Molecular Human Reproduction vol.1, Human Reproduction vol.10 no.6 pp. 1579–1602, 1995

# The signals and molecular pathways involved in implantation, a symbiotic interaction between blastocyst and endometrium involving adhesion and tissue invasion

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**Implantation is a complex process requiring the interaction of the blastocyst, and subsequently the developing embryo with the endometrium. Initially, the detailed cellular interactions implicated in this process were defined. More recently, many signals and molecular pathways are recognized that induce, or regulate the complex series of interactions required for implantation. In this review, the cellular and molecular interactions that take place during implantation are discussed.**

*Key words:* cytokines/endometrium/epithelial cells/fertility/implantation

## Introduction

The term 'implantation' has been applied to a series of events initiated by fertilization of ovum which ultimately lead to the embedding of blastocyst in the endometrium. During this process, upon entry of the morula into the endometrial cavity, a blastocyst is formed that establishes a contact with the surface epithelium of endometrium. During a series of exquisitely controlled steps, the blastocyst is gradually implanted in the underlying stroma. Formation of placenta, the so-called 'placentation', completes the implantation process and establishes a means of supporting the embryo to the end of the pregnancy period (Figure 1).

## Sequences of implantation

Much of the information in humans regarding the phases and the orchestrated cellular events of implantation have been deduced or derived from the specimens available in the Carnegie collections. According to the available specimens, various stages have been defined (Figure 1). Stage 1 is initiated by fertilization of ovum. Division of the zygote marks the initiation of stage 2. At stage 3, the morula has entered the endometrial cavity and shortly thereafter the blastocyst is formed. In humans, the entry of the morula into the uterine cavity occurs about 72–96 h after ovulation/fertilization (Croxatto *et al.*, 1978; Buster *et al.*, 1985) (Figure 1). The zona pellucida seems to dissolve on the fifth day, about 110–120 h after ovulation/fertilization (Buster *et al.*, 1985). For ease of understanding, we have arbitrarily divided the implantation of human blastocyst into three phases. In phase I (stage 3), the blastocyst is free within the endometrial cavity and has not interacted with the surface epithelium. In phase II (stage 4), the blastocyst adheres and then penetrates the surface epithelium and subsequently the underlying stroma. Placentation is the predominant feature of phase III (stages 5–9) of implantation.

### *Phase I: free blastocyst*

#### *Stage 3*

Since implantation is the outcome of interaction of blastocyst with endometrium, it can be considered that this process is initiated as soon as a dialogue is established between the embryo and endometrium. The elegant studies of Das *et al.* (1994) recently demonstrated that before blastocyst adheres to the surface epithelium, the expression of a growth factor designated as heparin binding epidermal growth factor (EGF)-like growth factor (HB-EGF) is induced in the luminal epithelium of mouse endometrium. In view of this finding, it can be suggested that the interaction between the blastocyst and endometrium begins immediately after entry of the embryo into the endometrial cavity and prior to active penetration of endometrium by the developing blastocyst. It is, therefore, reasonable to refer to the period when the free blastocyst is found in the uterus as phase I of implantation. During stage 3, the blastocyst matures (stage 3a) and then loses the zona pellucida (stage 3b) (hatching). This period occurs on day 5 after ovulation. The extracellular layer of the blastocyst (zona pellucida) is shed 1–2 days prior to penetration of the surface epithelium. Due to the virtual absence of fluid in the endometrial cavity, the free blastocyst is probably in contact with surface epithelium. After loss of zona pellucida, the trophoblast cells near the inner cell mass develop surface processes and fuse to form the syncytial trophoblasts.

### *Phase II: adhesion and intrusion*

#### *Stage 4*

This stage includes attachment of the blastocyst to the surface epithelium (apposition phase) followed by the penetration of

