hours (this period allows cells to adhere and achieve confluence), at which time murine embryos were added. To continue passage of these cells a 75 cubic cm culture vessel was also seeded following plating out of the four-well dishes, and used for replicate experiments.

3.2.5 Fibroblast Cell Monolayers.

3.2.5.1 L Cell Monolayers.

L cells are an established cell line derived from murine fibroblasts (NTCC clone, ATCC CC1, 1988) and maintained in Ham's F10 medium (Flow Laboratories Inc., USA) supplemented with L-glutamine (1.5mM), HEPES (10mM), sodium bicarbonate (24mM), 10% heat-inactivated FCS and with penicillin (60mg/L) and streptomycin (100mg/L). For the purpose of these experiments the cells were gradually introduced to MEM and over a period of two weeks were finally cultured in MEM (Ham's F10:MEM; 4:1, 3:1, 2:1, 1:1, 0:1) in a 75 cubic cm culture flask in a humidified incubator, 5% CO₂ in air at 37°C, and were prepared as follows prior to the addition of embryos. The monolayer was washed in EBSS at 37°C (2 x 5 minutes) then the salt solution was replaced with EBSS-T-E and incubated for 5-10 minutes or until the cells had detached from the bottom of the culture vessel, at which time 2mL of heat-inactivated FCS was added. The mixture was then transferred to a 10mL tube and centrifuged for 10 minutes at 500g. The supernatant was discarded and the pellet of cells resuspended in 2 mL of MEM. L cells were plated out into four-well multidishes at a concentration of 5×10^5 cells per well in 1 mL of MEM, and the plates were placed in a humidified incubator at 37°C, 5% CO₂ in air for 24 hours, the

embryos were then added to the wells. To allow continuation of these cell lines, a 75 cubic cm culture flask was also seeded with L cells at the time the four-well dishes were plated out, and left in the incubator until needed for the next experiment.

3.2.5.2 3T3 Cell Monolayers.

3T3 cells are an established cell line derived from murine fibroblasts (ATCC CC92, 1988) and maintained in RPMI 1640 (GIBCO BRL, Life Technologies Inc., USA) medium supplemented with L-glutamine (1.5mM), HEPES (10mM), sodium bicarbonate (24mM), 10% heat-inactivated FCS and with penicillin (60mg/L) and streptomycin (100mg/L). To allow the use of these cells in these experiments the cells were gradually introduced to MEM (RPMI:MEM; 4:1, 3:1, 2:1, 1:1, 0:1), and over a period of two weeks were finally cultured in MEM, in a 75 cubic cm culture flask, in a humidified incubator at 37°C, 5% CO₂ in air, and prepared as follows. A flask of cells was washed in EBSS (2 x 5 minutes) and then EBSS-T-E added and the flask incubated for 5-10 minutes, or until the cells had detached from the bottom of the culture vessel, at which time approximately 2 mL of HI-FCS was added to stop the enzymatic reaction. The suspension of cells was then transferred to a 10 mL tube and centrifuged for 10 minutes at 500g, the supernatant discarded and the cell pellet resuspended in 2 mL of MEM. The 3T3 cells were plated out as described for the other cell lines, that is, at a concentration of 5×10^5 cells per well in 1mL of MEM in four-well multidishes and incubated under humidified conditions at 37°C, 5% CO₂ in air for 24 hours prior to the addition of embryos. These cells were also placed in a large culture flask and allowed to continue growth in the incubator until required for further experiments.

3.2.6 Uterine monolayers.

As described earlier, the procedure for the production of uterine monolayers is briefly as follows: following collection of the embryos from day 3 pregnant females, uteri

were dissected free of remaining oviductal and other tissues and washed in a balanced salt solution, cut into smaller pieces and placed in a petri dish containing EBSS-T-E for 15 minutes at 37°C. The enzyme solution was replaced with fresh EBSS-T-E, the uterine tissue further dissociated by mincing with sterile scissors for approximately 1 minute and the mixture subsequently incubated for 30 minutes. After the incubation period 5% heat-inactivated FCS was added and the cell suspension then centrifuged for 20 minutes at 500g. The supernatant was then discarded and the pellet of cells was resuspended in culture media supplemented with progesterone ($3.2 \times 10^{-6} \text{M}$) and oestradiol ($3.7 \times 10^{-5} \text{M}$), and the cells plated out at approximately 1.0×10^6 cells per well in a four-well multidish and incubated under humidified conditions at 37°C, 5% CO₂ in air for 24 hours prior to the addition of embryos.

3.2.7 Conditioned Media.

Conditioned medium was prepared from mixed uterine, primary amnion cells, FL amnion, 3T3 cells and L cells. Cells were grown to confluence in MEM, then washed once in MEM for 5 minutes and fresh media added. Conditioned media was harvested after a 24-48 hour culture period, centrifuged at 500g for 10 minutes immediately following collection and either (a) aliquoted out into four-well multidishes (1mL/well) for embryo culture, or (b) aliquoted out into 5 mL sterile tubes and stored at -80°C for later assay.

3.2.8 Culture of Embryos.

Eight-cell embryos were randomly allocated to a control or experimental group, with each group consisting of three to five replicates, with embryos from four to six mice per replicate. Embryos were added 10-15 per well, 4 wells per replicate, approximately 15-20 minutes after being collected from the uterus and were maintained *in vitro* for a period of 5 days in wells containing 1 mL of culture medium alone (control group), conditioned medium, or culture media and one of the cell monolayers as indicated.

3.2.9 ^3H -thymidine incorporation into hatched blastocysts in co-culture and conditioned media.

Eight-cell embryos were placed into co-culture with either uterine, primary amnion, FL amnion or L cell monolayers or cultured alone in MEM and allowed to develop to the hatched blastocyst stage, in a humidified incubator at 37°C , 5% CO_2 in air. Once they attained this developmental stage they were placed one embryo per well into microtitre tray wells (96 well plate, LINBRO, Flow Laboratories Inc., USA) containing $1\ \mu\text{Ci}$ ^3H -thymidine (Amersham, U.K.) in $150\ \mu\text{l}$ HEPES buffered MEM. Embryos were then incubated for 6 hours, harvested using a Titretech automated cell harvester on glass fibre paper, left to dry overnight, scintillant was added to each sample and using a liquid scintillation counter incorporation of ^3H -thymidine was assessed.

3.2.10 Assessment of Morphological Development.

Daily observations on embryo development were made using an inverted microscope and the numbers of embryos achieving morula, blastocyst or hatching blastocyst stage were recorded according to Hsu, (1979). Developmental changes of the embryos associated with implantation (Sherman, 1978) were observed and recorded on day 4-5 of culture and for this series of experiments post-hatching embryos were recorded as achieving stage 1 when they displayed proliferating trophectoderm cells and stage 2 when they showed outgrowth of trophectoderm cells on the bottom of the culture vessel when in media alone or invasive outgrowth when cultured with one of the cell monolayers.

3.2.11 Statistics.

All data were analysed using Chi-square tests, except for ^3H -thymidine incorporation experiments where student's t-test was used.

3.3 RESULTS.

3.3.1. The effect of embryos co-cultured with primary amnion monolayer compared with mixed uterine monolayer, on development and survival *in vitro*.

Embryos co-cultured on a mixed uterine monolayer in the presence of progesterone and oestradiol showed increased developmental capacity compared to embryos cultured in medium alone. In order to determine whether embryos were responding to the presence of the cell monolayer as a more favourable substratum to develop on compared to a plastic dish or, whether the mixed uterine cells in response to steroid stimulation were producing an embryotrophic substance, embryos were also co-cultured with a selected number of somatic cell types, of which one was a primary amnion monolayer.

The percentage of embryos developing from the 8-cell stage to the blastocyst stage did not differ significantly in any of the groups, that is, whether in media alone, on steroid supplemented uterine monolayers or with primary amnion monolayers (107/120, 89.2%; 121/122, 99.2%; 159/163, 97.5%; respectively). The total number of co-cultured embryos on amnion monolayers that completely hatched (152/163, 93.3%) was not significantly different from the number of embryos co-cultured with uterine monolayers (110/122, 90.2%), but was significantly different from the media alone group (82/120, 68.3%) $p < 0.01$. Subsequent development of these embryos to the implantation stage (post-hatch stage 2) continued to show co-culture with amnion monolayers as an equally favourable culture system for murine development and survival as uterine monolayers *in vitro*. This was evident as 143/163 (87.7%) of 8-cell embryos co-cultured with amnion monolayers developed to post-hatch stage 2, as did 102/122 (83.6%) of embryos co-cultured with uterine monolayers and both of these groups were significantly different compared to the media alone group (67/120, 55.8%), $p < 0.01$. (Table 10).

Table 10. Development of embryos *in vitro*: the effect of co-culture with amnion monolayers compared to uterine monolayers on development and survival.

| Developmental Stage | Media Alone | Uterine Monolayer | Amnion Monolayer |
|---------------------|-------------|-------------------|------------------|
| 8-cell* | 120 | 122 | 163 |
| Morula | 95.0 | 100 | 100 |
| Blastocyst | 89.2 | 99.2 | 97.5 |
| Hatched Blastocyst | 68.3 | 90.2+ | 93.3+ |
| Post-hatch Stage 1 | 59.2 | 84.4+ | 88.3+ |
| Post-hatch Stage 2 | 55.8 | 83.6+ | 87.7+ |

* Number of 8-cell embryos. All other values expressed as a percentage of initial numbers of 8-cell embryos achieving developmental stage cited.

+ Significantly different from media alone $p < 0.01$, Chi-square.

3.3.2 The effect of embryos co-cultured with FL amnion monolayer compared with mixed uterine monolayer, on development and survival *in vitro*.

As referred to earlier, a number of selected somatic cell monolayers were used to determine the suitability of other cells to support embryo development and survival *in vitro*, and FL amnion was one of these. When 8-cell embryos were cultured in media alone (15/24, 62.5%) or with uterine monolayers (20/25, 80.0%) or FL amnion monolayers (19/25, 76.0%) no significant difference was evident between groups in the number of embryos that attained the hatched blastocyst stage. Once development of embryos proceeded on further it became apparent that there was no significant difference between the media alone group (13/24, 54.2%) and when embryos were co-cultured with FL amnion monolayers (14/25, 56.0%), but both groups were significantly different to the uterine monolayer group (20/25, 80.0%) $p < 0.01$, at post-hatch stage 2. (Table 11.)

Table 11. Development of embryos *in vitro*: the effect of co-culture with FL amnion monolayers compared to uterine monolayers on development and survival.

| Developmental Stage | Media Alone | Uterine Monolayers | FL amnion Monolayers |
|---------------------|-------------|--------------------|----------------------|
| 8-cell* | 24 | 25 | 25 |
| Morula | 100 | 100 | 100 |
| Blastocyst | 100 | 100 | 96.0 |
| Hatched Blastocyst | 62.5 | 80.0 | 76.0 |
| Post-hatch Stage 1 | 54.2 | 80.0+ | 64.0 |
| Post-hatch Stage 2 | 54.2 | 80.0+ | 56.0 |

* Number of embryos. All other values expressed as a percentage of initial numbers of 8-cell embryos achieving developmental stage cited.

+ Significantly different from other groups for that developmental stage, $p < 0.01$ Chi-square.

3.3.3 The effect of embryos co-cultured with L cell monolayer compared with mixed uterine monolayer, on development and survival *in vitro*.

Embryos were co-cultured with L cell monolayers in order to investigate their suitability as a substratum for embryo growth and development, *in vitro*. Eight-cell embryos were cultured in media alone or with either uterine monolayers or L cell monolayers for a period of 5 days and development and survival was scored. Of the 80 embryos cultured in media alone, 56 (70.0%) hatched which was significantly different from both those co-cultured with uterine monolayers (82/87, 94.3%) and L cell monolayers (97/106, 91.5%), $p < 0.01$. As development proceeded towards implantation no differences were observed between the two co-culture groups, that is, at post-hatch stage 2, 85.1% (74/87) of embryos co-cultured with uterine monolayers attained this stage compared to 85.8% (91/106) of embryos co-cultured with L cell monolayers, and both groups were significantly different from the embryos cultured in media alone (47/80, 58.8%), $p < 0.01$ (Table 12).

Table 12. Development of embryos *in vitro*: the effect of co-culture with L cell monolayers compared to uterine monolayers on development and survival.

| Developmental Stage | Media Alone | Uterine Monolayers | L cell Monolayers |
|---------------------|-------------|--------------------|-------------------|
| 8-cell* | 80 | 87 | 106 |
| Morula | 96.3 | 98.9 | 97.2 |
| Blastocyst | 91.3 | 98.9 | 95.3 |
| Hatched Blastocyst | 70.0 | 94.3+ | 91.5+ |
| Post-hatch Stage 1 | 60.0 | 87.4+ | 87.7+ |
| Post-hatch Stage 2 | 58.8 | 85.1+ | 85.8+ |

* Number of embryos. All other values expressed as a percentage of initial numbers of 8-cell embryos achieving developmental stage cited.

+ Significantly different from control group (media alone), $p < 0.01$ Chi-square.

3.3.4. The effect of embryos co-cultured with fibroblast cell lines on development and survival *in vitro*.

Once it was established that L cell monolayers were a suitable substratum for embryos to develop on, the development and survival of embryos co-cultured with another fibroblast cell line (3T3 cells) was investigated. Eight-cell embryos were cultured in media alone or co-cultured with either L cell monolayers or 3T3 cell monolayers and development observed and scored over a 5 day culture period. There were no differences observed between groups from the 8-cell to blastocyst stage of development (Table 13). Of the 32 embryos co-cultured with 3T3 cell monolayers, 21 (65.6%) attained the hatched blastocyst stage which was significantly different from the L cell monolayer group (24/26, 93.2%) $p < 0.01$, but was not considerably different to the media alone group (19/28, 67.9%) at the hatched blastocyst stage of development. Of the hatched blastocysts cultured on L cell monolayers 88.5% (23/26) attained post-hatch stage 1 and continued through to post-hatch stage 2 which was significantly different ($p < 0.05$) from both the control group (13/28, 46.4%) and the group of embryos cultured on 3T3 cells (18/32, 56.3%).

Table 13. Development of embryos *in vitro*: the effect of co-culture with fibroblast cell lines (L cells and 3T3 cells) and fibroblast conditioned media (CM) on development and survival.

| Developmental Stage | Control (Media alone) | Monolayers | | Conditioned Media | |
|---------------------|-----------------------|------------|------|-------------------|------|
| | | L cells | 3T3 | L cells | 3T3 |
| 8-cell* | 28 | 26 | 32 | 26 | 31 |
| Morula | 100 | 100 | 100 | 96.2 | 96.8 |
| Blastocyst | 100 | 100 | 93.8 | 96.2 | 87.1 |
| Hatched Blastocyst | 67.9 | 92.3 | 65.6 | 80.8 | 54.8 |
| Post-hatch Stage 1 | 46.4 | 88.5+ | 56.3 | 73.1 | 45.2 |
| Post-hatch Stage 2 | 46.4 | 88.5+ | 56.3 | 73.1 | 45.2 |

* Numbers of embryos. All other values expressed as a percentage of initial numbers of 8-cell embryos achieving developmental stage cited.

+ Significantly different from media alone, $p < 0.05$.

3.3.5 Embryo development following culture in primary amnion conditioned media.

Co-culture of 8-cell embryos on amnion monolayers significantly increased development of these embryos to post-hatch stage 2 when compared to uterine monolayer co-culture as can be seen in Table 10. In order to establish whether embryo-cell contact is essential or whether enhanced embryo development is due to the availability of specific growth and developmental factors produced by these cells, embryos were cultured in amnion conditioned media.

When 8-cell embryos were cultured in amnion conditioned media 88.8% (71/80) attained the hatched blastocyst stage which was not significantly different from 8-cell embryos cultured in media alone (52/71, 73.2%) or on amnion monolayers (70/71, 98.6%). Of the 80 embryos cultured in amnion conditioned media 63 (78.8%) proceeded to post-hatch stage 1 compared to 60.6% (43/71) of those cultured in media alone and 94.4% (67/71) of embryos cultured on amnion monolayers. By post-hatch stage 2 only 56.3% (40/71) of embryos cultured in media alone continued their development to post-hatch stage

2 which was significantly different from both those embryos cultured on amnion monolayers (67/71, 94.4%) $p < 0.01$, and the embryos cultured in amnion conditioned media (63/80, 78.8%), $p < 0.10$ (Table 14).

Table 14. Development of embryos *in vitro*: the effect of co-culture with amnion monolayers compared with amnion monolayer conditioned media (CM) on development and survival.

| Developmental Stage | Media Alone | Amnion Monolayer | Amnion CM |
|---------------------|-------------|------------------|-----------|
| 8-cell* | 71 | 71 | 80 |
| Morula | 98.6 | 100 | 100 |
| Blastocyst | 93.0 | 100 | 97.5 |
| Hatched Blastocyst | 73.2 | 98.6 | 88.8 |
| Post-hatch Stage 1 | 60.6 | 94.4+ | 78.8 |
| Post-hatch Stage 2 | 56.3 | 94.4++ | 78.8 |

* Numbers of embryos. All other values expressed as a percentage of initial numbers of 8-cell embryos achieving developmental stage cited.

+ Significantly different from media alone, $p < 0.05$.

++ Significantly different from media alone, $p < 0.01$.

3.3.6 Embryo development following culture in L cell conditioned media.

L-cell monolayers proved to be a suitable substratum for embryo co-culture as can be seen in Table 12. Production of specific growth and developmental factors by L cells in monolayer culture and their subsequent availability to embryos was investigated by embryo culture in L cell conditioned medium.

When 8-cell embryos were cultured in either media alone, on L cell monolayers or in L cell conditioned media, no significant differences were observed between these groups until post-hatch stage 1. Of the 8-cell embryos cultured in L cell CM 88.1% (111/126)

attained the hatched blastocyst stage compared with 72.8% (59/81) in the media alone group and 88.2% (90/102) in the group cultured on L cell monolayers. By post-hatch stage 1 the differences between these groups became greater, 77.0% (97/126) of embryos cultured in L cell CM attained this stage and continued to post-hatch stage 2 and was not significantly different to those cultured on L cell monolayers at post-hatch stage 1 (83.3%, 85/102) or post-hatch stage 2 (81.4%, 83/102) nor significantly different to the media alone group (60.5%, 49/81) at both post-hatch stage 1 and stage 2 (Table 15).

Table 15. Development of embryos *in vitro*: the effect of co-culture with L cell monolayers compared with L cell conditioned media (CM) on development and survival.

| Developmental Stage | Media Alone | L cell Monolayer | L cell CM |
|---------------------|-------------|------------------|-----------|
| 8-cell* | 81 | 102 | 126 |
| Morula | 100 | 98.0 | 98.4 |
| Blastocyst | 96.3 | 98.0 | 97.6 |
| Hatched Blastocyst | 72.8 | 88.2 | 88.1 |
| Post-hatch Stage 1 | 60.5 | 83.3+ | 77.0 |
| Post-hatch Stage 2 | 60.5 | 81.4+ | 77.0 |

* Numbers of embryos. All other values expressed as a percentage of initial numbers of 8-cell embryos achieving developmental stage cited.

+ Significantly different from media alone, $p < 0.05$.

3.3.7 Embryo development following culture in uterine cell conditioned media.

Production of specific growth and developmental factors by uterine cells in monolayer culture and the subsequent availability of these factors to embryos was investigated by embryo culture in uterine cell conditioned media.

8-cell murine embryos were cultured in either media alone, uterine cell conditioned media or on uterine cell monolayers and no differences were observed between these groups

until the hatched blastocyst stage of development. Of the 98 8-cell embryos cultured in uterine cell CM 78 (79.6%) reached the hatched blastocyst stage which was not significantly different from the media alone group (59.6%, 53/89) or from those cultured on uterine monolayers (90.8%, 69/76). By post-hatch stage 1 67.3% (66/98) of embryos cultured in uterine CM attained this stage compared with only 52.8% (47/89) in the media alone group and 84.2% (64/76) in the group of embryos cultured with uterine cells. Subsequent development of the embryos cultured in uterine cell CM to post-hatch stage 2 resulted in 62.2% (61/98) of these embryos attaining this stage compared to 48.3% (43/89) of the embryos cultured in media alone, and 82.9% (63/76) of the embryos cultured with uterine cells (Table 16).

Table 16. Development of embryos *in vitro*: the effect of co-culture with uterine monolayers compared with uterine monolayer conditioned media (CM) on development and survival.

| Developmental Stage | Media Alone | Uterine Monolayer | Uterine CM |
|---------------------|-------------|-------------------|------------|
| 8-cell* | 89 | 76 | 98 |
| Morula | 98.9 | 100 | 100 |
| Blastocyst | 92.0 | 92.1 | 90.8 |
| Hatched Blastocyst | 59.6 | 90.8 | 79.6 |
| Post-hatch Stage 1 | 52.8 | 84.2** | 67.3 |
| Post-hatch Stage 2 | 48.3 | 82.9*** | 62.2** |

* Number of embryos. All other values expressed as a percentage of 8-cell embryos achieving developmental stage cited.

** Significantly different to media alone, $p < 0.01$.

*** Significantly different to media alone, $p < 0.001$.

3.3.8 Embryo development following culture in 3T3 cell conditioned media.

It was established earlier that 3T3 cells cultured in monolayer were not advantageous as a substratum for embryos to develop on in co-culture, and although this was the case, it seemed necessary to investigate if these cells were producing factors that were detrimental or inhibitory to the development of these embryos. Therefore embryos were cultured in 3T3 cell conditioned media to determine this.

8-cell embryos were cultured in media alone, 3T3 cell conditioned media or on 3T3 cell monolayers and development observed and scored over a 5 day period. Of the 31 8-cell embryos cultured in 3T3 cell CM 17 (54.8%) reached the hatched blastocyst stage which was not significantly different from either the embryos cultured in media alone (19/28, 67.9%) or the embryos co-cultured with 3T3 cell monolayers (21/32, 65.6%). Subsequent development showed a similar trend in all three groups; 45.2% (14/31) of embryos cultured in 3T3 cell CM attained the post-hatch stage 1 of development and the same number continued developing to post-hatch stage 2 which was not significantly different to the embryos cultured in either media alone (46.4%, 13/28) or in co-culture with 3T3 cell monolayers (56.3%, 18/32). (Table 13).

3.3.9 Embryo development following culture in FL amnion conditioned media.

As with 3T3 cell monolayers, FL amnion did not prove to be an exceptional substratum for embryo co-culture. It was necessary therefore to investigate the possibility that these cells were producing detrimental or inhibitory factors that were affecting the developmental potential of the embryos in culture. This was accomplished by culturing 8-cell embryos in FL amnion conditioned media (CM). Embryos were divided into three groups, and cultured in either media alone, FL amnion CM or on FL amnion monolayers, development was observed and scored over a 5 day culture period.

At the hatched blastocyst stage of development no significant differences were observed between the group of embryos cultured in FL amnion CM (80.0%, 20/25) and

those cultured in either the media alone (62.5%, 15/24) or co-cultured with FL amnion monolayers (76.0%, 19/25). Significant differences between the groups were not apparent as development progressed. In the media alone group 54.2% (13/24) of embryos continued through post-hatch stage 1 to post-hatch stage 2 of development compared to 76% (19/25) of embryos cultured in FL amnion CM attaining post-hatch stage 1 and only 64% (16/25) attaining post-hatch stage 2, and of the embryos co-cultured with FL amnion monolayers 64% (16/25) reached post-hatch stage 1 and only 56% (14/25) progressed to post-hatch stage 2 of development (Table 17).

Table 17. Development of embryos *in vitro*: the effect of culture with FL amnion conditioned media (CM) on development and survival.

| Developmental Stage | Media Alone | FL amnion | FL CM |
|---------------------|-------------|-----------|-------|
| 8-cell* | 24 | 25 | 25 |
| Morula | 100 | 100 | 100 |
| Blastocyst | 100 | 96.0 | 100 |
| Hatched Blastocyst | 62.5 | 76.0 | 80.0 |
| Post-hatch Stage 1 | 54.2 | 64.0 | 76.0 |
| Post-hatch Stage 2 | 54.2 | 56.0 | 64.0 |

*Number of embryos. All other values expressed as a percentage of 8-cell embryos achieving developmental stage cited.

3.3.10 The effect of co-culture and culture in conditioned media on mouse embryonic development *in vitro* as assessed by ^3H -thymidine incorporation into hatched blastocysts.

In order to determine if the different culture conditions were contributing to the capacity of the embryo to proliferate, embryos were cultured under the specified conditions then pulsed with ^3H -thymidine and incorporation assessed. When embryos were cultured in media alone, then pulsed, it was found that the mean number of counts were 437 ± 15 and this was significantly different to the mean number of counts calculated for the group of

embryos co-cultured with both uterine cell monolayers (526 ± 34) and amnion monolayers (609 ± 22) as well as for the embryos cultured in L cell conditioned media (369 ± 23), but was not significantly different to the other culture conditions (Table 18).

Table 18. Effect of co-culture and conditioned media on mouse embryonic development *in vitro* as assessed by ^3H -thymidine incorporation into hatched blastocysts.

| Cell Type | Co-culture* | Conditioned Media* |
|---------------|--------------------|--------------------|
| Media alone | 437 ± 15 (15) | - |
| Uterine cells | 526 ± 34 (16)+ | 395 ± 23 (18) |
| Amnion | 609 ± 22 (20)+ | 407 ± 16 (20) |
| FL amnion | 515 ± 17 (13) | 385 ± 29 (16) |
| L cells | 482 ± 42 (13) | 369 ± 23 (18)+ |

* Results are expressed as mean counts \pm SEM incorporated per embryo, where (n) is the number of embryos assessed.

+ Significantly different from media alone value ($p < 0.05$).

3.4 DISCUSSION.

Early studies by Cole and Paul (1965) demonstrated that mouse embryos could develop and implant on HeLa and L cell monolayers, and these embryos more closely resembled those at similar developmental stages *in vivo* than the embryos cultured in simple media. The use of mixed uterine cell monolayers as a substratum for embryo development *in vitro* (Saloman and Sherman, 1975; Sherman and Wudl, 1976) provided support for these early observations. Subsequent work using uterine epithelial cell monolayers has also provided a useful culture system to gain a better understanding of the cellular biology of implantation (Kubo *et al.*, 1981; Van Blerkom and Chavez, 1981; Lindenberg *et al.*, 1989).

The results presented here indicate that primary human amnion epithelial cell monolayers and L cell monolayers were equally effective as a substratum for embryo development and survival *in vitro*. In addition, conditioned media from these cells also enhanced embryo development to post-hatching stages of development. Human amnion which is of embryonic origin (Perry, 1981) has been used by other workers in co-culture systems to maintain neuronal axon development *in vitro* (Davis *et al.*, 1987), amniotic fluid has also been used as a culture medium for human embryos *in vitro*, and is known to contain hormones and other growth factors (Kirby and Trounson, 1986). L cells were found to consistently enhance embryo development *in vitro* as did L cell conditioned media. It is of interest that L cells have been recognised as a potent source of macrophage-colony stimulating factor (M-CSF) (Stanley *et al.*, 1983).

Rettenmier *et al.* (1987) have described a number of molecular species of M-CSF, including a membrane bound form that may function via cell-cell contact. Recent work by Pollard *et al.* (1987) has shown that one species of M-CSF produced by uterine tissues accumulates in fetal tissues and in amniotic fluid.

In contrast, the FL amnion cell line and the monolayer of 3T3 cells proved an unsuitable substratum for embryo development. It seems possible that, just as primary amnion and L cells are producing growth promoting factors *in vitro* which support embryo development, FL amnion and 3T3 cells could be producing inhibitory factors or producing growth factors at concentrations which are actually inhibitory to embryos *in vitro*. Other ways in which a particular cell type could prove to be either inhibitory or embryotrophic could be by adjusting the O₂ tension, altering the pH, removal of waste, or removal or binding of growth regulators within the culture system.

It becomes apparent from these studies that particular cell monolayers such as primary amnion and L cells act in an embryotrophic capacity to promote embryo growth and development *in vitro*, others such as FL amnion and 3T3 cells do not support or are ineffective as suitable substrata for embryo development *in vitro*. The determining factor as to whether a particular cell monolayer is either effective or ineffective as a substratum might reflect the kind or amounts of growth factors that are being produced *in vitro* under

the culture conditions specified, and whether these growth factors and the relative amounts of growth factors are important to the embryo during its development from the 8-cell stage to the implantation stage. The following question also arises; what changes have occurred to the FL amnion epithelial cells in order for these cells to be transformed into a cell line, and have these changes produced oncogenes which may in some way lessen the capacity of this cell line to support embryo growth? This and other questions need to be answered and further investigation is necessary to determine exactly what growth factors are being produced by each of the cell monolayers studied here. This would allow a better understanding of the importance of various growth factors to the embryo at the peri-implantation stage of development.

CHAPTER 4.

**PRODUCTION OF CYTOKINES AND PROSTAGLANDINS BY SELECTED
SOMATIC CELL MONOLAYERS USED FOR EMBRYO CO-CULTURE *IN VITRO*.**

4.1 EXPERIMENTAL SERIES 1. PRODUCTION OF CYTOKINES BY SELECTED SOMATIC CELL MONOLAYERS USED FOR EMBRYO CO-CULTURE *IN VITRO*.

4.1.1 INTRODUCTION.

Recently cytokines have emerged as important factors within the reproductive tract, influencing key events such as the growth and the implantation of the embryo, (reviewed by Pampfer *et al.*, 1991).

A number of studies have shown that the cytokine Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) is embryotrophic (Robertson *et al.*, 1991) and although no expression of GM-CSF was evident in embryos (Murray *et al.*, 1990), this cytokine was found to be produced by uterine luminal and glandular epithelium (Robertson and Seamark, 1990,1991). The role or involvement of other cytokines such as Interleukins (IL) IL-2, IL-3 and IL-6, during the peri-implantation stage of development has not as yet been fully determined (Murray *et al.*, 1990).

In view of the results described in chapters 2 and 3, that is, the suitability of uterine cell monolayers and other somatic cell lines as a substratum for enhanced embryo development *in vitro* during the pre-implantation stage of development, conditioned media from these cell monolayers were assayed for a number of cytokines, with the aim of obtaining a better understanding of the possible role of these growth factors during the peri-implantation stage of development.

4.1.2 MATERIALS AND METHODS.

4.1.2.1 Production of conditioned media.

Conditioned medium was prepared from amnion cells, FL amnion cells, L cells and uterine cells in monolayer culture as described previously, and assayed for IL-2, IL-3, GM-CSF and total CSA. In addition whole uteri from day 3 pregnant female mice (obtained as

previously described) were used to prepare epithelial and stromal cell monolayers and the subsequent collection of conditioned media which were assayed for GM-CSF and IL-6. The procedure used was a modification of the protocol described by Sherman (1978), and is briefly as follows (see Chapter 2 for more detailed description): individual uteri were trimmed, slit lengthwise to expose the luminal surface and washed twice in cold phosphate buffered saline (PBS) pH 7.2, within 10-15 minutes of collection, under sterile conditions. Uteri were then placed in a tube containing 0.5% trypsin, 2.5% pancreatin in PBS (1 uterine horn per ml) for 1 hour on ice followed by 1 hour at room temperature. Approximately 10% MEM containing 10% FCS was added to final volume of enzymatic solution. The mixture was then agitated by passage up and down through a sterile plastic pipette for a minute. The supernatant which contained epithelial and other detached cells was centrifuged at 200g for 10 minutes, the cell pellet was washed once in MEM. The stromal population was obtained from the remaining tissue by placing it into a petri dish containing EBSS-T-E (1 uterine horn per ml) for 45 minutes at 37°C. The epithelial fraction was similarly agitated, following addition of MEM+FCS, and the supernatant containing the stromal cells was pelleted at 200g for 10 minutes, then washed again in MEM. Both the epithelial and stromal cells were plated out in medium alone or in medium supplemented with either oestradiol (3.7×10^{-5} M) or progesterone (3.2×10^{-6} M) or a combination of both at a concentration of 1.0×10^6 cells per well in four well multidishes, and incubated for at least 24 hours at 37°C in 5% CO₂ in air. The conditioned media was harvested after a 24-48 hour culture period, centrifuged at 500g for 10 minutes immediately following collection, stored at -80°C until assayed.

4.1.2.2 Interleukin-2 (IL-2) Assay.

Conditioned media from amnion cells, FL amnion, L cell and uterine monolayers were assayed for IL-2 using the IL-2 dependent T-cell line CTLL (Gillis and Watson, 1980). Assays were performed by incubating duplicate serial dilutions of aliquots of conditioned media with 5×10^3 cells in 200 μ l of RPMI with 10% FCS overnight, in 96

well microtitre trays (Costar). Cultures were then pulsed with 1 μCi of ^3H -thymidine for 6 hours, harvested using a Titretech automated cell harvester onto glass fibre paper, then scintillation fluid was added to each sample and using a counter incorporation of label was assessed. IL-2 assays were standardized against recombinant IL-2, minimum detectable amounts were 0.5 'BRMP' (NIH Biological Response Modifiers Program) U/mL.

4.1.2.3 Interleukin-3 (IL-3) Assay.

Conditioned media from amnion cells, FL amnion cells, L cell and uterine cell monolayers were assayed for IL-3 as described by Kelso and Owens (1988) using the IL-3 dependent cell line 32D c13 (Greenberger *et al.*, 1983). Assays were done by incubating duplicate serial dilutions of aliquots of conditioned media with 2×10^3 cells in 200 μl of RPMI with 10% FCS for 2 days, in 96 well microtitre trays. Cultures were then pulsed with 1 μCi of ^3H -thymidine for 6 hours, and incorporation of label was quantified as for the IL-2 assays. Standardization for IL-3 assays was against recombinant IL-3 and minimum detectable amounts were 0.5 CFUc U/mL.

4.1.2.4 Interleukin-6 (IL-6) Assay.

Medium alone (control) and conditioned media from uterine, epithelial and stromal cell monolayers with or without steroids were assayed for IL-6 using the IL-6 dependent cell line 7TD1 (Van Snick *et al.*, 1986). Assays were performed by incubating duplicate serial dilutions of aliquots of samples with 2×10^3 7TD1 cells in 200 μl RPMI plus 1% NS (Nutridoma, Boehringer Mannheim), for 3 days. Using a modification of the method described by Mossman (1983), the number of cells was estimated by colourimetric determination of conversion of MTT (Sigma, USA) to formazan. In brief, 25 μl of 4mg/ml MTT in RPMI was added to each well (96 well microtitre tray) followed by a 4 hour incubation at 37°C, subsequently medium was aspirated and formazan product was solubilized by the addition of 100 μl of 95% ethanol, and absorbance at 570 nm measured.

A dose response curve was used to determine IL-6 titre and the minimum detectable amount of IL-6 was 5 U/ml where 50 U/ml was defined as producing half maximal 7TD1 growth.

4.1.2.5 Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) Assay.

Amnion, FL amnion, L cell and uterine monolayer conditioned media were assayed for GM-CSF using a GM-CSF dependent cell line FD 5/12 (Duhrsen 1988), by a procedure described by Kelso and Owens (1988). Briefly, assays were done by incubating duplicate serial dilutions of aliquots of conditioned media with 2×10^3 cells in 200 μ l of RPMI with 10% FCS in 96 well microtitre trays, for 2 days. Cultures were then pulsed with 1 μ Ci of 3 H-thymidine for 6 hours, harvested on glass fiber and counted as for the interleukin assays. Using recombinant GM-CSF to standardize results, GM-CSF titres were determined from dose response curves, and results were expressed in CFUc units/ml where 50 U equals the concentration of GM-CSF stimulating half maximal colony numbers to develop in a bone marrow assay, with 0.5 U/ml of GM-CSF being the minimum detectable amount.

4.1.2.6 Total Colony Stimulating Activity (CSA) assay.

Conditioned media from amnion, FL amnion, L cell and uterine cell monolayers were assayed for total colony stimulating activity, it is known as such because it includes Macrophage-Colony Stimulating Factor (M-CSF), Granulocyte-Colony Stimulating Factor (G-CSF), GM-CSF and IL-3 activity. Total CSA was assayed by stimulation of colony formation in agar of mouse bone marrow cells virtually as described by Metcalf (1970). The procedure was performed as follows: bone marrow cells were obtained from the femur of adult female Balb-C mice, washed and subsequently all adherent cells were removed by incubation for 1 hour in a plastic flask. Agar medium was prepared from double strength Iscove's modified DMEM supplemented with 20% FCS and antibiotics, mixed with an equal volume of 0.6% volume/weight Bacto-agar. Triplicate 1.0 ml cultures were set up in 35mm plastic Petri dishes and contained 5×10^4 bone marrow cells and 100 μ l of serially

diluted conditioned medium in agar medium and allowed to set. Cultures were then incubated for 12 days at 37°C in a humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂. Cultures were then fixed in 2.5% glutaraldehyde in PBS pH 7.2 for 24 hours, floated onto glass slides and left to dry completely, then stained. Slides were scored for monocyte, neutrophil, eosinophil and mixed colonies. CSF titres were determined from dose response curves and expressed as U/ml where 50 U is the CSF concentration stimulating half maximal colony numbers to develop.

4.1.3 RESULTS.

4.1.3.1 IL-2, IL-3, GM-CSF and total CSA in conditioned media from selected somatic cell monolayers.

As described in the previous chapter not all somatic cell monolayers provide a suitable substratum for embryo growth and development in a co-culture system; it was therefore necessary to assay the conditioned media obtained from these cell monolayers for specific growth and developmental factors in order to ascertain which could be considered as embryotrophic and which inhibitory to the embryo. Subsequently, IL-2, IL-3, GM-CSF and total CSA were assayed for in the conditioned media. When all of the conditioned media were assayed it became evident that any IL-2 and IL-3 present in the conditioned media was below the detectable levels as can be seen in Table 19. GM-CSF on the other hand was present at a level of 5.2 U/ml in the uterine conditioned media compared to undetectable levels in all other conditioned media tested. Total CSA was found in the uterine CM (8U/ml) and also in L cell CM (30U/ml), with undetectable levels in the remaining conditioned media. (Table 19).

Once it was established that GM-CSF was produced by uterine cells in monolayer culture and released into the media it was possible to assay conditioned media from epithelial and stromal cells in monolayer culture in the presence or absence of steroid hormone to determine which cell type was responsible for this production and also see if

this production was steroid hormone dependent. In the absence of steroids GM-CSF production by epithelial cells was considerably greater (138.8 U/ml) than the level detected in stromal CM (23.0 U/ml) as can be seen in Table 20. The levels of GM-CSF detected in epithelial CM in the presence of oestradiol (46.1 U/ml), progesterone (61.7 U/ml), and when the two steroids were combined (66.5 U/ml), are lower than the levels produced in the absence of steroids. In the case of the stromal CM the levels of GM-CSF detected remained low in the presence of oestradiol (27.5 U/ml), progesterone (38.9 U/ml) and also when the two steroids were combined (19.4 U/ml), compared to the level detected in the absence of steroids. (Table 20).