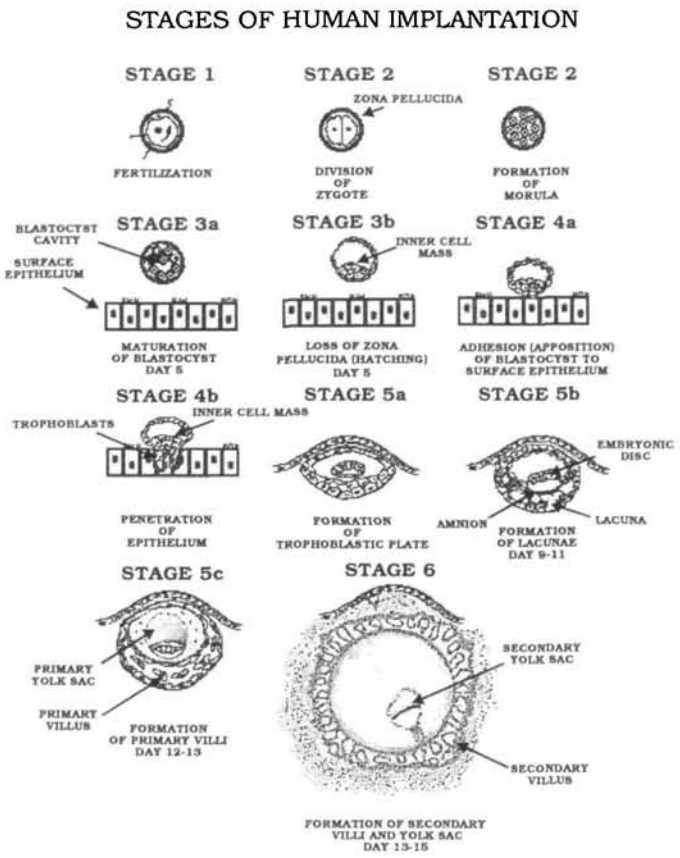


**Figure 1.** Stages 1–6 of human implantation. Stage 1 is initiated with fertilization of the ovum. Through the subsequent stages, the developing embryo migrates into the endometrial cavity, adheres to the surface epithelium and implants in the endometrium (Enders and Schlafke, 1977; Enders, 1991).

the surface epithelium (penetration stage). The attachment of the blastocyst and penetration of the surface epithelium have never been described in humans. However, based on other events in humans or the available information in primates, they have been deduced to occur on days 5–6 after ovulation–fertilization (Enders, 1991; Psychoyos, 1993). The initial contact that is made between the trophoblasts and the endometrial surface epithelium is close apposition of the plasma membranes of the trophoblasts with the apical plasma membranes of the surface epithelial cells. The plasma membranes of these cells run parallel to each other and are separated by a distance of 20 nm. A specialized submembranous filamentous network supports stable cell–cell binding between these cells (Denker, 1993). This phase of the trophoblast–endometrial interaction which is also called the 'apposition phase' is followed by penetration of the surface epithelium. Trophoblasts in different species use one of the following modes of invasion of endometrium (Schlafke and Enders, 1975): (i) displacement penetration—this mode of endometrial invasion is seen in the mouse and rat, where a number of surface epithelial cells detach from their basement membrane and from each other; these cells degenerate and then they are phagocytized by trophoblasts. As a consequence, the trophoblasts are exposed to the bare basement membrane. Processes of the decidual cells penetrate the basement membrane (Figure 2); (ii) Fusion penetration—this type of endometrial invasion is seen in

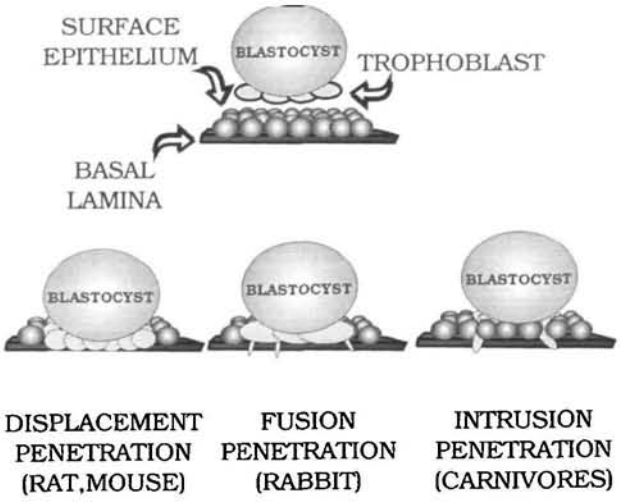


**Figure 2.** Various modes of penetration of surface epithelium of endometrium by trophoblasts in various species. In some species, the surface epithelial cells detach from their basement membrane and are phagocytized by the trophoblasts (displacement penetration). In other species, the trophoblasts either fuse with the surface epithelial cells (fusion penetration), or interpose themselves among the epithelial cells (intrusion penetration) (Denker, 1977, 1993).

the rabbit, and it involves binucleate cells in ruminants. Syncytiotrophoblasts fuse with the surface epithelial cells and form a syncytium that penetrates the basement membrane of the surface epithelium (Figure 2); (iii) intrusion penetration—this mode of endometrial invasion is seen in carnivores but has also been demonstrated in the rhesus monkey (Enders, 1991). This type of invasion includes penetration of processes of the syncytiotrophoblasts between the surface epithelial cells. This leads to a loss of junctions between neighbouring epithelial cells and formation of junctions between the trophoblasts and the epithelial cells. The trophoblasts interpose themselves among epithelial cells and then penetrate through the basement membrane underlying the surface epithelium (Figure 2).

### *Phase III: placentation*

During this phase of implantation, the placenta is formed. This phase ends when tertiary villi have been formed.

#### *Stage 5*

This stage occurs 7–13 days after ovulation and is ended by the development of primary villi. In stage 5a, the trophoblasts at the implantation site in humans have expanded into masses of both cytotrophoblasts and syncytial trophoblasts. In stages 5b and 5c, the trophoblasts have invaded the vessel walls and form part of their walls. The blood flows from these maternal vessels into spaces formed between the trophoblasts called lacunae. In stage 5c, the cytotrophoblasts have formed the inner surface of the implantation site and face the blastocyst cavity.