Table 19. Assay of Amnion, FL amnion, L cell and Uterine cell monolayer conditioned media for IL-2, IL-3, GM-CSF and total CSA.

| Monolayer | IL-2 | IL-3 | GM-CSF | CSA |
|-----------|------|------|--------|-----|
| Amnion | <0.1 | <0.1 | <1 | <5 |
| FL amnion | <0.1 | <0.1 | <1 | <5 |
| L cell | <0.1 | <0.1 | <1 | 30 |
| Uterine | <0.2 | <0.2 | 5.2 | 8 |

Table 20. Assay of epithelial and stromal cell monolayer conditioned media in the presence or absence of steroids for GM-CSF activity (CFUc U/ml).

| Monolayer | GM-CSF (U/ml) | | | |
|------------|---------------|------|------|------|
| | 0 | E | P | PE |
| Epithelial | 138.8 | 46.1 | 61.7 | 66.5 |
| Stromal | 23.0 | 27.5 | 38.9 | 19.4 |

KEY: 0 indicates the absence of steroids in the media used.

E indicates media was supplemented with 3.7×10^{-5} M oestradiol.

P indicates media was supplemented with 3.2×10^{-6} M progesterone.

PE indicates media was supplemented with both steroids at above concentrations.

4.1.3.2 IL-6 in conditioned media from mixed uterine, epithelial and stromal cell monolayers.

In order to determine whether mixed uterine cell monolayers produce IL-6, therefore contributing to the embryotrophic effect seen in steroid supplemented embryo-uterine co-culture (seen in Chapter 2), CM collected from mixed uterine monolayers was assayed for the presence of IL-6. In addition, CM from epithelial and stromal cell monolayers, with or without steroids, were also assayed to identify which cell type, if any, was responsible for any IL-6 production and whether this production was steroid hormone dependent. All CM assayed showed a considerable amount of IL-6 compared to media alone (< 10 U/ml). The amount of IL-6 detected in mixed uterine CM (9,500 U/ml) in the absence of steroids was less than the amount found in epithelial cell CM (17,400 U/ml) and stromal cell CM (58,900 U/ml) under the same conditions. In the presence of oestradiol, the amount of IL-6 detected was reduced in both the epithelial CM (9,100 U/ml) and stromal CM (8,500 U/ml) compared to the levels detected in epithelial and stromal CM in the absence of steroids. The amount of IL-6 produced by epithelial cells in the presence of progesterone (17,200 U/ml) was equivalent to that produced in the absence of steroids, and in the presence of both progesterone and oestradiol there was a considerable increase in the amount of IL-6 produced (78,600 U/ml). When stromal cells were cultured in the presence of progesterone or progesterone and oestradiol there was a considerable decrease in the amount of IL-6 produced by these cells (< 1,000 U/ml). (Table 21).

Table 21. Assay of uterine, epithelial and stromal cell monolayer conditioned media with/without steroids for IL-6 activity (U/ml).

| Monolayer | IL-6 (U/ml) | | | |
|-------------|-------------|-------|---------|---------|
| | 0 | E | P | PE |
| Media alone | < 10 | *N/D | N/D | N/D |
| Uterine | 9,500 | N/D | N/D | 13,300 |
| Epithelial | 17,400 | 9,100 | 7,200 | 78,600 |
| Stromal | 58,900 | 8,500 | < 1,000 | < 1,000 |

*N/D not done.

4.1.4 DISCUSSION.

There is mounting evidence to suggest that a number of growth factors, particularly the cytokines play a crucial role in the formation and maintenance of extraembryonic tissues during pregnancy (see reviews by Pollard, 1990., Pampfer *et al.*, 1991). Uzumaki *et al.* (1989) have identified receptors for granulocyte-colony stimulating factor (G-CSF) on human trophoblast cells and placental membranes. Wegmann *et al.* (1989) have shown that GM-CSF is synthesized by decidual cells, and stimulates ^3H -thymidine uptake by murine placental cells as does IL-3 (Athanasakis *et al.*, 1987). Colony stimulating factor-1 (CSF-1) synthesis by murine uterine glandular epithelium, regulated by the synergistic action of oestradiol-17B and progesterone, is elevated 10 000 fold during pregnancy (Pollard *et al.*, 1987), and uterine CSF-1 mRNA expression is seen as early as day 3, peaking at days 14-15 of pregnancy with CSF-1 mRNA expression evident in decidual cells and trophoblast at day 7.5 (Arceci *et al.*, 1989).

The results presented here clearly reveal that IL-2 and IL-3 could not be detected in any of the conditioned media assayed, indicating very little production of these factors *in vitro*. Recent work by Boehm *et al.* (1989), suggests that IL-2 production by the placenta could be important in the regulation of production of immunoregulatory substances by the placenta or it could have a role as a growth or maturation factor for the placenta, and

although a number of possible roles have been postulated, no definite role(s) has as yet been determined. The possible role of IL-2 or IL-3 in early pregnancy has not been studied but from the work presented here it appears as though their importance in early pregnancy may be minimal.

GM-CSF and CSA activity was evident in uterine cell conditioned media, with epithelial cell conditioned media showing the highest GM-CSF levels, although there was no evidence for a marked increase in output following inclusion of either oestradiol or progesterone or the steroids in combination. These results are consistent with other *in vitro* studies (Robertson *et al.*, 1990, Robertson and Seamark, 1990), although no GM-CSF has been detected in uterine flushings collected from Day 3-6 pregnant mice, the author suggests that this could be due to the extensive dilution of the collected uterine fluid from these mated mice.

Interestingly, the level of IL-6 activity found in epithelial cell conditioned media in the presence of both oestradiol and progesterone in combination was >8 fold higher than the level of IL-6 activity seen in the mixed uterine cell conditioned media both in the presence and absence of steroids, and the level of activity observed in the stromal cell conditioned media was high in the absence of steroids and considerably reduced in the presence of steroids. Others have also investigated the possible involvement of IL-6 during gestation. Work by Nishino *et al.* (1990) suggests that synthesis of IL-6 by human placental trophoblast cells which also express IL-6 receptors, could also be an intermediary in the autocrine regulation of hCG synthesis of these cells. A recent study by Robertson *et al.* (1992) has demonstrated epithelial cells in the endometrium as a primary source of IL-6 and this synthesis continues following mating and also later in pregnancy, in addition leukocytes and stromal cells also contribute to this synthesis. This work is supported by the findings of Tabidzadeh *et al.* (1989) who also found that human endometrial stromal cells synthesize IL-6. Even though a number of studies including the work described here, have reported the synthesis of IL-6 within the reproductive tract, its role in early embryogenesis is yet to be determined.

Production of GM-CSF and IL-6 during the preimplantation period by uterine cells may contribute in part to the enhanced embryo development and survival observed in the co-culture with mixed uterine cells and also may contribute to the stimulation of earlier hatching and implantation of blastocysts observed in the co-culture with epithelial and stromal cells, as described in chapters 2 and 3. The CSA observed in conditioned media from L cells could also in part explain the enhanced embryo development and survival observed in co-culture with these cells. A large number of events accompany decidualization including cellular proliferation, tissue remodelling and vascularization, which are important in the preparation of the uterus for embryo implantation. It is clear that cytokines play an important role in these events, as well as embryo development (Robertson *et al*, 1990). The precise role for these and other cytokines is yet to be determined, and certainly warrants further investigation.

4.2 EXPERIMENTAL SERIES 2. PRODUCTION OF PROSTAGLANDINS BY UTERINE, EPITHELIAL AND STROMAL CELL MONOLAYERS.

4.2.1 INTRODUCTION.

A number of early reports suggested that both Prostaglandin E₂ (PGE₂) and Prostaglandin F₂ α (PGF₂ α) are involved in the process of implantation in both mice (Holmes and Gordashko, 1980) and rats (Kennedy, 1977, Phillips and Poyser, 1981). Prostaglandins have also been reported to be secreted by blastocysts; a summary of these studies was recently reviewed by Lewis (1989) and implicates possible functions of prostaglandins in early development. The uterus also secretes prostaglandins which have a role in implantation and decidualization in early pregnancy (Evans and Kennedy, 1977).

This study was undertaken to determine whether prostaglandins were being produced by mixed uterine, epithelial and/or stromal cell monolayers *in vitro*, and whether this production was steroid dependent.

4.2.2 MATERIALS AND METHODS.

4.2.2.1 Production of conditioned media from mixed uterine, epithelial and stromal monolayers.

Conditioned media were prepared from mixed uterine, epithelial and stromal cell monolayers in the presence or absence of steroids as previously described. Once the conditioned media were harvested they were aliquoted out and stored at -80°C until assayed.

4.2.2.2 PGE2 assay.

Samples of conditioned media were thawed and an equivalent volume of methyl oximating reagent was added to samples and left to oximate at room temperature overnight. All samples were assayed for PGE2 (by Dr Robert Norman and his staff at TQEH) following the procedure described by Kelly *et al.* (1986). Briefly the procedure is as follows: all tubes for both standards and samples were set up in duplicate and contained $100\ \mu\text{l}$ of standard (maximum concentration of PGE2 was $1000\ \text{pg}/100\ \mu\text{l}$, other tubes were serial dilutions) or sample, $100\ \mu\text{l}$ ^3H -PGE ([5,6,8,11,12,14,15(n)- ^3H]Prostaglandin E2, Amersham; diluted in TRIS/EDTA/gelatin buffer, $5000\ \text{cpm}/100\ \mu\text{l}$), $100\ \mu\text{l}$ PGE2 antiserum, all tubes were then vortexed and incubated at 37°C for 1 hour. Following this incubation all tubes were incubated at 4°C overnight, and then $500\ \mu\text{l}$ of cold Dextran-Charcoal was added, each tube was vortexed and incubated at 4°C for a further 10 minutes. The assay tubes were then centrifuged for 10 minutes, the supernatant decanted and 1 ml scintillant added per tube. Tubes were vortexed, counted and the results calculated using the LKB (Wallac)-Logit Log Plot program.

4.2.2.3 PGF₂ α assay.

Samples of conditioned media were thawed and assayed for PGF₂ α (by Dr Robert Norman and his staff at TQEH) following the procedure described by Norman *et al.* (1981). Briefly the procedure is as follows: A volume of 100 μ l of standards (at a maximum concentration of 4624 pg/100 μ l, and serially diluted down to 36.1 pg/100 μ l) and samples were added to tubes in triplicate, 100 μ l of tracer ([5,6,8,9,11,12,14,15(n)-³H]PGF₂ α , 20,000 cpm/100 μ l) was added to all tubes and 100 μ l of PGF antiserum (working dilution 1:250) was added to all tubes except total and Nonspecific binding tubes. The tubes were vortexed and incubated at 4°C overnight. Following the incubation, 500 μ l of Dextran-Charcoal was added to all tubes, all tubes were vortexed and incubated at 4°C for 10 minutes. The assay tubes were then centrifuged for 10 minutes at 4000g the supernatant decanted and 1 ml scintillant added per tube. Tubes were vortexed, counted and the results calculated using the LKB (Wallac)-Logit Log Plot program.

4.2.3 RESULTS.

4.2.3.1 PGE₂ activity in conditioned media from mixed uterine, epithelial and stromal cell monolayers.

In order to determine whether PGE₂ was important to embryos in the pre-implantation period of development, production of PGE₂ by uterine mixed and individual cell types, and also whether this production was steroid dependent was investigated.

Following the assay of all the conditioned media, it became apparent that the mixed uterine cell population produced the highest level of PGE₂ (268 μ g/L) in the presence of both oestradiol and progesterone (Table 22). When conditioned media from epithelial monolayers were assayed in the absence of steroids, the level of PGE₂ activity detected was 17.16 μ g/L, in the presence of oestradiol the level of PGE₂ activity detected was 7.80 μ g/L, in the presence of progesterone the level of PGE₂ detected was 5.86 μ g/L, and in the

presence of both steroids the level of PGE2 detected was 4.42 $\mu\text{g/L}$ (Table 22). Levels of PGE2 activity detected in conditioned media from stromal cell monolayers was considerably higher than those measured for conditioned media from epithelial cell monolayers under all conditions. In the absence of steroids the level of PGE2 activity detected in stromal cell monolayer conditioned media was comparable to the levels seen in mixed uterine monolayer conditioned media in the presence of both steroids (229 $\mu\text{g/L}$) as can be seen in Table 22. In the presence of oestradiol the level of activity decreased to 109 $\mu\text{g/L}$ and in the presence of progesterone the amount of PGE2 detected dropped down even further to 42.0 $\mu\text{g/L}$. The amount of PGE2 activity detected in the presence of both steroids was 58.8 $\mu\text{g/L}$ (Table 22).

Table 22. Assay of mixed uterine, epithelial and stromal cell monolayer conditioned media in the presence or absence of steroids for PGE2 activity ($\mu\text{g/L}$).

| | PGE2 ($\mu\text{g/L}$) | | | |
|---------------------|--------------------------|------|------|------|
| | 0 | E | P | PE |
| MEM (control media) | 0.11 | N/D | N/D | N/D |
| Mixed uterine | N/D | N/D | N/D | 268 |
| Epithelial | 17.16 | 7.80 | 5.86 | 4.42 |
| Stromal | 229 | 109 | 42.0 | 58.8 |

N/D not done.

4.2.3.2 PGF2 α activity in conditioned media from mixed uterine, epithelial and stromal cell monolayers.

In order to determine whether PGF2 α is being produced by the uterus at the pre-implantation period of development, and its importance to embryo development, conditioned media from mixed uterine, epithelial and stromal monolayers were assayed for PGF2 α activity.

When control media was assayed 0.65 $\mu\text{g/L}$ PGF 2α activity was detected which was considered as the baseline activity for all conditioned media assayed. Conditioned media from mixed uterine monolayers in the presence of both oestradiol and progesterone showed a PGF 2α activity of 37.54 $\mu\text{g/L}$ (Table 23). To identify which cell type in the uterus was responsible for PGF 2α production and whether this activity was steroid dependent, both epithelial and stromal conditioned media were also assayed. Conditioned media from epithelial monolayers in the absence of steroids showed 44.16 $\mu\text{g/L}$ PGF 2α activity, in the presence of oestradiol, PGF 2α activity was 35.08 $\mu\text{g/L}$, in the presence of progesterone was 27.05 $\mu\text{g/L}$ and in the presence of both steroids PGF 2α activity was 31.57 $\mu\text{g/L}$ (Table 23). PGF activity was the highest in conditioned media from stromal monolayers in the absence of steroids at 116.80 $\mu\text{g/L}$, this decreased in the presence of oestradiol to 98.2 $\mu\text{g/L}$ and in the presence of progesterone PGF 2α activity was 81.9 $\mu\text{g/L}$, and in the presence of both steroids the activity from the stromal population of cell was at its lowest at 60.0 $\mu\text{g/L}$ (Table 23).

Table 23. Assay of mixed uterine, epithelial and stromal cell monolayer conditioned media in the presence or absence of oestradiol and progesterone for PGF 2α activity ($\mu\text{g/L}$).

| | PGF 2α ($\mu\text{g/L}$) | | | |
|---------------------|-----------------------------------|-------|-------|-------|
| | 0 | E | P | PE |
| MEM (control media) | 0.65 | *N/D | N/D | N/D |
| Mixed uterine | N/D | N/D | N/D | 37.54 |
| Epithelial | 44.16 | 35.08 | 27.05 | 31.57 |
| Stromal | 116.8 | 98.2 | 81.9 | 60.0 |

* N/D not done.

4.2.4 DISCUSSION.

Evidence is accumulating to suggest a possible role for prostaglandins in early pregnancy particularly at the time of implantation. A number of studies have suggested that

the source of prostaglandins in the reproductive tract at the time of implantation can be from the blastocyst or from the endometrium (see review by Kennedy and Armstrong, 1981). Prostaglandins are also known to play an important role later in pregnancy and during parturition in humans (Green *et al.*, 1981; Zakar and Olson, 1992), and throughout the reproductive tract in the male and female (Karim and Hillier, 1979; Kelly, 1981).

Phillips and Poyser (1981) have shown that PGE 2 and PGF2 α production by the uterus peaks at the time of implantation *in vitro* in the rat. Early work by Holmes and Gorgashko (1980) and Yee and Kennedy (1988) also postulated a stimulatory role for prostaglandins in the decidual cell reaction in the mouse and rat. Others have indicated that prostaglandins may play a part in the immunosuppression observed throughout pregnancy (Matthews and Searle, 1987; Wood *et al.*, 1988).

The data presented here demonstrate PGE 2 and PGF2 α production by uterine cells *in vitro* and that production is primarily by the stromal population, but there was a decrease in production of PGF2 α by the stromal cells with the inclusion of steroids, suggesting that production is not steroid dependent. It is possible that the prostaglandins produced by these cells contribute to the enhanced embryo development observed when embryos are co-cultured with the mixed uterine cells *in vitro* as described in chapter 2, and could also contribute to the enhanced hatching, also demonstrated by Biggers *et al.* (1978), and attachment seen when embryos were co-cultured with stromal cells as described in chapter 2.

In addition to these results, earlier studies strongly implicate both PGE 2 and PGF2 α as important factors in regulating and initiating implantation of blastocysts in the reproductive tract in a number of species. Similar to other growth factors within the uterus at this early period in development, prostaglandins could act in an autocrine fashion, initially to upregulate production of prostaglandin synthesis by the uterine cells, and in addition to stimulate vascularization of the endometrium (reviewed by Kennedy and Armstrong, 1981) and also acting in a paracrine fashion by stimulating important changes within the blastocyst, such as blastocoele expansion, hatching (Biggers *et al.*, 1978), glucose metabolism of preimplantation embryos (Khurana and Wales, 1987) and also

stimulate changes in the trophoblast cells necessary for implantation (Holmes and Gordashko, 1980). The precise mechanisms involved in these processes and the role prostaglandins play require further investigation.

CHAPTER 5.

**PRODUCTION OF INSULIN-LIKE GROWTH FACTOR-I (IGF-I),
INSULIN-LIKE GROWTH FACTOR-II (IGF-II) AND INSULIN-LIKE GROWTH
FACTOR-BINDING PROTEINS (IGF-BP) BY SELECTED SOMATIC CELL
MONOLAYERS USED FOR EMBRYO CO-CULTURE *IN VITRO*.**

5.1 INTRODUCTION

The role of growth factors in the growth and development of the conceptus is the focus of active research, in particular, the role of insulin and insulin-like growth factors (IGFs) in the regulation of fetal growth (Gluckman, 1986). Less is known about the role of these growth factors during early embryogenesis.

Although many studies have been conducted to determine the structure, function and physiology of insulin and IGFs (Moses *et al.*, 1980, Marquardt *et al.*, 1981, Dafgård *et al.*, 1985, Hey *et al.*, 1987, Francis *et al.*, 1989) and their receptors (Bala *et al.*, 1983, Nissley *et al.*, 1985, Lin *et al.*, 1986, Morgan *et al.*, 1987, Kiess *et al.*, 1988), few studies have investigated the possible role of IGFs in early embryonic development *in vitro*. IGF-II has been identified as the major fetal somatomedin in humans and rats, both IGF-I and IGF-II receptors being present in fetal tissues (Sara and Hall, 1984). Low levels of IGF-II mRNA have been found in early organogenesis (Pratten *et al.*, 1988). *In vitro* the source of these factors could be provided to developing embryos from serum supplemented media. There appear to be few reports of studies done supplementing culture media with IGFs, which would allow us to examine the possible role of these factors in early embryonic events.

This study was undertaken to examine whether the various somatic cell monolayers used for co-culture with 8-cell embryos *in vitro* produce IGFs and subsequently gain a better understanding into their possible role in the pre-implantation period in early development.

5.2 MATERIALS AND METHODS.

5.2.1 Production of serum-free conditioned media.

Fetal calf serum contains IGFs and since IGF RIAs are sensitive to these levels of growth factors, it was necessary to assay serum free conditioned media for IGF production. Subsequently, serum-free conditioned media was prepared from uterine cells, primary amnion cells, FL amnion and L cells. Cells were grown to confluence in MEM+FCS, then washed twice for 5 minutes each in MEM without FCS (MEM-SF) and fresh serum-free media added. Conditioned media was harvested after a culture period of 48 hours, centrifuged at 200g for 10 minutes to pellet any cells which could contaminate the conditioned media, and aliquoted out in 5 ml tubes, stored at -80°C until assayed.

5.2.2 IGF-I Radioimmunoassay (RIA).

All samples of conditioned media were freeze dried, dissolved in 0.5-1.0 ml acid column mobile phase, microfuge ultrafiltrated through a 0.45 μm filter and 200 μl injected onto a 1 x 30 cm Waters 125 Protein Pak size exclusion chromatography column (254nm), and after 5 ml, 60 200 μl fractions were collected. Once collected, fractions were assayed. The procedure followed can be described briefly as: all tubes for both standards (recombinant h IGF-I) and samples were set up in triplicate, and the RIA was conducted in two incubations. The first incubation was set up with tubes for the standard curve containing 200 μl standard (maximum concentration of standard 10ng/ml, other tubes were serial dilutions) in RIA buffer (0.03M phosphate, pH 7.5, 0.2% (w/v) protamine sulphate, 0.2% (w/v) sodium azide and 0.05% (v/v) Tween 20), 50 μl mobile phase, 30 μl 0.4M Tris, 50 μl of the first antibody (rabbit anti-h-IGF serum at a final dilution of 1/10,000) except in total and blank tubes, and finally 50 μl Tracer (^{125}I -iodo-hIGF, diluted to approx 20,000 cpm/ 50 μl). The sample tubes were also set up to contain: 50 μl of sample fractions, 30 μl 0.04M Tris, 200 μl RIA buffer, 50 μl of first antibody, and 50 μl Tracer.

Total tubes were then capped and all tubes placed at 4°C overnight. The second incubation was performed the following day. To all tubes except totals, 10 µl normal rabbit serum and 50 µl of second antibody (goat anti-rabbit serum) were added, and incubated for 30 minutes at 4°C. At the end of the incubation period 1 ml 5.5% PEG in PBS was added to all tubes except total tubes, and then all tubes were centrifuged at 4,000 rpm for 30 minutes, the supernatant carefully aspirated and remaining pellet counted on appropriate counter, standard curve drawn and levels of IGF-I/fraction/ml evaluated for each sample.

5.2.3 IGF-II Radioreceptorassay (RRA).

All samples of conditioned media were freeze dried, dissolved in 0.5-1.0 ml acid column mobile phase, microfuge ultrafiltrated through a 0.45 µm filter and 200 µl were injected into a size exclusion chromatography column and fractions collected (as for the IGF-I RIA) were assayed for IGF-II by RRA. The procedure used can be described as follows: all tubes were set up in triplicate, and the method of assay involved two incubations. The first incubation was set up with tubes for standards containing 200 µl standard (with maximum concentration of standard being 20ng/ml recombinant ovine IGF-II, and other tubes being serial dilutions) in RRA buffer (1) (0.01M Tris, 0.5% BSA and 10mM CaCl₂), 30 µl 0.4M Tris (except in total tubes), 50 µl mobile phase (except in total tubes), 100 µl oPM (lyophilized sheep placental membranes, with final assay dilution of 1/301) (except in total and blank tubes), 50 µl Tracer (¹²⁵I-rhIGF-II, diluted to 10,000 cpm/50 µl). The sample tubes were also set up to contain: 50 µl of fraction from each sample, 200 µl RRA buffer (1), 30 µl 0.4M Tris, 100 µl oPM, and 50 µl Tracer. Following addition of the tracer all tubes were vortexed, the total tubes capped, and all tubes placed at 4°C overnight. The following day the second incubation was set up, which involved adding 1 ml of RRA buffer (2) (0.01M Tris, 0.5% BSA and 0.1M CaCl₂) to all tubes except the total tubes, all tubes were then centrifuged for 30 minutes at 4200 rpm at 4°C, the supernatant carefully aspirated and the pellet counted in an appropriate counter. A standard curve was plotted and levels of IGF-II/fraction/ml for each sample determined.

5.3 RESULTS.

5.3.1 IGF-I and IGF-BP in conditioned media from selected somatic cell monolayers.

Following IGF-I radioimmunoassay of media alone (control) and conditioned media from uterine cells, L cells, primary amnion cells, and FL amnion cells under serum-free conditions, it was possible to plot pg IGF-I/fraction/ml vs elution volume (mls). As can be seen from Figure 5.1., the level of IGF-I in MEMSF is less than 10pg/fraction/ml and is below the limit of detection for the RIA, throughout all fractions, which provides a good base line. Collectively, the total IGF-I detected in the control media was 32.3pg/ml and 11.7pg/ml IGF-BP (Table 24). The level of IGF-I in pg/fraction/ml increased from this base line for fractions of uterine conditioned media (Figure 5.2.), and the total IGF-I detected was 403.7pg/ml and 646.2pg/ml IGF-BP. Similarly, for fractions of L cell conditioned media (see Figure 5.3. for plot of pg IGF-I/fraction/ml vs elution volume), total IGF-I was 316.8pg/ml and 47.4pg/ml IGF-BP, as can be seen in Table 24. Comparable levels of IGF-I were also seen for conditioned media from primary amnion, where total IGF-I detected was 484.0pg/ml and 1857.8pg/ml IGF-BP, (see Figures 5.4. and Table 24). Fl amnion conditioned media exhibited the highest levels of both IGF-I (3342.3pg/ml) and IGF-BP (4066.5pg/ml), as can be seen in Figure 5.5. and Table 24.

Figure 5.1. IGF-I activity (pg/fraction/ml) in Minimal Essential Medium-Serum Free (MEMSF).

MEMSF was freeze dried, dissolved in 0.5ml acid column Mobile Phase, microfuge ultrafiltrated through a 0.45 μm filter and 200 μl injected onto a Protein Pak size exclusion chromatography column (see Chapter 5.2.2 for details), fractions collected and assayed for IGF-I activity.

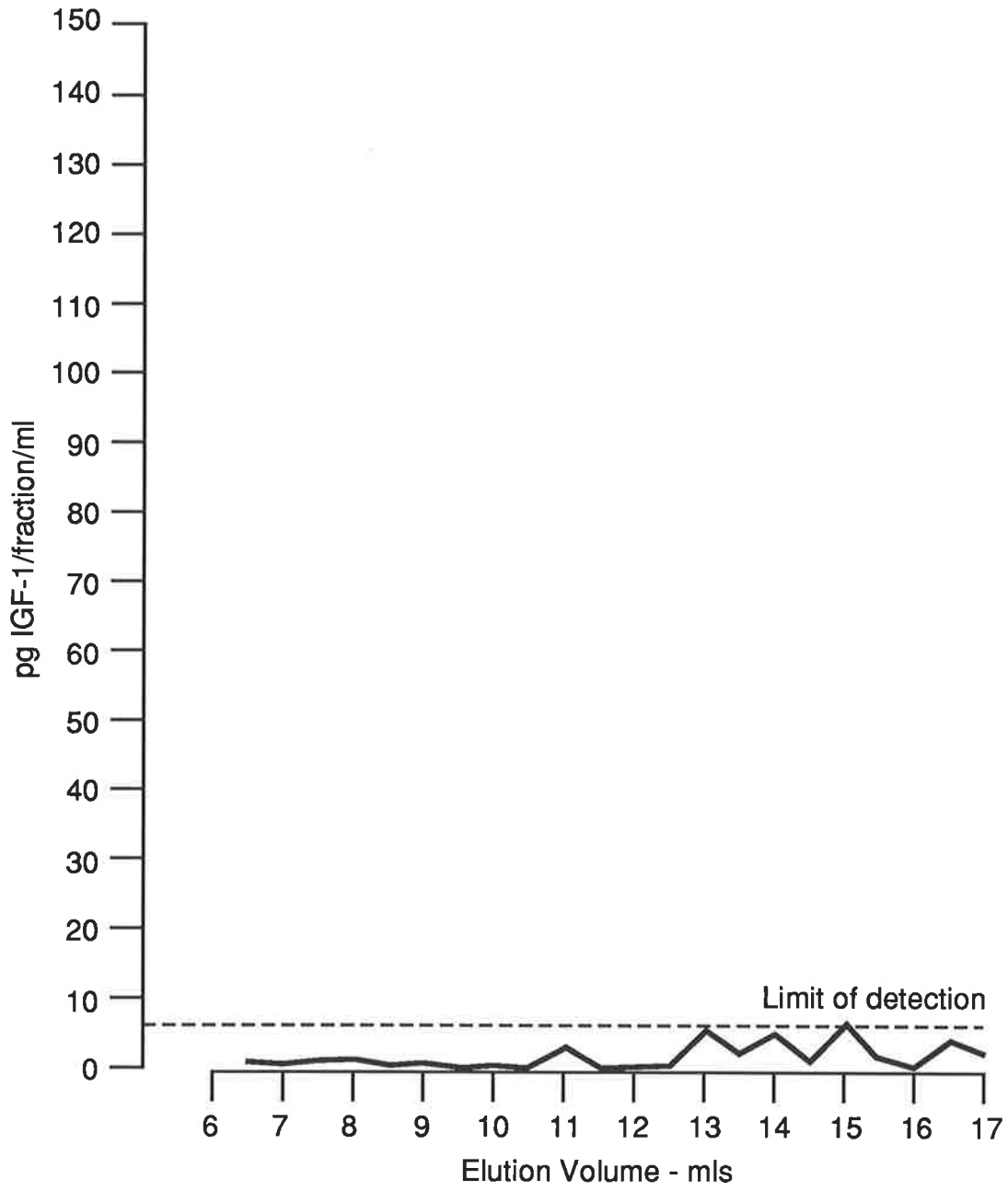


Figure 5.2. IGF-I activity (pg/fraction/ml) in uterine monolayer conditioned media under serum free conditions.

Uterine monolayer conditioned media-serum free was freeze dried, dissolved in 0.5ml acid column Mobile Phase, microfuge ultrafiltrated through a 0.45 μm filter and 200 μl injected onto a Protein Pak size exclusion chromatography column (see Chapter 5.2.2 for details), fractions collected and assayed for IGF-I activity.

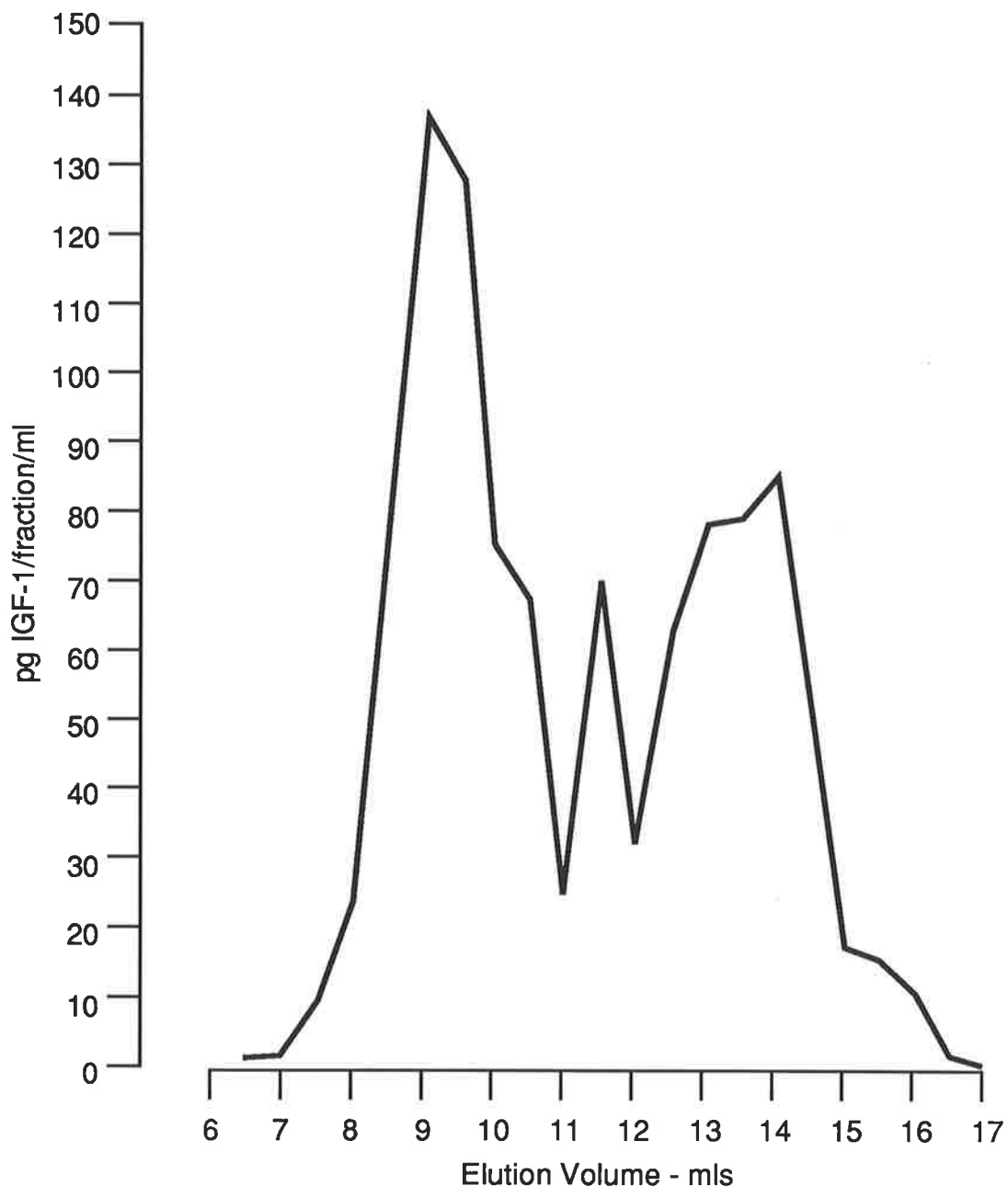


Figure 5.3. IGF-I activity (pg/fraction/ml) in L-cell monolayer conditioned media under serum free conditions.

L-cell monolayer conditioned media-serum free was freeze dried, dissolved in 0.5ml acid column Mobile Phase, microfuge ultrafiltrated through a 0.45 μ m filter and 200 μ l injected onto a Protein Pak size exclusion chromatography column (see Chapter 5.2.2 for details), fractions collected and assayed for IGF-I activity.

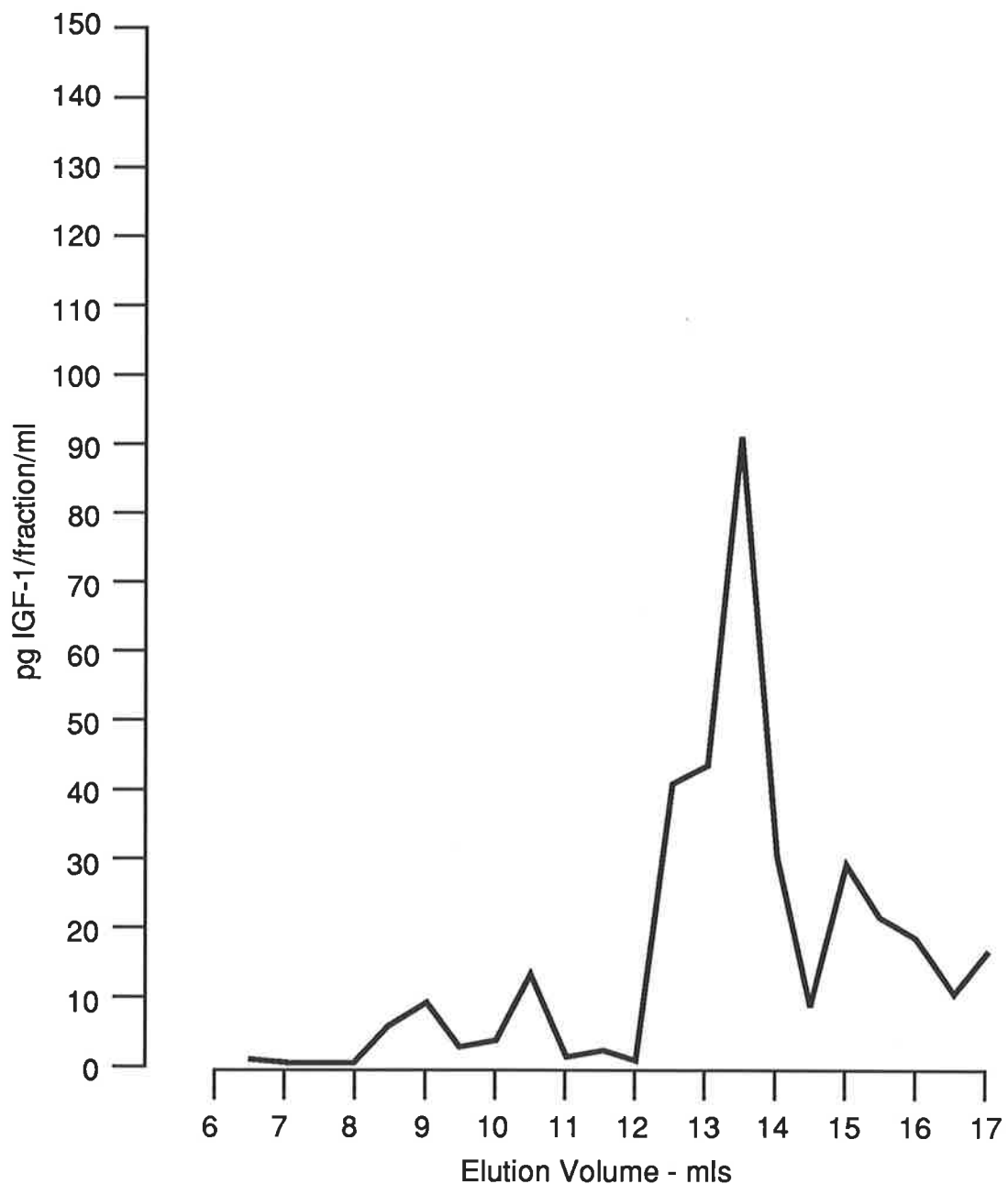


Figure 5.4. IGF-I activity (pg/fraction/ml) in Primary Amnion monolayer conditioned media under serum free conditions.

Primary amnion monolayer conditioned media-serum free was freeze dried, dissolved in 0.5ml acid column Mobile Phase, microfuge ultrafiltrated through a 0.45 μ m filter and 200 μ l injected onto a Protein Pak size exclusion chromatography column (see Chapter 5.2.2 for details), fractions collected and assayed for IGF-I activity.