#### *Stage 6*

Formation of secondary placental villi and secondary yolk sac characterizes this stage.

#### *Stage 7*

Branching of the villi and formation of anchoring villi occurs in this stage of implantation.

### *Stages 8–9*

It is during these stages that the tertiary villi develop.

## Endometrial receptivity and blastocyst intrusivity

In order for the blastocyst to implant in endometrium, this tissue should be appropriately prepared and the trophoblastic cells of the developing blastocyst should have attained the ability to invade tissues. A successful implantation is therefore the outcome of interaction of a receptive endometrium with an intrusive blastocyst. Various experimental evidence has shown that endometrium is well prepared for implantation during a defined period called the 'implantation window' (Psychoyos 1973a,b, 1986, 1988; Psychoyos and Casimiri, 1980). Successful implantation not only depends on a receptive endometrium, it requires a blastocyst with the ability to bind to the surface epithelium and then in sequential manner to pass the surface epithelium and implant in the underlying endometrium. In the mouse, the first sign that trophoblast cells have developed an intrusive behaviour appears about 10–15 h after the embryo hatches from the zona pellucida. The initial in-vivo sign of this behaviour, however, is the ability of the trophoblasts to adhere to the surface epithelium. The following is a summary of the sequential steps involved in the transit of the blastocyst through the surface epithelium and into the underlying endometrial stroma, as well as the cellular and molecular signals that have been implicated. The cellular processes that occur during implantation are elucidated in several species and are discussed below. Some aspects of the implantation process bear a striking resemblance to inflammatory events as well as to tumour invasion. However, in contrast to inflammatory response and tumour invasion, implantation consists of a precisely controlled series of events.

### *Endometrial factors contributing to endometrial receptivity and to implantation*

Endometrial receptivity signifies the development of specific cellular and molecular events whose expression is coordinately induced in expectation of blastocyst arrival. Perhaps, some of these events occur even in the absence of a blastocyst within the endometrial cavity or endometrium, whereas induction of others requires establishment of a dialogue between blastocyst and the receptive endometrium. Various architectural, cellular and molecular events in endometrium are coordinated with the 'implantation window' and thus may be the essential elements in the repertoire that signifies endometrial receptivity. This includes formation of pinopodes (Ciocca *et al.*, 1983; Martel *et al.*, 1991; Massai *et al.*, 1993; Psychoyos, 1993), as well as changes in the expression of adhesion molecules (Kadokawa *et al.*, 1989; Tabibzadeh, 1992; Lessey *et al.*, 1992, 1994; Denker, 1993), cytokines (Paria and Dey, 1990; Bhatt *et al.*, 1991; De *et al.*, 1993) and other endometrial proteins (Julian *et al.*, 1994). On the other hand, some endometrial changes seem to occur only in response to the blastocyst. For example, the permeability of the subepithelial capillaries is significantly increased in the endometrium surrounding the blastocyst (Psychoyos, 1973b, 1993). This change occurs in all species studied thus far and therefore is the universal response of endometrium to as yet unidentified signals from the blastocyst (Psychoyos, 1973b, 1993). This increased permeability can be easily seen at the implantation site by injection of a blue dye such as Evans Blue or Protamine Blue (Psychoyos, 1973b, 1993). In the rat, this event occurs about 110 h after ovulation/fertilization. During this period, the increased permeability is seen not only towards the blastocyst but towards other non-specific signals such as traumatic stimuli (Psychoyos, 1973b). However, by the end of day 5 of pregnancy this increased responsiveness of the endometrial vasculature ends and uterus becomes an unfavourable environment to the blastocyst. Blastocysts that are transferred to the endometrial cavity on day 6 of pregnancy do not survive and are expelled within a few hours from the endometrial cavity (Psychoyos, 1973b, 1993). In the following section various cellular and molecular events associated with development of endometrial receptivity and which are thought to be involved in the implantation are discussed.

### *Steroid hormones*

Presence of endometrial receptivity was first established in the rat and later was validated in other species (Psychoyos, 1973b, 1976, 1986, 1993). These studies showed that endometrium could be maintained in various states including a neutral phase, a receptive phase and a non-receptive or refractory phase. In the rat, induction of endometrial receptivity requires a minimum of 3 days of priming of endometrium with progesterone and with minute amounts of oestrogen at the end of this period (Psychoyos, 1993). In the rat, under normal conditions, the 48 h priming of the endometrium by progesterone is reached in the evening of day 4 of pregnancy or pseudopregnancy. During this time, the endometrium is in a 'neutral' state. This neutral state can also be induced by the daily administration of progesterone in rats ovariectomized early in pregnancy. Eggs of the pre-blastocyst stage can develop *in utero* to blastocysts and survive for long periods of time in a state of dormancy as if the process of implantation is frozen at the apposition phase. Availability of oestrogen at the end of the progesterone priming in pregnant or pseudopregnant rats, makes the endometrium 'receptive' around noon on day 5. The significance of oestrogen in fertility has now been clearly established in mice whose oestrogen receptor has been deleted. Both males and females with a disrupted oestrogen receptor gene were found to be infertile (Korach, 1994). In the rat, the entry of morulae into the endometrial cornu occurs on day 4 of pregnancy when the endometrium is in a neutral phase. However, this phase is quickly switched to the receptive phase on day 5 of pregnancy. This period is followed by a 'non-receptive' state on day 6 (Psychoyos, 1973b, 1993). This non-receptive state can also be artificially induced. If progesterone is administered to induce a neutral state and then oestrogen is administered, first the receptive phase is induced and then the non-receptive state is reached within 36 h. Continuous administration of progesterone can prolong this non-receptive period indefinitely. Re-programming of endometrial receptivity requires a minimum of 2 days withdrawal of progesterone administration (Psychoyos, 1973b, 1976, 1986). It is postulated

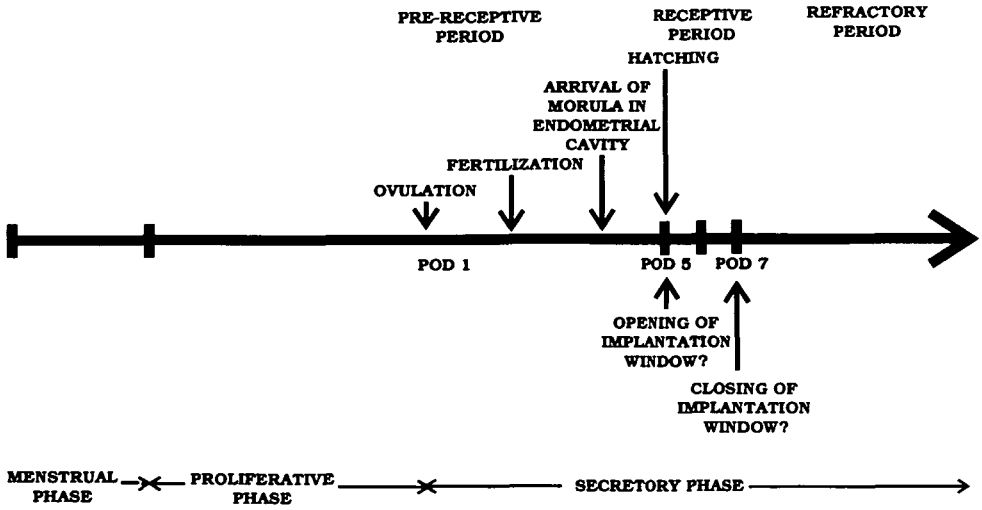


**Figure 3.** Receptive and non-receptive periods of the human menstrual cycle. Menstrual cycles consist of a menstrual phase, a proliferative period followed by the secretory phase. The endometrium of humans seems to be receptive to implantation during a defined interval. The dates and durations that correspond to the period of receptivity have not been clearly defined. However, according to some authors this period seems to fall on the post-ovulatory days (POD) 5–7. Endometrium does not seem to act as a proper host to the blastocyst beyond the confines of this period (Psychoyos, 1973a,b, 1976, 1986, 1988, 1993; Psychoyos and Casmiri, 1980).

that an 'implantation window' also exists in human endometrium during which the endometrium is receptive to implantation by the blastocyst. This phase is followed by a 'non-receptive' phase when the endometrium is refractory to the implantation process (Strauss and Gurpide, 1991; Psychoyos, 1993). However, there is no general agreement as to the dates and duration of such an implantation window. For example, it has been suggested that duration of the implantation window is confined to the post-ovulatory days 5–7 of the normal menstrual cycle (Psychoyos, 1993) (Figure 3). Rogers and Murphy (1989) concluded that the duration of the human implantation window must be at least 3.5 days, whereas Formigli *et al.* (1987) suggest that the period of endometrial receptivity may be as long as 7 days. In-vitro fertilization (IVF) trials have also shown that there is a period of receptivity for the endometrium (Figure 4). For the 4–12-cell stage conceptus, the optimal period of transfer seems to be on days 17–19 of the artificial cycles, with day 15 being the first day of administration of progesterone (Rosenwaks, 1987; Navot *et al.*, 1991). In some IVF trials pregnancies were established when the concepti were transferred on days 16–19 but not on days 20–24 of the artificial cycles (Navot *et al.*, 1986; Rosenwaks, 1987). Estimated implantation times calculated by regression analysis of serial human chorionic gonadotrophin (HCG) measurements were estimated to be between days 7–11 after embryo transfer (Tur-Kaspa *et al.*, 1990). The timing of this 'transfer window' seems to vary, depending on the developmental state of the transferred conceptus and the method of hormone treatment. The shorter duration for the embryo transfer in some artificially induced cycles may be attributable to the rapidity by which the endometrium is prepared. For example, the dates of endometrial biopsies were found to be more advanced than expected after ovulation induction by a human menopausal gonadotrophin (HMG)/HCG regimen (Garcia *et al.*, 1984; Martel *et al.*, 1987). Endometrial receptivity is susceptible to changes by steroid hormones. For example, pinopodes whose presence on the surface epithelium coincides with the period of endometrial receptivity can be induced by administration of progesterone in the rat. These structures remain on the surface epithelium as long as administration of progesterone continues. However, when progesterone is administered along with low doses of oestrogen, the pinopodes appear on day 4 and disappear on day 5 of daily administration of hormones (Martel *et al.*, 1987). RU 486, a progesterone antagonist, displaces the time of appearance of pinopodes from day 5 to day 6 or 7 in the rat (Sarantis *et al.*, 1988).