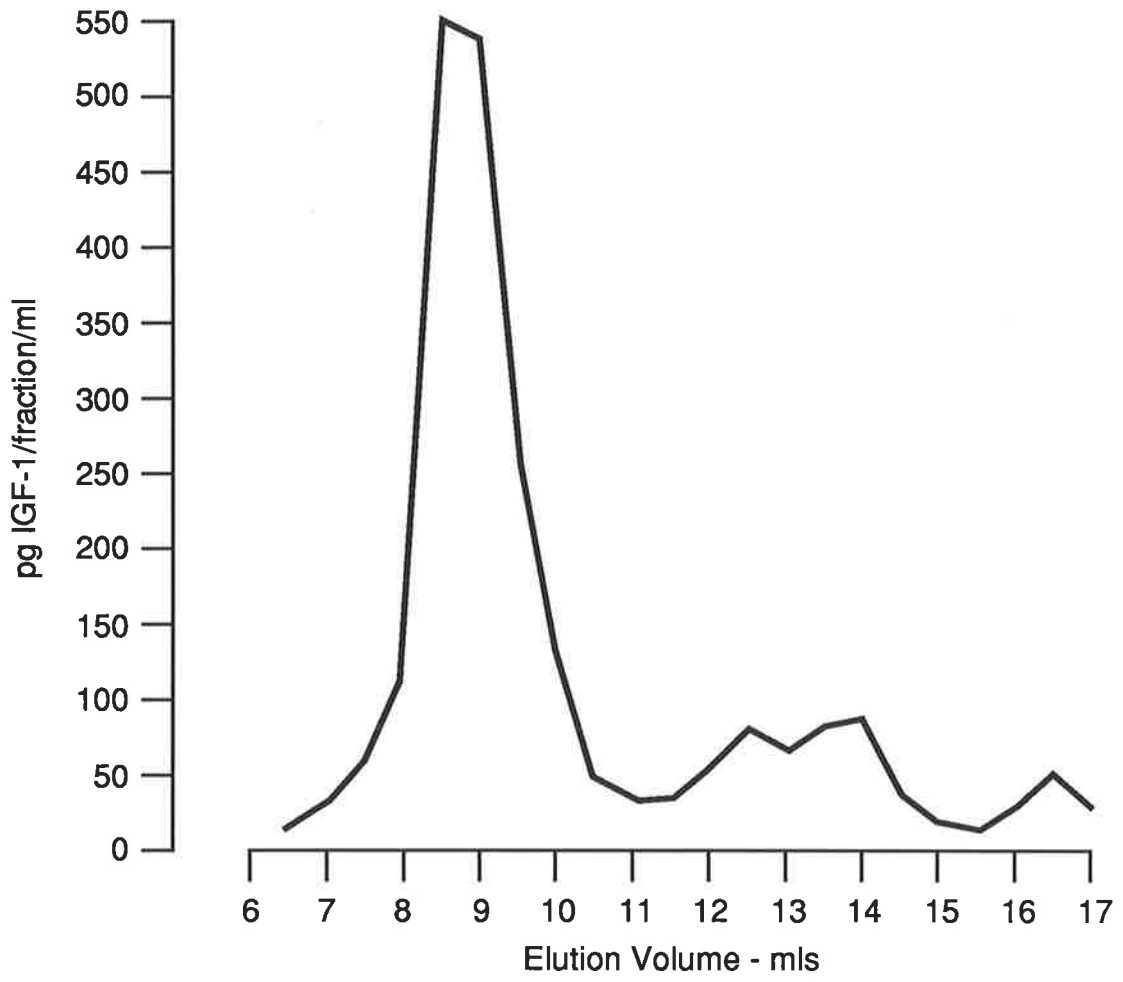
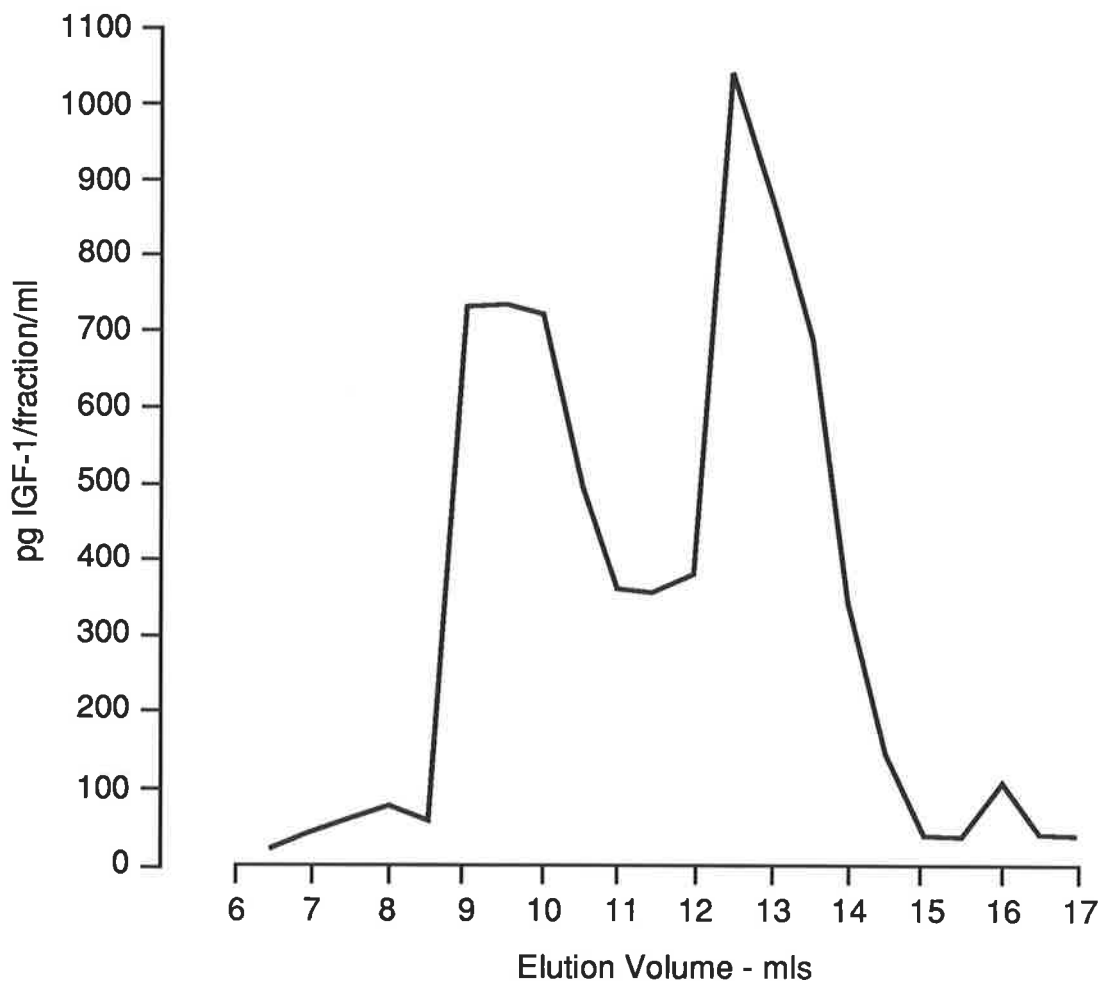
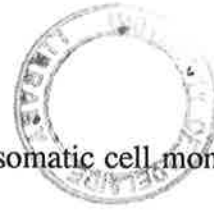


Figure 5.5. IGF-I activity (pg/fraction/ml) in FL amnion monolayer conditioned media under serum free conditions.

FL amnion monolayer conditioned media-serum free was freeze dried, dissolved in 0.5ml acid column Mobile Phase, microfuge ultrafiltrated through a 0.45 μm filter and 200 μl injected onto a Protein Pak size exclusion chromatography column (see Chapter 5.2.2 for details), fractions collected and assayed for IGF-I activity.





5.3.2 IGF-II and IGF-BP in conditioned media from selected somatic cell monolayers.

Once the IGF-II radioreceptor assays were conducted on media alone (control) and conditioned media from uterine cells, L cells, primary amnion and FL amnion cells under serum-free conditions, it was possible to plot ng IGF-II/fraction/ml vs elution volume (mls). As can be seen from Figure 5.6., all of the fractions of media alone assayed contained less than 0.1 ng IGF-II/fraction/ml which was below the limit of detection of the assay, with the total IGF-II detected being 0.365ng/ml CM and 0.275ng/ml IGF-BP, these levels were therefore considered as the base levels of IGF-II and IGF-BP in the media for this group of assays (Table 24). Higher levels of both IGF-II and IGF-BP for fractions of uterine cell CM were detected with total IGF-II being 2.34ng/ml and total IGF-BP being 2.4ng/ml, (Figure 5.7., Table 24). Slightly lower levels were present in fractions of conditioned media from L cells, (Figure 5.8.), with total IGF-II detected being 1.69ng/ml and IGF-BP totalling 0.61ng/ml, (Table 24). From the fractions of primary amnion (Figure 5.9.) the total IGF-II detected was 2.026ng/ml and the total IGF-BP 3.422ng/ml, (Table 24). The highest levels of IGF-II and IGF-BP were detected in the fractions of FL amnion conditioned media, (Figure 5.10.). The total IGF-II in this CM was 4.445ng/ml and the total IGF-BP detected was 5.938ng/ml, (Table 24).

Table 24. The amount of IGF-I (pg/ml), IGF-II and IGF-BP in serum-free conditioned media from selected somatic cell lines used to support embryonic development *in vitro*.

| Conditioned Media | RIA IGF-I (pg/ml) | | RRA IGF-II (ng/ml) | |
|-------------------|-------------------|--------|--------------------|--------|
| | IGF-I | IGF-BP | IGF-II | IGF-BP |
| MEMSF | 32.3 | 11.7 | 0.365 | 0.275 |
| Uterine | 403.7 | 646.2 | 2.34 | 2.4 |
| L cell | 316.8 | 47.4 | 1.69 | 0.61 |
| Primary amnion | 484.0 | 1857.8 | 2.026 | 3.422 |
| FL amnion | 3342.3 | 4066.5 | 4.445 | 5.938 |

Figure 5.6. IGF-II activity (ng/fraction/ml) in Minimal Essential Medium which is serum free (MEMSF).

MEMSF was freeze dried, dissolved in 0.5ml acid column Mobile Phase, microfuge ultrafiltrated through a 0.45 μ m filter and 200 μ l injected onto a Protein Pak size exclusion chromatography column (see Chapter 5.2.3 for details), fractions were collected and assayed for IGF-II activity.

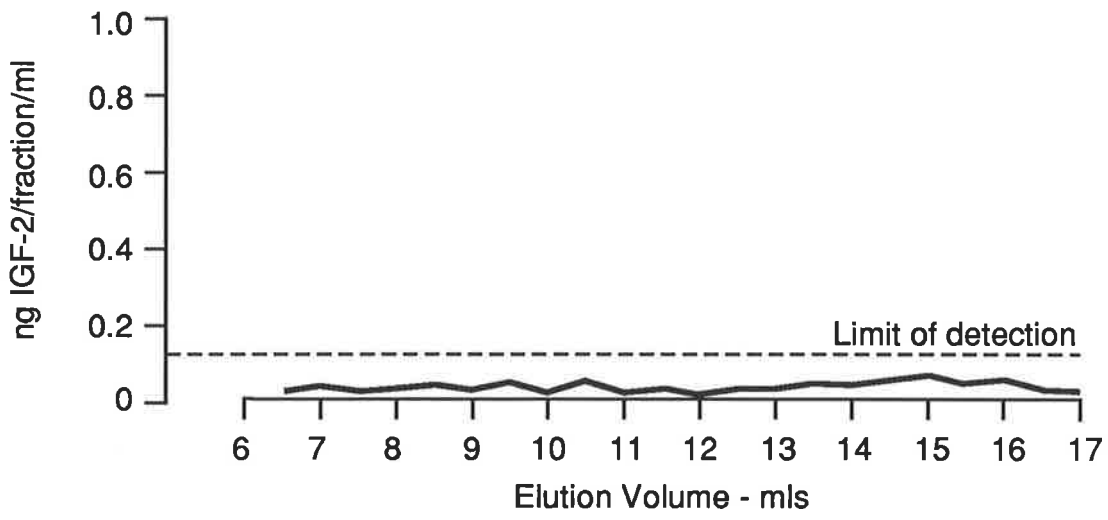


Figure 5.7. IGF-II activity (ng/fraction/ml) in Uterine cell monolayer conditioned media under serum free conditions.

Uterine cell monolayer conditioned media-serum free was freeze dried, dissolved in 0.5ml acid column Mobile Phase, microfuge ultrafiltrated through a 0.45 μm filter and 200 μl injected onto a Protein Pak size exclusion chromatography column (see Chapter 5.2.3 for details), fractions were collected and assayed for IGF-II activity.

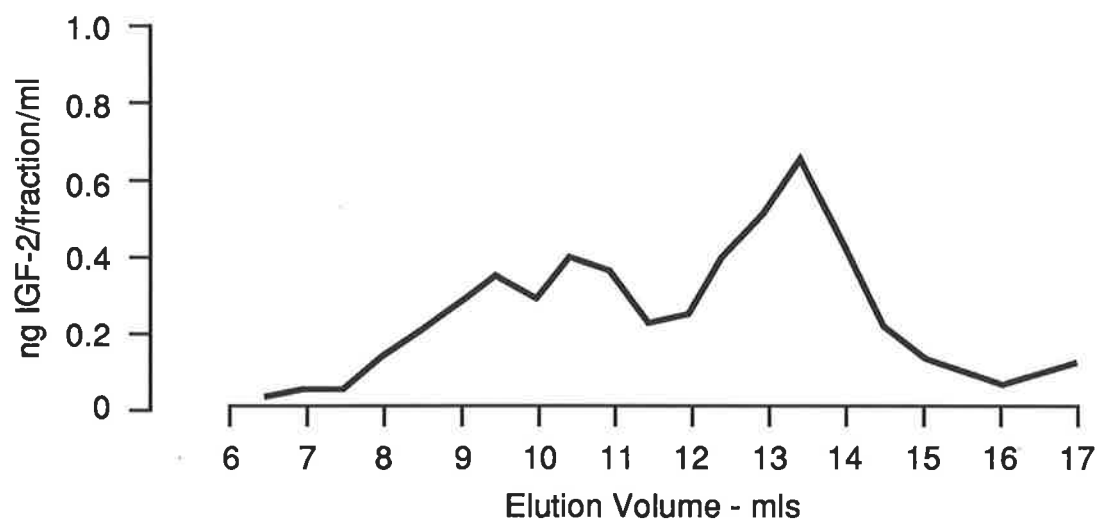


Figure 5.8. IGF-II activity (ng/fraction/ml) in L-cell monolayer conditioned media under serum free conditions.

L-cell monolayer conditioned media-serum free was freeze dried, dissolved in 0.5ml acid column Mobile Phase, microfuge ultrafiltrated through a 0.45 μm filter and 200 μl injected onto a Protein Pak size exclusion chromatography column (see Chapter 5.2.3 for details), fractions were collected and assayed for IGF-II activity.

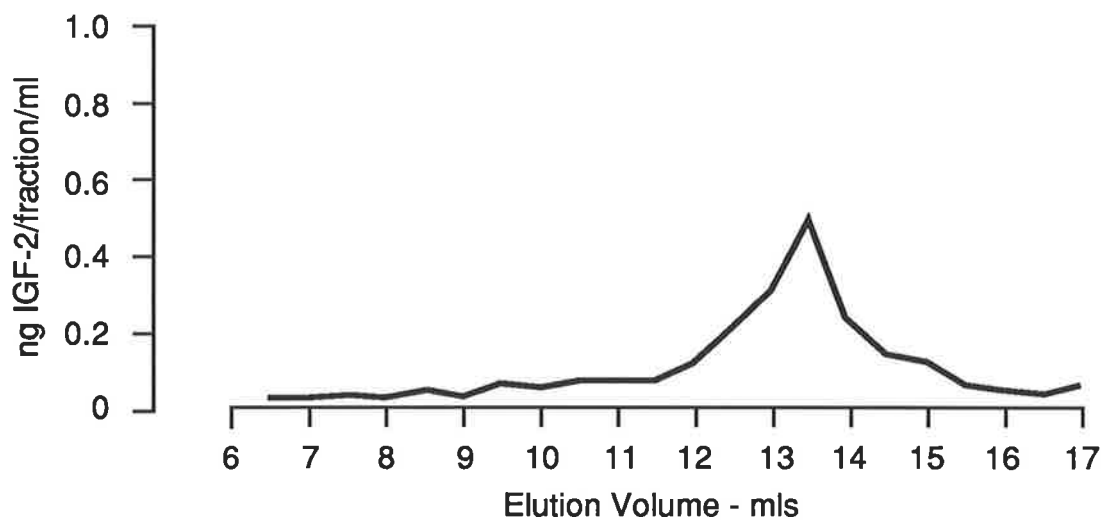


Figure 5.9. IGF-II activity (ng/fraction/ml) in Primary Amnion monolayer conditioned media under serum free conditions.

Primary amnion monolayer conditioned media-serum free was freeze dried, dissolved in 0.5ml acid column Mobile Phase, microfuge ultrafiltrated through a 0.45 μm filter and 200 μl injected onto a Protein Pak size exclusion chromatography column (see Chapter 5.2.3 for details), fractions were collected and assayed for IGF-II activity.

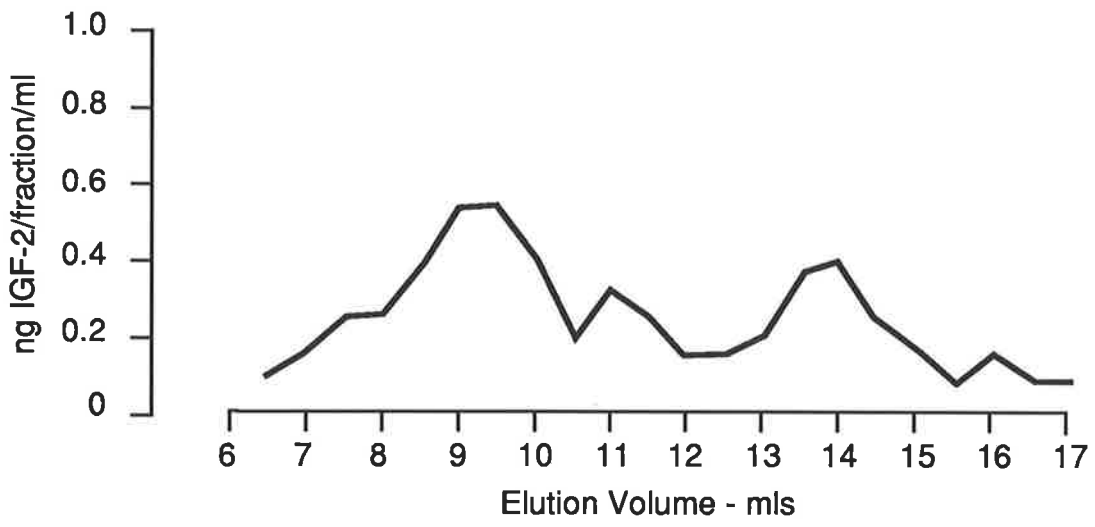
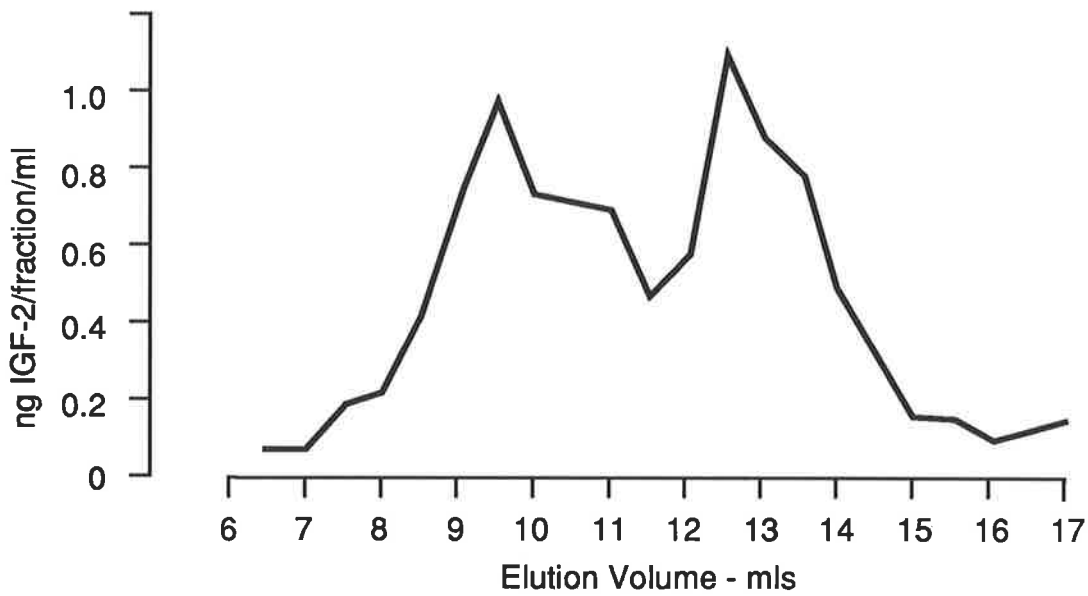


Figure 5.10. IGF-II activity (ng/fraction/ml) in FL amnion monolayer conditioned media under serum free conditions.