### Cytokines

In contrast to the systemic steroids, a number of cytokines whose action is primarily exerted in autocrine, paracrine, or juxtacrine fashion have been reported to be implicated in implantation. In the following section, the role of certain cytokines likely to be implicated in human implantation is discussed.

*Leukemia inhibitory factor* (LIF). The strategy to specifically delete genes by a gene targeting technique has opened the possibility of addressing the function of a given gene in the whole animal (Capecchi, 1989). Examination of the effect of most of the genes that have been deleted thus far has not focused on fertility. However, the indirect evidence emerging from these studies has revealed the significance of these genes in fertility, and viability of the embryos (Tables Ia–Id). The effect of gene deletion may range from prenatal to postnatal mortality (Tables Ic–Id). In other instances, gene deletion results in implantation failure or reduced fertility (Table Ib). However, deletion of some genes is without any apparent or pronounced effect on the implantation or viability of the embryo or newborn mice (Table Ia). For obvious reasons, when homologous recombination results in lethality, the impact of that gene in implantation cannot be studied further (Tables Ic–Id). Although deletion of a large number of genes may have some effects on implantation, the ability to maintain and breed mice with the knockout gene suggests that the effect of

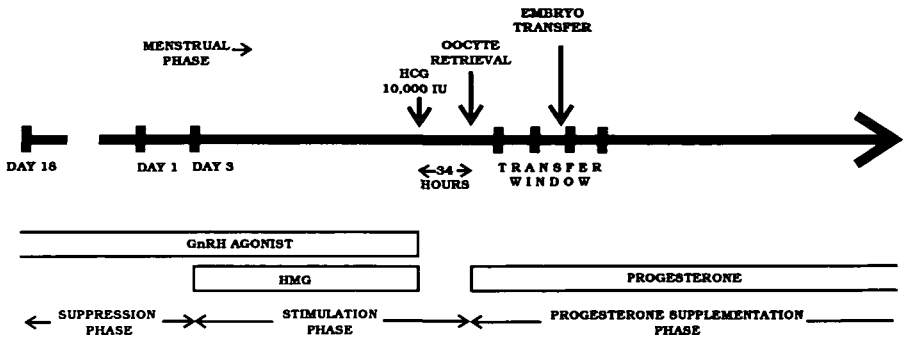


**Figure 4.** Transfer window during an artificial cycle. An artificial cycle is induced first by suppression of the release of the pituitary hormones by the gonadotrophin-releasing hormone (GnRH) agonist during a period called the suppression phase. This period is initiated on day 18 of a menstrual cycle and continues until the third day of menstruation of the subsequent cycle. The amount of GnRH is tapered off on this day and human menopausal gonadotrophin (HMG) is added to stimulate the development of follicles. The co-administration of GnRH and HMG continues and the amount of HMG is progressively increased starting on days 8 or 9 of the artificial cycle until the diameter of the follicles is at least 20 mm or the serum oestrogen concentration reaches at least 500 pg/ml. When these criteria are met, 10 000 IU of human chorionic gonadotrophin (HCG) is administered which simulates the action of luteinizing hormone in a regular cycle. About 34 h later, oocytes are retrieved and administration of progesterone is initiated during a period called luteal support or progesterone supplementation phase. Oocytes are artificially fertilized by spermatozoa *in vitro*. The embryo is generally transferred 48–52 h after oocyte retrieval (Tur-Kaspa *et al.*, 1990). The appropriate time for the transfer of the in-vitro grown embryo (transfer window) seems to coincide with days 1–4 after the progesterone administration is initiated (Navot *et al.*, 1986; Rosenwaks, 1987; Psychoyos, 1993).

these genes on implantation is not significant (Table Ia). LIF is unique: of all the genes that have been studied, deletion of the gene for this cytokine results in implantation failure in homozygous mutant mice.

The role of cytokines in proliferation and differentiation of endometrial cells is now well established (Tabibzadeh, 1991, 1994). In particular, LIF was shown to be indispensable to the process of implantation. The expression of this cytokine was found in the endometrial glands specifically on the fourth day of pregnancy in mice. This increased expression was under maternal control and preceded implantation of the blastocyst (Bhatt *et al.*, 1991). Later, the role of LIF in this process was clearly established in mice that were rendered genetically deficient in the *LIF* gene (Stewart *et al.*, 1992). It was shown that mice in which the expression of the *LIF* gene was eliminated by homologous recombination were infertile (Stewart *et al.*, 1992). These animals ovulated and their ova actively fertilized; however, the developed blastocysts failed to implant in these animals but successfully implanted in the normal surrogate female mice (Stewart *et al.*, 1992). LIF selectively inhibited the formation of primitive ectoderm while permitting the differentiation of primitive endoderm, providing some clue as to how this cytokine participates in preimplantation mouse development (Shen and Leder, 1992).

LIF expression in human endometrium is also consistent with the notion that this cytokine may be implicated in human implantation. The expression of LIF mRNA and protein has been demonstrated in human endometrium (Charnock-Jones *et al.*, 1994). The expression of LIF mRNA and protein was found to be grester during the secretory phase than the proliferative phase (Charnock-Jones *et al.*, 1994). The amount of LIF mRNA in human endometrium is increased about six times during the mid- to late secretory phases as compared to that from the proliferative phase (Charnock-Jones *et al.*, 1994). Quantitative reverse transcriptase–polymerase chain reaction (RT–PCR) demonstrated that expression of LIF mRNA is more abundant in the epithelial than stromal cell fraction of human endometrium (Kojima *et al.*, 1994). Consistent with these findings, LIF protein could be detected primarily in the glandular epithelium rather than the stroma in the mid- to late secretory human endometria (Charnock-Jones *et al.*, 1994). Glandular epithelium showed prominent immunoreactivity for LIF (Charnock-Jones *et al.*, 1994). In addition, the expression of this cytokine was enhanced during the secretory phase (Charnock-Jones *et al.*, 1994). These findings suggest that the expression of LIF mRNA and protein are precisely controlled over a period of time when implantation is expected to take place.

*Interleukin-6* (IL-6). In humans, interleukin-1 (IL-1), IL-6, transforming growth factor (TGF)-β and soluble tumour necrosis factor (TNF) receptors have been found in the human embryo culture fluids during IVF (Austgulen *et al.*, 1995). In the mouse uterus, the expression of IL-1, IL-6 and TNF-α mRNA temporally coincides with the post-mating period which is associated with an acute inflammatory-like response (De *et al.*, 1993). Implantation occurs late on day 4 of pregnancy in this species. Northern blotting, bioassays and immunoenzymatic localization showed that the expression of mRNA and proteins of IL-1, IL-6 and TNF-α were detectable on days 3–9 of pregnancy in uterus. The bioactivity of IL-6 was high on days 3–9 and peaked on days 5 and 6 of pregnancy. The bioactivity for TNF-α progressively increased from day 3 to day 8 of gestation. The bioactivity for IL-1 which was low on day 3 of gestation, peaked on days 4–5, and decreased on days 7 and 8 of gestation (De *et al.*, 1993). The IL-1 increase in gestational uterus was associated with the preimplantation surge of oestrogen (Yoshinaga *et al.*, 1969; Psychoyos, 1973b). This association suggests that the production of IL-1 may be regulated by steroid hormones.

LIF is structurally related to and shares a common receptor transducing signal with a family of related cytokines including IL-6, oncostatin-M, ciliary netrotrophic factor, and IL-11. After

**Table Ia.** The effect of gene disruption on fertility and viability of mouse embryo and newborn; Group I genes. Genes whose deletion is not associated with prenatal or postnatal mortality in the homozygous mice[a]