FL amnion monolayer conditioned media-serum free was freeze dried, dissolved in 0.5ml acid column Mobile Phase, microfuge ultrafiltrated through a 0.45 μm filter and 200 μl injected onto a Protein Pak size exclusion chromatography column (see Chapter 5.2.3 for details), fractions were collected and assayed for IGF-II activity.



5.4 DISCUSSION.

As reported earlier (Robertson *et al.*, 1991) a number of the cell lines considered in this study (uterine, L cell and primary amnion) provide a suitable substratum for 8-cell murine embryos to develop on *in vitro*. The results presented here seem to indicate that all conditioned media from cell lines assayed produce substantial amounts of IGF-I, IGF-II and associated IGF-BP compared to control media. It is interesting to note that the cell lines that provide an embryotrophic environment for embryos to develop in, produce relatively less amounts of IGFs than does FL amnion which is considered as a unsuitable substratum for embryo growth and development *in vitro*.

A recent study by Rao *et al.* (1990), investigated the effect of culturing murine 2-cell, 8-cell, morula and blastocyst stage embryos in media supplemented with either insulin, IGF-I or IGF-II for 1 hour and assessed the effect of these growth factors on the rate of DNA, RNA and protein synthesis, and in addition their effect on the rate of macromolecular transport into intact embryos. They found that insulin but not IGF-I and IGF-II stimulated incorporation of label, and also postulated a role for insulin in early development, although possible roles for IGF-I and IGF-II remain unclear. Culturing early embryos in the presence of IGF-I, IGF-II and various IGF-BP may give us a better understanding of the role these growth factors play.

Receptors for insulin and IGF-I have been demonstrated on 8-cell and morula stage mouse embryos with an increase in numbers of these receptors being expressed by the blastocyst stage (see review by De Pablo *et al.*, 1990). In addition mRNA transcripts for insulin and IGF-I receptors were found at the 8-cell, blastocyst and day 7.5 post-implantation embryos, suggesting that the embryo is receptive to these factors early in development. Limited studies have also been done and have shown that blocking the receptor-ligand binding has deleterious effects on the embryo. Therefore these factors appear to be important in early embryogenesis by stimulating growth and differentiation of embryonic cells, although the physiological role these factors play is still unclear as is the importance of IGF-II and IGF-BP to the embryo in early development.

From the results obtained in this study it appears that IGF-I, IGF-II and IGF-BP do play an important role at the time of implantation, but relative amounts of these growth factors also seem to specify whether these factors act in an embryotrophic or inhibitory capacity. It is feasible that the uterus would provide these factors to the embryo, but regulation of the concentration of these factors made available to the embryo seems to be the determining process as to whether these factor act in an embryotrophic or inhibitory capacity.

Many studies have identified a number of IGF-BP which are important to later stages of development and are under maternal control, as well as during the menstrual cycle, (Pekonen, *et al.*, 1988, Rutanen *et al.*, 1988, Waites *et al.*, 1988, Fazlebas *et al.*, 1989). Results presented here suggest that IGF-BP at high concentrations might be inhibitory to the embryo, as high levels (although these levels were below physiological levels) were found in conditioned media from FL amnion which proved to be unsuitable in supporting embryo development *in vitro*. The mode of action of IGF-BPs could be by limiting the availability of IGFs to the embryo at the precise time in development when receptors are expressed on the embryo, in particular, at the 8-cell to morula stage of development when the embryo enters the uterus and is beginning to differentiate. Not only the presence, but also the precise concentration of growth factors including the IGFs and IGF-BPs could determine whether the embryo proceeds to develop and subsequently implants into the uterus or whether the embryo deteriorates and pregnancy is not established. Many questions on the importance of insulin like-growth factors and their binding proteins and their role in early developmental events still remain unanswered. Most recently Harvey and Kaye (1992) provided evidence which showed insulin and IGF-I stimulate growth in murine blastocysts. Further investigations by a number of groups into the possible role of these growth factors in early embryogenesis are currently being undertaken.

CHAPTER 6.

**THE ROLE OF EPIDERMAL GROWTH FACTOR IN THE PERI-IMPLANTATION
PERIOD OF EARLY PREGNANCY.**

6.1 INTRODUCTION.

In whole animals and in cell culture systems Epidermal Growth Factor (EGF) is known to stimulate cell proliferation. EGF has been found in a wide variety of human fluids, in particular, amniotic fluid (Barka *et al.*, 1978) and in seminal fluid (Elson *et al.*, 1984).

Recent studies have shown that EGF does play an important role throughout gestation. EGF receptors have been identified on fetal mouse and rat tissues (Hortsch *et al.*, 1983) and also transforming growth factor α (TGF α) which shares the EGF receptor has been shown to be contained within mouse embryonic tissues (Twardzik, 1982), and have been identified also on uterine epithelial cells (Tomooka *et al.*, 1986). Elevated binding of radiolabelled EGF is reported to occur at preimplantation sites within murine uteri (Brown *et al.*, 1989). Exogenous EGF could be required in order to establish EGF and TGF α synthesis within the embryo (Rizzino, 1987).

Work by Pratten *et al.* (1988) has investigated the effect of adding EGF to a 9.5 day (early head fold stage) rat embryo culture system. The culture system used was suboptimal as the serum used was depleted of factors by repeated use. Improved growth of embryos was observed following the addition of EGF to the suboptimal culture medium in a dose dependent manner.

There appear to be no reports on the effect of supplementing earlier embryo culture systems with EGF. This study was therefore undertaken to examine the role of EGF in early embryogenesis, in particular, the pre-implantation period. In addition the role of EGF, within the co-culture system established, to support embryo development was examined.

6.2 MATERIALS AND METHODS.

6.2.1 Animals.

As described previously animals used throughout this study were Balb-C x C57 3-4 week old F1 females primed with PMSG and 48 hours later with hCG to achieve superovulation. At this point each female was placed in a cage with a fertile male and left overnight. The next morning each female was checked for the presence of a vaginal plug as evidence of mating and this was then considered as day 1 of pregnancy.

6.2.2 Media.

The culture medium used for this series of experiments was prepared as previously described, briefly; powdered Minimal Essential Medium is dissolved in Milli-Q water supplemented with sodium bicarbonate, FCS and penicillin, streptomycin and fungizone added. The pH and osmolarity of the media were adjusted to 7.4 and 280 mOsm respectively and sterilized by filtration. Where indicated the medium was supplemented with epidermal growth factor (provided by Leanna Read, Waite Institute of South Australia, 1989) at various concentrations.

6.2.3 Collection of embryos.

8-cell embryos were collected on day 3 of pregnancy as previously described from females killed 71-73 hours post-hCG injection. The whole reproductive tract was dissected out and placed in EBSS and the embryos teased or flushed out of the oviduct-uterus junction and washed once in unsupplemented culture medium prior to being placed into a control or experimental group and maintained in a humidified gas environment at 37°C.

6.2.4 Culture of embryos.

For experimentation, 8-cell embryos were randomly assigned to a control group (unsupplemented media) or experimental group (media supplemented with 1, 10 or 100 ng/ml of EGF). Each group consisted of five replicates, with embryos from four to six females per replicate. Embryos were added 10-20 per well in four well multidishes, approximately 15-20 minutes after being recovered from the uterus and were maintained *in vitro* for a period of 5 days in wells containing either unsupplemented media or EGF supplemented media at the concentration indicated.

6.2.5 Assessment of morphological development.

All embryos were assessed daily, using an inverted microscope, and details of development scored over the 5 day period. Numbers of embryos were recorded attaining various stages from 8-cell through to hatched blastocyst stage (Hsu, 1979). Further development of these embryos connected with implantation were also monitored (Sherman, 1978) and based on the assessment of morphological development as described in earlier chapters.

6.2.6 Production of conditioned media.

Conditioned media was prepared as previously described from uterine cells, L cells, primary amnion cells and FL amnion cells. Briefly, cells were grown to confluence in MEM, washed once and fresh media added. Conditioned media was harvested after a 48 hour culture period, centrifuged at 500g for 10 minutes immediately following collection and aliquoted out into 5 ml sterile tubes and stored at -80°C until assayed for EGF.

6.2.7 Epidermal growth factor (EGF) assay.

All the conditioned media samples were assayed by Dr. Leanna Read and her staff from the Waite Campus, University of Adelaide. The procedure followed for the EGF radioimmunoassay is as follows: all tubes for both standards (50 ng/50 μ l hEGF) and samples were set up in triplicate, and the assay was conducted in two incubations. The first incubation was set up with tubes for the standard curve containing 100 μ l standard (with the maximum concentration of standard 50 ng/50 μ l, other tubes were serial dilutions in EGF RIA buffer plus 0.5% BSA), or 100 μ l sample, as well tubes were set up to determine total counts and non-specific binding. To all tubes, 100 μ l tracer (125 I-EGF, fraction 26; where 100 μ l equals 5,500 cpm) was added and only to tubes containing standards 50 μ l primary antibody (a hEGF rabbit). Tubes were mixed and incubated overnight at 4°C. The following day 50 μ l of 1/10 dilution (in EGF RIA buffer plus 0.5% BSA) of secondary antibody (sheep a rabbit) was added to all tubes except those for total counts, followed by 50 μ l of 1/40 dilution (in buffer) rabbit serum (carrier protein) in all tubes except those for total counts. Finally, to all tubes excluding those for total counts was added 1 ml 6% PEG. Tubes were mixed and incubated for 1 hour at room temperature, centrifuged at 3750 rpm for 30 minutes, the supernatant aspirated and the pellet counted. A standard curve was produced and levels of EGF in the samples determined from the standard curve.

6.3 RESULTS.

6.3.1 The effect of EGF on 8-cell embryo development and survival *in vitro*.

Embryos were cultured in medium supplemented with 1, 10, or 100 ng/ml EGF in order to determine whether EGF is embryotrophic or inhibitory to 8-cell embryo growth, development and survival *in vitro*. There were no differences observed between embryos cultured in unsupplemented medium or in EGF supplemented media at all concentrations tried as can be seen in Table 25 as assessed by Chi-square analysis.

6.3.2 EGF in conditioned media from selected somatic cell monolayers.

In order to determine whether EGF was produced by the various cell monolayers used for embryo co-culture, unsupplemented culture medium as well as conditioned media obtained from uterine cells, L cells, primary amnion and FL amnion were assayed for the presence of EGF. No detectable levels of EGF were found in any of the samples assayed.

Table 25. The effect of EGF (1, 10, 100 ng/ml) on 8-cell embryo development and survival *in vitro*.

| Stage of Development | Unsupplemented Media | EGF (ng/ml) | | |
|----------------------|----------------------|-------------|------|------|
| | | 1 | 10 | 100 |
| 8-cell* | 72 | 73 | 73 | 73 |
| Morula | 100 | 100 | 100 | 100 |
| Blastocyst | 94.4 | 97.3 | 94.4 | 100 |
| Hatching Blastocyst | 83.3 | 87.7 | 80.8 | 79.5 |
| Hatched Blastocyst | 65.3 | 67.1 | 54.8 | 60.3 |
| Post-hatch stage 1 | 65.3 | 67.1 | 52.1 | 54.8 |
| Post-hatch stage 2 | 65.3 | 67.1 | 52.1 | 54.8 |

* Number of 8-cell embryos. All other values expressed as a percentage of initial numbers of 8-cell embryos cultured which developed to the stage cited.

6.4. DISCUSSION.

Epidermal growth factor is known to play a significant role throughout gestation. From this study it appears as though EGF does not play a significant role in the development of 8-cell embryos cultured under the conditions described in this chapter.

Recent work by Paria and Dey (1990) has provided evidence that strongly supports a significant role for EGF in preimplantation development. Their work showed that in the presence of EGF murine blastocysts hatched earlier, and as early as the 8-cell stage, receptors to EGF were detected.

Interestingly, no EGF was detected in the conditioned media assayed. Recent work has demonstrated EGF receptors in the uterus of the guinea pig (Sorrentino and Hendrix, 1984), rat (Mukku and Stancel, 1985), human (Chegini *et al.*, 1986 Hofmann *et al.*, 1984,) and mouse (Bosset *et al.*, 1990). In addition, others have shown stimulation of uterine epithelial cell division by EGF *in vitro* (Tomooka *et al.*, 1986), and EGF stimulation of prostaglandin production by porcine uterine stromal and epithelial cells *in vitro* (Zhang *et al.*, 1992).

From a number of reports we know EGF can enhance embryo growth in later stages of development (Pratten *et al.*, 1988), and it is possible that EGF acts in a paracrine/autocrine manner in the uterine environment during pregnancy. From the results presented here, it is not possible to conclude whether or not EGF has a role in the peri-implantation stages of development. It is clear, however, that EGF does not appear to play a role in the enhanced embryo development and survival observed whilst embryos are co-cultured *in vitro* with either uterine cells in the presence of steroid hormones or with primary amnion or L cell monolayers.

CHAPTER 7.

**THE EMBRYOTROPHIC EFFECT OF MYELOID LEUKAEMIA
INHIBITORY FACTOR (LIF) ON MURINE EMBRYOS *IN VITRO*.**

7.1 INTRODUCTION.

The establishment of an appropriate uterine environment during mammalian embryogenesis is dependent upon complex interactions between embryo and uterus. An increasing body of evidence indicates that peptide signalling molecules play an important role in this process. These include proteins originally identified as growth factors (see reviews by Hill *et al.*, 1987 and Brigstock *et al.*, 1989) and proteins such as cytokines which were identified in other cellular systems. These molecules are located in the uterus during pregnancy and have been shown to regulate the attachment, implantation, growth and development of the embryo and foetus (Arceci *et al.*, 1989, Pollard *et al.*, 1987, Robertson *et al.*, 1990, Wegmann, 1988, Wegmann *et al.*, 1989).

Myeloid leukaemia inhibitory factor (LIF) has pleiotropic activities in a number of mammalian cell systems (Gough *et al.*, 1989; Hilton *et al.*, 1988; Metcalf, 1989; Moreau *et al.*, 1988, Smith *et al.*, 1988, Williams *et al.*, 1988, Yamamori *et al.*, 1989). Second lines of evidence implicate this protein in the regulation of development. LIF was found by Williams *et al.*, (1989) to act as a differentiation restriction factor for embryonic cells and at a concentration of 1000U/ml could be used instead of embryonic fibroblast cells to maintain embryonic stem cells (ES cells), which are derived from the inner cell mass of the preimplantation embryo, *in vitro* in an undifferentiated state. Recent work by Bhatt *et al.* (1991) on the expression of LIF in the adult has identified uterine endometrial glands, specifically on the fourth day of pregnancy, as the site of extensive LIF expression and this expression is under maternal control. Furthermore they suggest that LIF may play a significant role in the process of implantation *in vivo*. A study by Shen and Leder (1992) has shown LIF expression peaks in the uterus at the preimplantation stage of embryogenesis at the time when blastocysts are present in the uterus and they also postulate a possible role for LIF in preimplantation development in the mouse. Others have shown that inappropriate expression of LIF can lead to inhibition of gastrulation (Conquet *et al.*, 1992) and the lack of maternal expression results in viable blastocysts which not only fail to implant but also do not continue to develop further (Stewart *et al.*, 1992).

The ability to culture early mammalian embryos *in vitro* in simple defined media is routine in many laboratories, although embryo viability for extended periods of time is compromised after culture in simple media. The advantage of such an *in vitro* assay system is that it allows the effects of addition of putative embryotrophic factors to be studied. The aim of this study is to further explore the possible role of LIF in early embryonic development *in vitro*.

7.2 MATERIALS AND METHODS.

7.2.1 Animals.

3-4 week old F1 (Balb/C x C57 Blk or CBA x C57 as indicated) female mice were administered 7.5 i.u. PMSG (Folligon, Intervet, Australia) and 7.5 i.u. hCG (Chorulon, Intervet, Australia) 48 hours later to achieve superovulation. Once hCG injected, each female was placed immediately with one male (CBA x C57) overnight. The following morning each female was examined for evidence of mating; those which exhibited a vaginal plug were then considered as day 1 pregnant. Pseudo pregnant females, used as recipients in embryo transplantation experiments were prepared by placing two females with one vasectomised male (CBA x C57). The next morning each female was checked for the presence of a vaginal plug as evidence of mating, this was then considered as day 1 of pseudo pregnancy.

7.2.2 Collection and culture of embryos.

(a) One-cell embryos were recovered from the ampulla of 4-6 day 1 pregnant CBA x C57 females per replicate, washed twice in culture media and randomly assigned to either a control or experimental group within 20 minutes of collection. All one-cell embryos were cultured in human tubal fluid medium (HTF) which was prepared according to Quinn *et al.* (1985b). Each group consisted of approximately 10-15 embryos placed in a 20 μ l drop of

either (a) HTF media alone (control group) or (b) LIF-supplemented HTF media, the concentration of LIF varying from 2000U/ml to 62.5U/ml. Both control and experimental groups were repeated 5-6 times. Embryos were cultured for approximately 5 days under paraffin oil (BDH limited, England), in a humidified 37°C incubator, in 5%CO₂, 5%O₂, 90%N₂ and development scored as described later.

(b) Eight-cell embryos were teased or flushed out of the oviduct-uterus junction of 4-6 day 3 pregnant Balb-C x C57 females per replicate, washed twice in culture media and randomly allocated to either a control group (culture media alone) or experimental group (LIF supplemented culture media, 1000U/ml) within 20 minutes of collection.

The culture medium was prepared as previously described in Chapter 2, from powdered Minimal Essential Medium (MEM; Eagle, with Earle's salts, with L-glutamine, without Sodium Bicarbonate, Flow Laboratories, U.K.). Embryos (10-20) were cultured in 1ml of media per well (four-well multidish; Nunc, West Germany), 1-2 wells per group, 4-8 replicates per group (according to the experiment). All 8-cell embryos were cultured for 5-7 days in a humidified 37°C incubator, 5%CO₂ in air, and growth and development observed and scored as described later.

7.2.3 Uterine transfers.

Once collected, 8-cell embryos were cultured in either media alone (controls) or in LIF-supplemented media (1000U/ml) to the (a) hatching blastocyst stage or (b) hatched blastocyst stage. At each of these stages 4 embryos from the control group were transferred to one uterine horn of a day 2½ pseudo pregnant recipient female, and 4 embryos from the LIF-treated group were transferred to the other uterine horn of the same female, with 13 females for group (a) embryos and 8 females for group (b) embryos. Uterine horns were scored on day 15 for the number of embryos reabsorbed and number of foetuses.

7.2.4 ^3H -thymidine incorporation into blastocysts following culture with LIF for specified periods of time.

Eight-cell embryos were placed into culture with either media alone or medium supplemented with 1000U/ml LIF and allowed to develop over a 5 day culture period in 96 well microtitre plates (1 embryo per well) in a humidified incubator at 37°C, 5% CO₂ in air. Once embryos were cultured for 48, 72, 96 or 120 hours, the culture medium was carefully aspirated, wells checked to make sure embryos were not lost and 1 μCi ^3H -thymidine in 150 μl HEPES buffered MEM added per well. Embryos were then incubated for 6 hours, harvested using a Titretech automated cell harvester on glass fibre paper, left to dry overnight, scintillant added to each sample and using a liquid scintillation counter incorporation of ^3H -thymidine was assessed.

7.2.5 Assessment of Morphological Development.

All embryos were assessed daily, using an inverted microscope, and details of development scored over a 5-7 day culture period, depending on the experiment.

Numbers of embryos attaining various stages from 1-cell for one experiment, or 8-cell through to hatching blastocyst stage (Hsu, 1979) and rates of development in hours post hCG were recorded.