| Disrupted gene | Phenotype | Reference |
|---|---|---|
| **Leukocytes** | | |
| CD45-exon6 protein tyrosine phosphatase | Impaired T cell, maturation normal B cell development, and normal fertility of homozygous mutant mice | Kishihara *et al.*, 1993 |
| **T cells** | | |
| CD4 | Decreased helper cell activity and normal fertility of homozygous mutant mice | Rahemtulla *et al.*, 1991; Locksley *et al.*, 1993; Killeen *et al.*, 1993 |
| CD8 | Normal helper cell activity and lack of CD8+ positive cells in homozygous mutant mice. Normal fertility of homozygous and heterozygous mice | Fung-Leung *et al.*, 1991 |
| TCRαβ | Loss of thymic medullae, increase in splenic B cells in homozygous mutant mice[a] | Philpott *et al.*, 1992; Mombaerts *et al.*, 1992 |
| TCR delta | Normal T cell development and normal fertility of homozygote mutant female mice | Itohara *et al.*, 1993 |
| TdT | Normal lymphocyte differentiation and breeding in homozygous mutant mice | Komori *et al.*, 1993; Gilfillan *et al.*, 1993 |
| **B cells** | | |
| Immunoglobulin μ chain | Arrest in B cell development in homozygous mutant mice[a] | Kitamura *et al.*, 1991; Kitamura and Rajewsky, 1992 |
| Immunoglobulin κ chain | Blockage of κ chain gene rearrangement in homozygous mutant mice[a] | Zou *et al.*, 1993; Cheng *et al.*, 1993 |
| Immunoglobulin heavy chain | Blockage of B cell development and production of antibody in homozygous mutant mice[a] | Jakobovitz *et al.*, 1993 |
| **Proteins of the major histocompatibility class (MHC) class** | | |
| MHC class II | Near complete elimination of CD4+ lymphocytes in spleen and thymus. Complete inability to respond to T cell-dependent antigens. Reduced growth, impaired–normal breeding performance in homozygous mutant mice | Cosgrove, *et al.*, 1991; Grusby *et al.*, 1991, 1993; Ladel *et al.*, 1994 |
| MHC class II invariant chain | Deficiency in CD4+ cells, and normal fertility of homozygous mutant mice | Viville *et al.*, 1993 |
| **Cytokines and their receptors** | | |
| IL-2 | Normal T cell maturation in homozygous mutant mice[a] | Schorle *et al.*, 1991 |
| IL-4 | Blockage of Th2 cytokine response in homozygous mutant mice[a] | Kopf *et al.*, 1993; Kuhn *et al.*, 1991 |
| IL-6 | Protection from bone loss after oestrogen depletion. Impairment of immune and acute phase responses, and normal fertility of homozygous mutant mice | Poli *et al.*, 1994; Kopf *et al.*, 1994 |
| IL-10 | Anaemia, growth retardation, and development of chronic enterocolitis in homozygous mutant mice[a] | Kuhn *et al.*, 1993 |
| Insulin growth factor II | Growth deficiency[a] | DeChiara *et al.*, 1990 |
| CNTF | Progressive atrophy of motor neurons in homozygous mutant mice[a] | Masu *et al.*, 1993 |
| TGF-α | Abnormalities in hair follicles and eye, and normal fertility in homozygous mutant mice. | Luetteke *et al.*, 1993 |
| IFN-γ | Multiple immune dysfunction, normal development and fertility in homozygous mutant mice | Dalton *et al.*, 1993 |
| IFN-γ receptor | Deficiency in natural resistance, normal development[a] | Huang *et al.*, 1993 |

**Table Ia.** *Cont.*

| Disrupted gene | Phenotype | Reference |
|---|---|---|
| TNF receptor I | Alteration of signalling to specific (p55/60) stimuli and unaltered development of thymocyte and lymphocyte populations and normal fertility of homozygous mutant mice | Pfeffer *et al.*, 1993; Rothe *et al.*, 1993 |
| Lymphotoxin | Abnormal development of peripheral lymphoid organs in homozygous mutant mice[a] | De Togni *et al.*, 1994 |
| G-CSF | Chronic neutropenia, impaired neutrophil mobilization, deficiency in granulocyte and macrophage progenitor cells in homozygous mutant mice[a] | Lieschke *et al.*, 1994 |
| GM-CSF | Progressive accumulation of surfactant and proteins in alveolar spaces, and extensive lymphoid hyperplasia in airways in homozygous mutant mice[a] | Dranoff *et al.*, 1994 |
| **Adhesion molecules** | | |
| Platelet selectin | Defects in leukocyte behaviour (elevated number of circulating neutrophils, total absence of leukocyte rolling, delayed recruitment of neutrophils) and normal fertility in homozygous mutant mice | Mayadas *et al.*, 1993 |
| ICAM-1 | Homozygous mutant mice exhibit moderate granulocytosis, prominent abnormalities in inflammatory responses, normal growth and fertility | Sligh *et al.*, 1993 |
| **Others** | | |
| p-53 | Susceptibility to spontaneous tumours in homozygous mutant mice[a] | Doenhower *et al.*, 1992 |
| α1(IX) collagen | Non-inflammatory degenerative joint disease in homozygous mutant mice[a] | Fassler *et al.*, 1994 |
| mdr1a Permeability-glycoprotein | Deficiency in blood brain barrier, normal phenotype, and normal fertility in homozygous mutant mice | Schinkel *et al.*, 1994 |
| Tenascin | No anatomical or histological abnormalities, and normal reproduction in male and female homozygous mutant mice | Saga *et al.*, 1992 |
| Vimentin | Normal phenotype and fertility in homozygous mutant mice | Colucci-Guyon *et al.*, 1994 |
| Low density lipoprotein receptor | Increased total plasma cholestorol concentrations, normal triglyceride concentrations, and normal fertility of homozygous mutant mice | Ishibashi *et al.*, 1992 |
| tPA | Impairment of clot lysis and normal fertility in homozygous mutant mice | Carmeliet *et al.*, 1994 |
| uPA | Occasional fibrin deposition and normal fertility in homozygous mutant mice; mice mutant for both tPA and uPA are subfertile | Carmeliet *et al.*, 1994 |