Further development of these embryos connected with implantation were also monitored (Sherman, 1978). As described in earlier chapters post-hatching embryos showing proliferating trophoctoderm cells were recorded as attaining stage 1, with the appearance of trophoctoderm cells attached to the bottom of the dish designated stage 2. Embryos exhibiting further growth or spreading out of the trophoctoderm cells on the bottom of the dish were classed as attaining stage 3. Percent survival was calculated as the number of embryos which attained stage 3. (See Figure 7.1 for example of stage 3 embryos).

Area measurements were made using the Bio-Quant System image analyser and a Leitz microscope. Approximately 25-30 embryos were cultured with or without LIF (1000U/ml) for 5 days and 7 days, fixed and stained with toluidine blue. The area of the inner cell mass (ICM) and trophoblast outgrowth of each embryo was measured in microns squared (μm^2).

7.2.6 Statistics.

All results were analysed by Chi-square tests except for the data on the area of embryo inner cell mass and trophoblast following *in vitro* culture with LIF, which was analysed using the Student's t-test, and the data on DNA synthesis which was analysed using ANOVA.

7.2.7 Production of mixed uterine, epithelial and stromal monolayers.

Mixed uterine monolayers were produced as described earlier following the protocol as detailed in Chapter 2. Epithelial and stromal monolayers were produced using a modification of the procedure described by Sherman (1978), of which complete details can be found in Chapters 2 and 4.

7.2.8 Expression of LIF in cultures of mixed uterine, epithelial and stromal cells in the presence or absence of steroids.

Once mixed uterine, epithelial and stromal monolayers were produced, and cultured for 24 hours in the presence or absence of either oestradiol, progesterone or both steroids, and allowing for attachment of cells, media was aspirated and fresh media added. Cultures were then incubated for a further 24 hours, media aspirated and cells detached from the plastic dish by incubating monolayers in EBSS-T-E for approximately 5 minutes, cells were

then centrifuged at 200g for 10 minutes, supernatant was carefully aspirated and tubes containing cells stored at -80°C until assayed for LIF expression.

Cytoplasmic RNA (10mg) from each group of cells was assayed for LIF expression by ribonuclease protection assay as described by Rathjen *et al.* (1990). Assays were performed by Dr P. Rathjen, Department of Biochemistry, University of Adelaide.

7.3 RESULTS.

7.3.1 Effect of LIF on the development of one-cell embryos.

The addition of LIF (1000U/ml) did not significantly change the number of embryos developing from the one-cell stage through to the blastocyst stage, compared to the unsupplemented group (84.1%, 58/69 and 90.1%, 64/71 respectively). Media supplemented with a range of LIF concentrations (2000U/ml to 62.5U/ml) gave similar results (Table 26). Following daily observation and scoring, no significant difference in the rate of development and the total number of embryos reaching a particular developmental stage was evident between the unsupplemented and LIF-supplemented groups throughout the culture period.

Table 26. The effect of increasing concentrations of LIF (62.5 - 2000 U/ml) on the development of 1-cell Murine embryos *in vitro*.

| Conc of LIF | n | % at Indicated Stage of Development | | | |
|-------------|----|-------------------------------------|------|--------|------------|
| | | 4 | 8 | Morula | Blastocyst |
| 0 (Control) | 71 | 97.2 | 95.8 | 95.8 | 90.1 |
| 62.5 | 67 | 97.0 | 97.0 | 97.0 | 94.0 |
| 125 | 73 | 98.6 | 91.8 | 90.4 | 87.7 |
| 250 | 72 | 95.8 | 91.7 | 91.7 | 86.1 |
| 500 | 87 | 90.8 | 87.4 | 87.4 | 83.0 |
| 750 | 78 | 94.8 | 92.3 | 87.2 | 84.6 |
| 1000 | 69 | 95.7 | 89.9 | 87.0 | 84.1 |
| 2000 | 72 | 97.2 | 95.8 | 95.8 | 87.5 |

7.3.2 The effect of LIF on 8-cell embryo development.

Culture of 8-cell embryos in media supplemented with LIF (1000u/ml) for a period of 5 days significantly increased ($p < 0.01$) the number of embryos attaining stage 3 development to 80% (156/195) compared to 64% (223/349) of control embryos cultured without LIF, ($p < 0.01$) (Table 27). A significantly greater number of LIF-treated embryos were hatching at 96 hours post-hCG (33.8%) compared to the control group (7.6%), ($p < 0.001$). The total number of LIF-treated embryos that completely hatched (85.1%) was significantly greater ($p < 0.05$) than the number observed in the control group (62.1%), (Table 28). Additionally, it was apparent that a significant proportion (13.5%) of LIF-treated embryos had attained stage 2 (trophectoderm cells attached to the bottom of the dish) as early as 120 hours post-hCG compared to 0% of control embryos at the same point in time, ($p < 0.01$). By 144 hours post-hCG implantation of LIF-treated embryos (85.1%) was complete (stage 3) compared with controls where only 47% had reached stage 3 ($p < 0.01$); the control group required a further 24 hours for the maximum number of embryos to reach stage 3. (Table 29).

Table 27. The effect of LIF (1000U/ml) on the development of 8-cell Murine embryos *in vitro*.

| | n | % of 8-cell embryos developing to Stage 3. |
|----------------------|-----|---|
| Control ¹ | 349 | 64 |
| LIF | 195 | 80* |

* denotes a significant difference from control group, $p < 0.01$.

TABLE 28. The effect of LIF (1000U/ml) on the rate of hatching of Murine embryos *in vitro*.

| | n | % HATCHING | | | % HATCHED |
|---------|----|------------|-------|------|-----------|
| | | 96h | 100h | 120h | |
| Control | 66 | 7.6 | 68.9 | 70 | 62.1 |
| LIF | 74 | 33.8* | 93.2* | 98.6 | 85.1** |

* denotes a significant difference from the respective control group, $p < 0.001$.

** denotes a significant difference from the respective control group, $p < 0.05$.

Table 29. The effect of LIF on the rate of implantation (Stage 2 to Stage 3) of Murine embryos *in vitro*.

| | n | % IMPLANTING | | |
|---------|----|--------------|-------|--------|
| | | 120h | 144h | 168h |
| Control | 66 | 0 | 47.0 | 62.1 |
| LIF | 74 | 13.5* | 85.1* | 85.1** |

* denotes a significant difference from the respective control group, $p < 0.01$.

** denotes a significant difference from the respective control group, $p < 0.05$.

7.3.3 The Effect of LIF on Post-Blastocyst Development.

The effect of culturing 8-cell murine embryos in LIF supplemented media clearly demonstrates a significant increase in the total number of embryos implanting *in vitro*. This result does not identify the developmental stage at which embryos are sensitive to the presence of LIF. Although the effect of LIF on murine embryogenesis is only apparent at post-blastocyst stages of development it is possible that this reflects a delayed manifestation of LIF action at pre-blastocyst stages. To determine the developmental stage at which embryos are sensitive to the presence of LIF, 8-cell embryos were cultured *in vitro* in unsupplemented medium to the blastocyst stage and analysed for developmental effects of subsequent LIF treatment. The addition of 1000U/ml LIF resulted in a significant increase in the number of embryos reaching stage 3 (implantation) compared to embryos cultured throughout in supplemented medium (85.7% 30/35, 54.3% 19/35 respectively; $p < 0.01$). By contrast, addition of LIF to embryos between the 8-cell stage and the blastocyst stage did not affect subsequent development. Blastocysts were derived from 8-cell embryos cultured in the presence or absence of 1000U/ml LIF. Continued culture of these embryos in unsupplemented medium revealed no significant difference in the ability of these embryos to reach stage 3 of development (60.7% vs 54.3% as can be seen in Table 30). These results clearly indicate that murine embryogenesis *in vitro* is sensitive to the administration of LIF at post-blastocyst stages of development.

Table 30. The effect of varying the period of culture in LIF supplemented media on Post-blastocyst embryo development *in vitro*.

| Period of culture with LIF | n | % developing to Stage 3 |
|----------------------------|----|-------------------------|
| 0 (control) | 35 | 54.3 |
| 8-cell to Stage 3 | 35 | 77.1 |
| 8-cell to blastocyst | 35 | 65.7 |
| Blastocyst to Stage 3 | 35 | 85.7* |

* denotes a significant difference, $p < 0.01$.

7.3.4 The Affect of LIF on Embryo ICM and Trophoblast.

Image analysis was performed to determine whether culture in LIF resulted in an increase in the degree of trophoblastic outgrowth. At day 5 of culture, the mean trophoblastic area of embryos cultured in LIF (1000 U/mL) was 27% greater than controls ($111 \pm 30.7 \mu\text{m}$ vs $141 \pm 35.2 \mu\text{m}$ respectively, $p < 0.005$), (Figure 7.1). By day 7, the mean area of the inner cell mass (ICM) ($15.9 \pm 10.6 \mu\text{m}$) of LIF-treated embryos was significantly greater than that of the control embryos ($10.3 \pm 5.7 \mu\text{m}$) ($p < 0.025$), and a 43% increase in trophoblastic area was evident between controls ($207 \pm 67.8 \mu\text{m}$) and LIF-treated embryos ($296 \pm 67.2 \mu\text{m}$) ($p < 0.005$) (Table 31).

Table 31. The area of embryo ICM and trophoblast following *in vitro* culture with LIF (1000U/ml).

| | AREA ($\mu\text{m}^2 \pm \text{SD}$) | | | | |
|-------------|--|----------------|----|-----------------|---------------|
| | n | Control | n | LIF | *Significance |
| ICM | | | | | |
| Day 5 | 18 | 8.0 ± 3.5 | 18 | 8.3 ± 3.4 | NS |
| Day 7 | 11 | 10.3 ± 5.7 | 10 | 15.9 ± 10.6 | $p < 0.025$ |
| Trophoblast | | | | | |
| Day 5 | 18 | 111 ± 30.7 | 18 | 141 ± 35.2 | $p < 0.005$ |
| Day 7 | 11 | 207 ± 67.8 | 10 | 296 ± 67.2 | $p < < 0.005$ |

* denotes a significant difference between control group and respective LIF-treated group on the same day (Students t-test).

7.3.5 Embryo Viability Following Treatment with LIF.

Embryo transfer experiments were performed to determine whether culture in LIF would influence the potential of embryos to develop to later stages of pregnancy. Uterine transfers were carried out to assess the viability of embryos cultured in unsupplemented

transfers were carried out to assess the viability of embryos cultured in unsupplemented media and also embryos cultured in LIF-supplemented media. A group of embryos were cultured until they developed to either the hatching stage of development, or until they had completely hatched *in vitro*. Embryos from the unsupplemented media group and the LIF-supplemented group that had attained each of these stages of development were then transferred to pseudopregnant females. At day 15 of gestation the LIF-treated group of embryos transferred at the hatching blastocyst stage exhibited no significant difference to those from the unsupplemented group, as can be seen in Table 32. Furthermore, there were fewer reabsorptions in the LIF-treated group of embryos that were transferred at the hatched stage (19.2%) compared with controls (33.3%), $p < 0.001$, and in accordance with this, an increase in the number of foetuses was also seen in the LIF-treated group (80.8%) compared with the unsupplemented group (66.7%), $p < 0.05$.

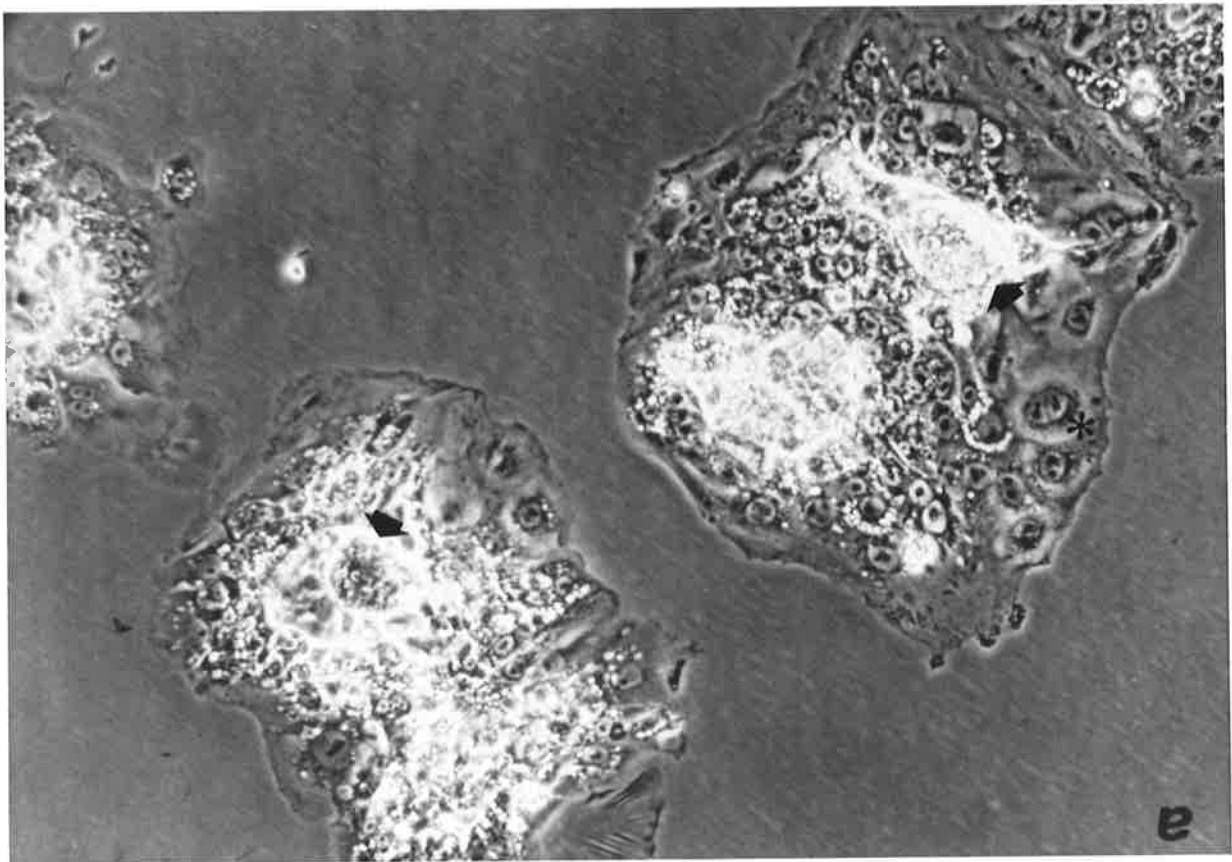
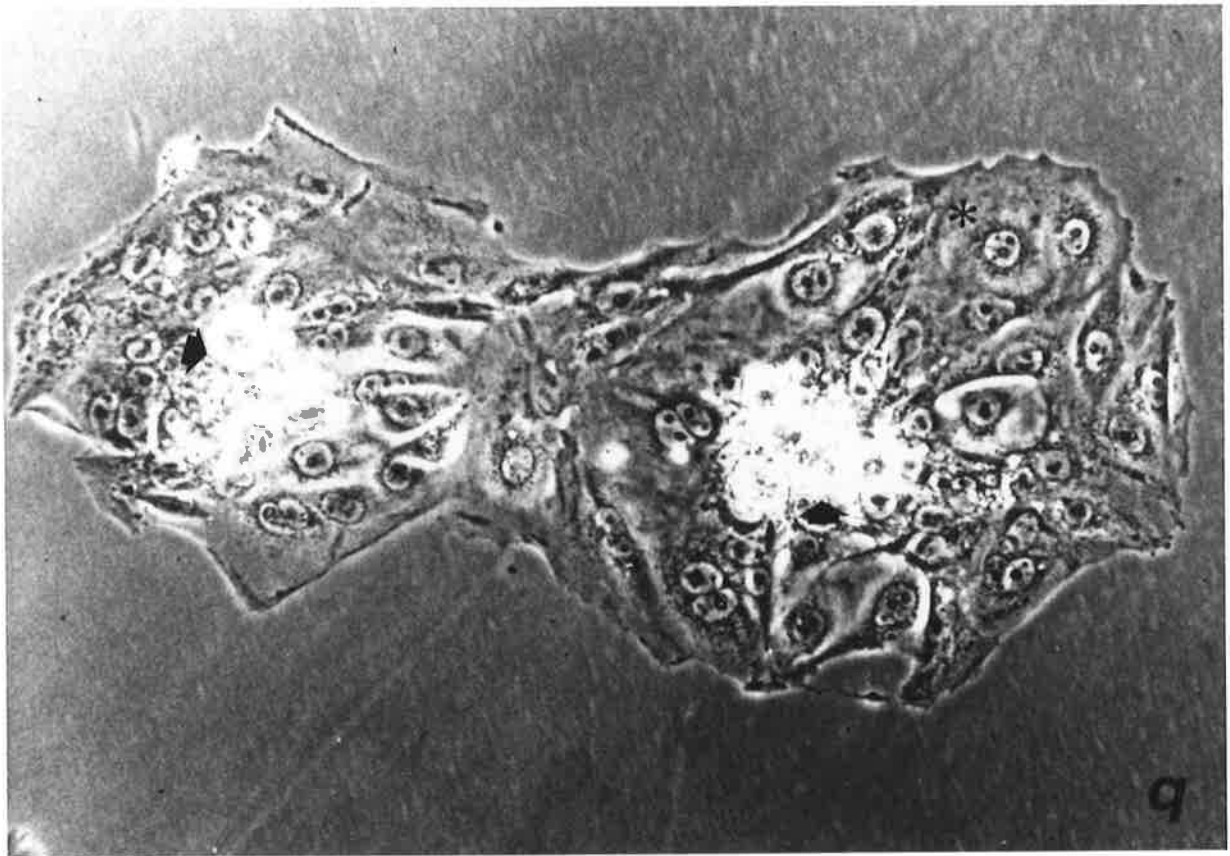
Table 32. The effect on embryo viability of including LIF (1000U/ml) during *in vitro* culture and subsequent uterine transfer to synchronised pseudopregnant females (see text for details).

| Addition of LIF | Embryo stage at time of Transfer | | | |
|-----------------|----------------------------------|------|---------|--------|
| | Hatching | | Hatched | |
| | - | + | - | + |
| No. transferred | 52 | 52 | 32 | 32 |
| % implanted | 96.2 | 90.4 | 84.4 | 81.3 |
| % reabsorbed | 30.0 | 29.8 | 33.3 | 19.2* |
| % foetuses | 70.0 | 70.2 | 66.7 | 80.8** |

* denotes a significant difference from the respective control group ($p < 0.001$, Chi-square analysis)

** denotes a significant difference from the respective control group ($p < 0.05$, Chi-square analysis)

Figure 7.1. Photograph of (a) Control embryos (unsupplemented media) and (b) LIF-treated embryos at Day 5 in culture, showing differences in the area of trophoblastic outgrowth (arrows identify ICM of each embryo).



7.3.6 The effect of LIF on DNA synthesis of 8-cell embryos over various culture periods *in vitro*.

In order to determine whether LIF was effecting DNA synthesis of embryos cultured in the presence of LIF compared with those embryos cultured in unsupplemented media, ^3H -thymidine was added to the culture system at various times throughout the culture period and incorporation of label used as a means of assessing this. Following 48, 72, 96 and 120 hours in culture, there appears to be no significant differences in the amount of label incorporated between the two groups, although there is an increase in the amount of label incorporated over time, and this would reflect the increase in cell number as development proceeds. (Figure 7.2).

7.3.7 LIF expression in uterine cells *in vitro*.

Development and survival of embryos is clearly enhanced under LIF-supplemented culture conditions. In order to determine whether the enhanced embryo development observed when co-cultured with uterine cells in the presence of steroids is associated with LIF production by the uterine cells. Mixed uterine, epithelial and stromal cells were examined for the expression of LIF.

Following RNAase protection assay, LIF expression was observed in Day 3 mixed uterine cells under all conditions tested, that is, without steroids, with progesterone alone, with oestradiol alone and with both steroids in combination, as can be seen in Figure 7.4, but was not evident in cells cultured from Day 2 pregnant uteri under the same conditions as mixed uterine cells from Day 3 pregnant uteri (Figure 7.5).

Once the results indicated that LIF expression was evident in the mixed uterine cells from Day 3 pregnant uteri, it was necessary to determine in which cell type LIF mRNA could be detected. No detectable levels of LIF expression were observed in epithelial cells from Day 3 pregnant uteri cultured in the absence or presence of steroids, (Figure 7.5). LIF expression was evident in stromal cells from Day 3 pregnant uteri in the presence of

steroids, and in the absence of steroids the expression appeared to increase 2-fold, as can be seen in Figure 7.6.

Figure 7.2 The effect of LIF (1000U/ml) on DNA synthesis of 8-cell Murine embryos over increasing culture periods *in vitro*.

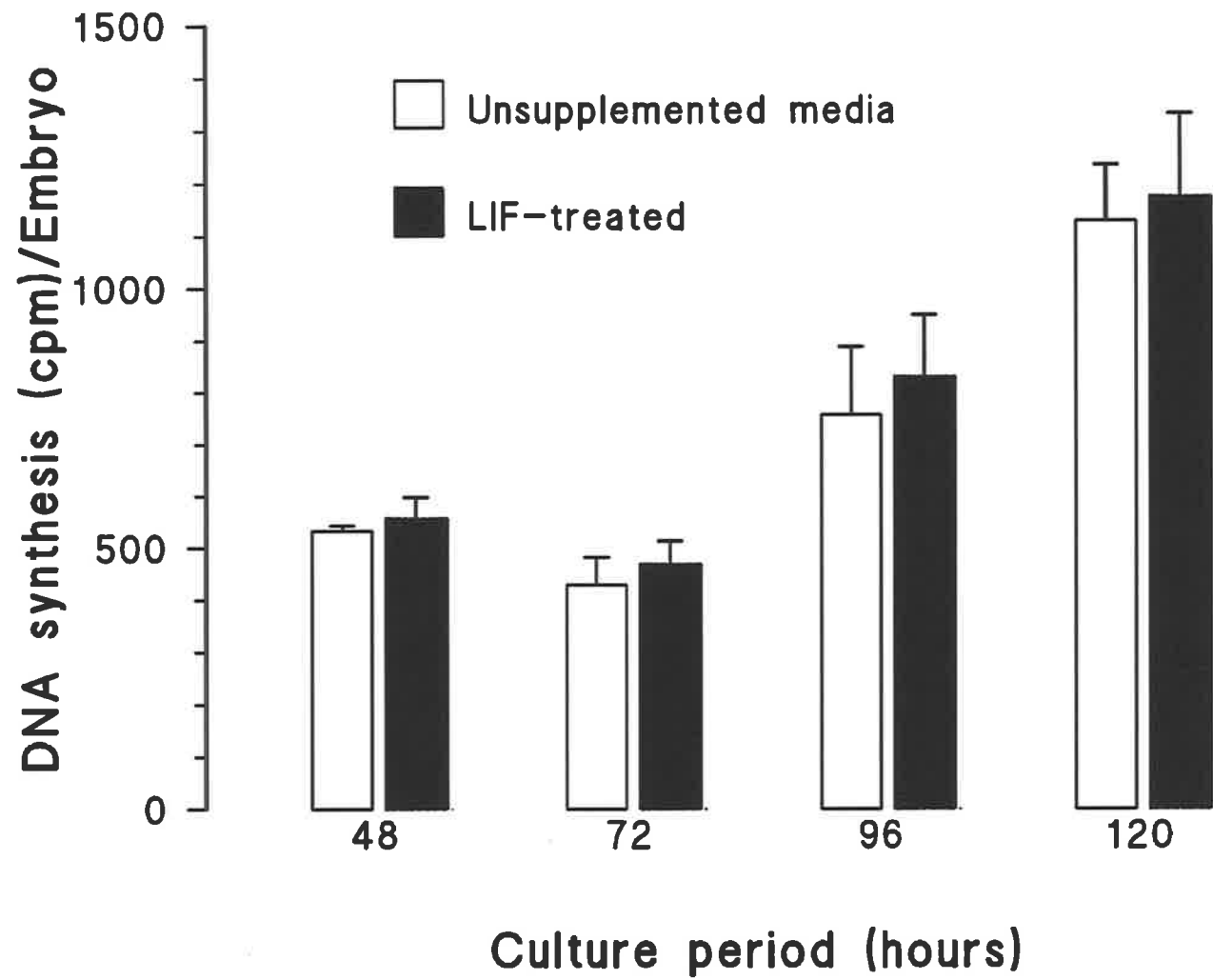


Figure 7.3. Autoradiogram of polyacrylamide-urea gel showing RNAase protected fragment of LIF mRNA in mixed uterine cells from Day 3 pregnant uteri following culture (see text for more details), with or without steroids.

- Legend:
- A. mGAP standard.
 - B. Day 3, mixed uterine cells, with progesterone and oestradiol.
 - C. Day 3, mixed uterine cells, with oestradiol.
 - D. Day 3, mixed uterine cells, with progesterone.
 - E. Day 3, mixed uterine cells, without steroids.

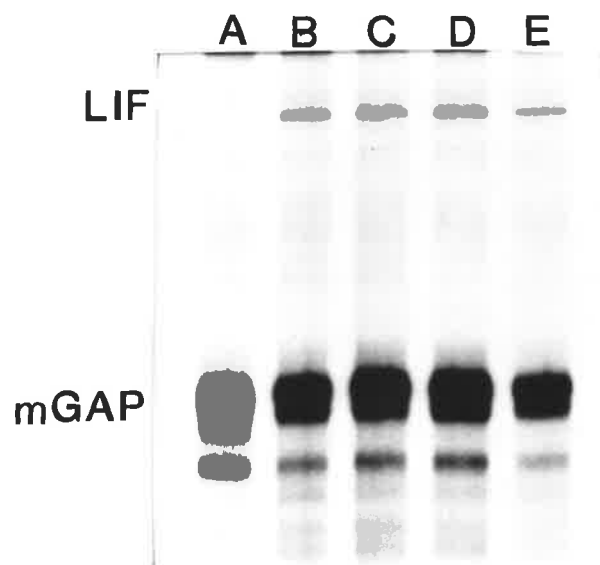


Figure 7.4. Autoradiogram of polyacrylamide-urea gel showing RNAase protected fragment of LIF mRNA in mixed uterine cells from Day 2 and Day 3 pregnant uteri following culture (see text for more details), with or without steroids.

- Legend:
- A. mGAP standard.
 - B. Day 3, mixed uterine cells, without steroids.
 - C. Day 3, mixed uterine cells, with progesterone and oestradiol.
 - D. Day 2, mixed uterine cells, without steroids.
 - E. Day 2, mixed uterine cells, with progesterone.
 - F. Day 2, mixed uterine cells, with oestradiol.
 - G. Day 2, mixed uterine cells, with progesterone and oestradiol.

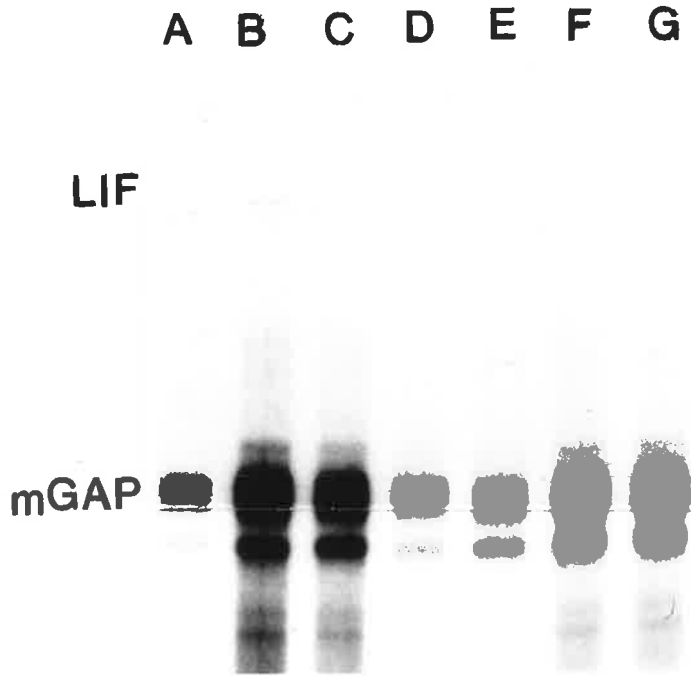


Figure 7.5. Autoradiogram of polyacrylamide-urea gel showing RNAase protected fragment of LIF mRNA in epithelial cells from Day 3 pregnant uteri following culture (see text for more details), with or without steroids.

- Legend:
- A. Day 3, epithelial cells, without steroids.
 - B. Day 3, epithelial cells, with progesterone.
 - C. Day 3, epithelial cells, with progesterone and oestradiol.
 - D. mGAP standard.

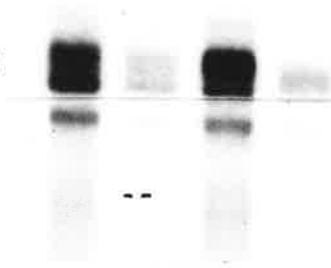
Figure 7.6. Autoradiogram of polyacrylamide-urea gel showing RNAase protected fragment of LIF mRNA in stromal cells from Day 3 pregnant uteri following culture (see text for more details), with or without steroids.

- Legend:
- A. Day 3, stromal cells, without steroids.
 - B. Day 3, stromal cells, with progesterone.
 - C. Day 3, stromal cells, with progesterone and oestradiol.
 - D. mGAP standard.

A B C D

LIF

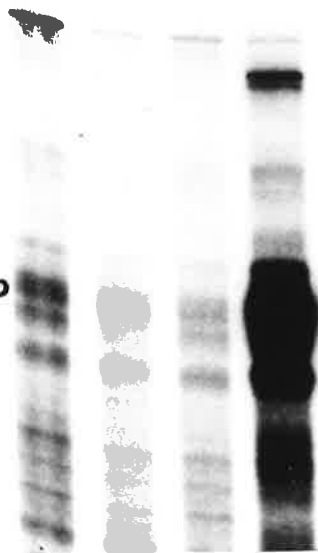
mGAP



A B C D

LIF

mGAP



7.4 DISCUSSION.

The present study clearly indicates that supplementing an otherwise limiting embryo culture media with LIF (1000U/ml) can markedly enhance embryo development. The culture medium utilized supports embryo development when embryos are co-cultured with uterine cells, but is limiting as a support for development of murine embryos in isolation. This therefore allows us to study the effects of addition of putative embryotrophic factors on embryo development and survival.

The data presented here indicate that LIF acts during uterine as opposed to oviductal stages of development to enhance the rate and proportion of embryos hatching from the zona pellucida and the number of embryos implanting onto the tissue culture dish. Clear evidence was also obtained to indicate that LIF promotes the proliferation of trophoblast cells and an increase in the inner cell mass. LIF is known to act on embryonic stem cells which are derived from the inner cell mass of the blastocyst, (Williams *et al.*, 1989). Whether LIF acts directly on trophoblast cells or indirectly through growth factors produced by the inner cell mass requires further investigation.

It is of great interest that recently it has been shown that LIF is produced by uterine glandular epithelial cells *in vivo* primarily on day 4 of pregnancy (Bhatt *et al.*, 1992). LIF mRNA transcripts have also been found in the pre-implantation blastocyst at 3.5 days post coitum and also post-implantation in the extraembryonic tissues and placenta (Conquet and Brulet, 1990). LIF may therefore be involved in early murine embryo development through both autocrine and paracrine mechanisms, either by preparing the uterine cells for the implantation process and/or by preparing the blastocyst to implant. Support for this hypothesis is obtained from the results presented here on the transfer of hatched embryos following culture in the presence of LIF which resulted in a significant decrease in the number of reabsorptions and a significant increase in the number of young. The data described here also show the rate of implantation and the final number of embryos implanting were greatly increased in the LIF-treated group. There was clear evidence of differences in the area of embryo inner cell mass at day 7 between unsupplemented and LIF

treated groups, and also in the area of trophoblast outgrowth on both day 5 and day 7. In particular, LIF-treated embryos exhibited a significantly larger trophoblast area than non-treated embryos, which suggests that LIF is acting on these particular cells. This study confirms that preimplantation embryos are receptive to the presence of LIF in culture media, and implicates LIF as an embryotrophin, this is further supported by the finding that in the absence of a functional LIF gene in females blastocysts in the uterus develop, are viable, but fail to implant, (Stewart *et al.*, 1992).

The importance of growth factors to events early in pregnancy only recently has become clear and our understanding of how the embryo and maternal tissues communicate during the preimplantation period is limited. Although LIF, now recognised as having pleiotrophic activity, has been studied extensively with respect to its actions and interactions on normal and leukaemic cells (Gough *et al.*, 1989., Hilton *et al.*, 1988., Metcalf, 1989., Moreau, 1988.), its actions on embryonic stem cells (Williams *et al.*, 1988., Smith *et al.*, 1988.) and also its ability to regulate growth and differentiation on the nervous system in the embryo and adult (Yamamori *et al.*, 1989.), its importance in early embryo-maternal interactions is only now becoming evident. The present study has identified LIF as a haemopoietic regulatory substance with embryotrophic activity. LIF could in part be important in the enhanced embryo development seen in the co-culture of embryos with uterine cells described in chapter 2. Further investigation of LIF's role in embryo-uterine interactions in early embryo development is warranted.

CHAPTER 8.

GENERAL DISCUSSION.

Embryonic loss occurs naturally *in vivo* during early pregnancy and it may be due to dysfunction of either the embryo, the reproductive tract or both. Embryonic loss also occurs following *in vitro* handling or culture of embryos, a procedure of importance to both human medicine and artificial animal reproduction techniques. Central to the successful manipulation of embryos outside the reproductive tract, is the ability to enable these embryos to continue their development *in vitro* as close as possible to the *in vivo* situation, thus ensuring maximum survival once these manipulated embryos are returned to their host. One of the biggest tasks at hand in this area still, is to develop a successful culture system which mimics the normal maternal environment at any particular stage of development. This is where the problem starts, what is considered normal? The time leading up to and including implantation is the least understood with respect to the intricate communication that occurs between the developing embryo and the maternal tissues. Therefore, the aims of the studies reported in this thesis were to establish a culture system which mimicked the *in vivo* environment during the peri-implantation period in early pregnancy and examine the interactions that occur within the maternal-embryo axis.

Firstly an *in vitro* co-culture system was established. It allowed murine 8-cell preimplantation embryos to develop in an environment which resembled the uterine environment *in vivo*. To accomplish this a number of points were addressed: (1) the source of uterine material used for the co-culture with embryos, (2) the inclusion of steroid hormones in the culture medium, and (3) a system to assess embryo viability following co-culture. Since the window for implantation is short within the developmental sequence of events, preparation of all monolayers of uterine cells were from day 3 pregnant uteri. This synchronised the developmental stage of the uterine cells with the developmental stage of the embryos whilst in co-culture. Co-culture of embryos with uterine cells did not enhance embryo development unless oestradiol and progesterone were added. Moreover the addition of these steroid hormones alone without uterine cells did not enhance embryo development, suggesting that the steroid hormones enhanced the capacity of the uterine cells to support pre-implantation stage embryos. In addition embryo viability was not compromised as assessed by uterine transfers of embryos following co-culture. This first study also found

that the epithelial and stromal populations of cells within the uterine population contributed with varying degrees to different developmental steps (ie hatching and attachment). Finally, the feasibility of establishing a co-culture system to facilitate investigation of pre-implantation events *in vitro* was confirmed.

Having established that co-culture of embryos with uterine cells enhanced embryo development and survival *in vitro*, the ability of other cell types to support embryo development and survival was examined. Primary amnion cells and L cells as monolayers were as effective in co-culture as uterine cells were in supporting 8-cell embryos. FL amnion cells and 3T3 cells in monolayer were unsuitable. The conditioned media from all five of the cell types, including uterine cells, also were tested for their ability to support or inhibit embryo development. Media from uterine cells, primary amnion cells and L cells supported embryonic development but that from FL amnion cells and 3T3 cells did not, just as co-culture of embryos with these cells had done. It would appear then that the cells which supported embryonic development were producing a soluble embryotrophic substance(s) that had the capacity to promote embryo growth and development, and in the case of the uterine cells, the production of an embryotrophic substance was stimulated by treatment with steroid hormones. The identity of this substance(s) was sought (chapters 4-7).

The *in vitro* production of selected cytokines, prostaglandins and IGFs and their associated binding proteins by uterine and other cells was examined. Of all conditioned media tested, production of the cytokines GM-CSF and IL-6 was detected. GM-CSF was produced by the uterine epithelial cells but this production was not dependent on steroid hormone treatment. However, the highest level of IL-6 activity was in the uterine epithelial cell conditioned media and this production was steroid hormone dependent. This suggested this growth factor may in part be responsible for the enhanced embryo development observed in co-culture. Production of both PGE₂ and PGF₂ α was identified in conditioned media. It was produced primarily by the stromal population of cells but was not steroid hormone dependent. Their presence suggested that they might have a role in implantation such as the decidual cell reaction that occurs in the uterine stroma. Interestingly an increase

in decidualization in these stromal cells was observed over the five day period in culture. Substantial amounts of IGF-I and IGF-II and their associated binding proteins were also detected in all conditioned media. It was evident that the cell types that could produce an embryotrophic medium or environment also produced relatively less IGFs and IGF-BPs than the cell types which were unsuitable for embryo co-culture. Thus the concentration of these factors may be important, such that elevated levels of these growth factors and in particular the IGF-BPs, may play a role in preventing the trophoblast from proliferating, and therefore disrupting the communication that occurs between the embryo and the maternal tissues just prior to the implantation process (reviewed by Bell, 1988, 1989). Future studies are necessary to identify which IGF-BPs contribute to this effect, and possibly supplementing culture media with these factors may help specify their precise role.

Using another approach to identify growth factors that may be of significance to pre-implantation events, was to examine the effects of growth factor addition to the culture medium on embryonic development and survival (chapter 6 and 7). Following culture of 8-cell embryos in unsupplemented media or in media supplemented with increasing doses of the growth factor EGF, no significant differences were observed in the rate of development or in the total number of embryos which attained post-hatching stages of development. In addition, no EGF was detected in any of the conditioned media assayed. From these results a role for EGF in the development of embryos cultured in the culture system established in this study could not be identified.

The studies reported in the final chapter addressed and published for the first time (Lavranos and Seamark, 1989) the importance of LIF in early embryogenesis *in vitro*. Enhanced embryo development was observed when 8-cell embryos were cultured in media supplemented with LIF. This enhanced development was comparable to that seen in co-culture with either uterine cells, L cells, or primary amnion cells. The presence of LIF had profound effects on the embryos. It stimulated earlier hatching, earlier attachment of blastocysts, and promoted greater outgrowth of trophoblast and subsequent proliferation of the ICM. Whether this is a fully direct or indirect effect still needs to be resolved. The embryotrophic effects of LIF were restricted to events after the 8-cell stages of

development. LIF did not compromise embryo viability in any way as assessed by uterine transfers and subsequent development and survival of embryos cultured in LIF supplemented medium, and interestingly reduced the number of embryo reabsorptions in the host uterine horns. LIF mRNA was detected in stromal cells cultured from day 3 pregnant uteri and not those of day 2, which confirms its involvement in the uterine as opposed to oviductal stages of development. The levels of LIF mRNA in cultured stromal cells was not altered by steroid hormone treatment and no LIF mRNA was detected in uterine epithelial cells. The results from this study identified the embryotrophic activity of this cytokine.

In conclusion, studies reported in this thesis have identified a co-culture system which will significantly enhance embryo growth, development and survival *in vitro* and will not compromise embryo viability following transfer of embryos to the mother. In addition they have contributed to the understanding of the communicative role of growth factors such as GM-CSF, IL-6, IGFs and LIF in the maternal-embryo interactions during the peri-implantation period in early murine development.

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