[a]Fertility is normal in homozygous mice or has not been reported.
CD = cluster designation; TCR = T cell receptor; MHC = major histocompatibility complex; IL = interleukin; CNTF = ciliary neutrotrophic factor; IFN = interferon; TNF = tumour necrosis factor; G-CSF = granulocyte colony stimulating factor; GM-CSF = granulocyte macrophage-colony stimulating factor; ICAM = intercellular adhesion molecule; PA = plasminogen activator.

binding of the ligand to its respective receptor, gp130 (the signal inducer common to all these cytokines), it dimerizes and in turn activates members of the Jak/Tyk family of tyrosine kinases (Boulton *et al.*, 1994). Therefore it is not surprising to find that considerable overlap exists in the biological actions of these cytokines in diverse target cells (Kishimoto *et al.*, 1994). Despite the common signal transducer for IL-6 and LIF, IL-6 deficient mice were fertile (Kopf *et al.*, 1994; Poli

**Table Ib.** The effect of gene disruption on fertility and viability of mouse embryo and newborn; group II genes: genes whose deletion leads to infertility, implantation failure or abnormal breeding of homozygous mice bearing embryo

| Disrupted gene | Phenotype | Reference |
|---|---|---|
| LIF | Homozygous females are fertile; failure in implantation and reduced growth occurs in homozygous mutant mice bearing embryo; normal fertility in homozygous male mice | Stewart *et al.*, 1992 |
| c-*mos* | Fertile homozygous males; reduced fertility in female due to failure of mature eggs to arrest during meiosis, resulting in parthenogenetic development of unfertilized eggs | Colledge *et al.*, 1994 |
| Oestrogen receptor | Both homozygous males and females are infertile | Korach, 1994 |

LIF = leukaemia inhibitory factor.

**Table Ic.** The effect of gene disruption on fertility and viability of mouse embryo and newborn; group III genes: genes whose deletion results in prenatal lethality in homozygous mice. Effect of homozygosity on fertility and implantation unknown

| Disrupted gene | Phenotype | Reference |
|---|---|---|
| N-*myc* | Defects in heart and lung; death occurs on days 10.5–12.5 of gestation in homozygous mutant mice | Moens *et al.* 1991,1993; Bernelot *et al.*, 1992; Charron *et al.*, 1992; Stanton *et al.*, 1992 |
| c-*myc* | Retarded growth of homozygotes with death 9.5–10.5 days after gestation; reduced fertility of the heterozygotes due to embryonic resorption prior to day 9.5 of gestation | Davis *et al.*, 1993 |
| WT-1 | Abnormal development of kidney, heart and lung in homozygous mutant mice; death on day 13–15 of gestation; heterozygous mutant mice appeared normal for 10 months | Kreidberg *et al.*, 1993 |
| DNA methyl transferase | Delayed development, stunted growth in homozygous mutant mice; death occurs prior to mid-gestation | Li *et al*, 1992 |
| CSK | Developmental arrest in the 10–12 somite stage; growth retardation and necrosis in neural tissues; enhanced p60c-src, p59fyn and p53/p56lyn; necrosis of homozygous embryos at E10.5 | Imamoto *et al.*, 1993; Nada *et al.*, 1993 |
| FGF-4 | Normal implantation and arrest of growth of inner cell mass in homozygous mutant mice; heterozygous mice are normal and fertile | Feldman *et al.*, 1995 |
| E-cadherin | Failure to form trophectoderm epithelium in homozygous mutant mice; death occurs around the time of implantation. | Larue *et al.*, 1994 |

WT-1 = Wilms tumour associated gene; CSK = c-SRC kinase; FGF = fibroblast growth factor; E-cadherin = epithelial cadherin.

*et al.*, 1994). These findings suggest that in contrast to LIF, IL-6 may be a dispensable cytokine during the peri-implantation period.

*Interleukin-1* (IL-1). Since IL-1 is secreted from the embryo (Zolti *et al.*, 1991), and immunoreactivity for the IL-1 receptor antagonist was found in the endometrial luminal epithelium of the mouse uterus (Dang and Polan, 1994), some view IL-1 as being important in implantation. This concept is supported by the fact that injection of recombinant IL-1 receptor antagonist protein to pregnant female mice resulted in implantation failure. The IL-1 receptor antagonist protein seems to interfere with embryonic attachment. However, this cytokine does not seem to have an adverse effect on the formation of blastocyst, hatching, in-vitro attachment of blastocyst to fibronectin or its in-vitro outgrowth and migration (Dang and Polan, 1994).

Other experimental evidence favours the view that IL-1 may be detrimental to embryonic development. For example, IL-1 partially inhibited the attachment of blastocysts to fibronectin-coated Petri dishes; however, it enhanced the outgrowth of trophoblastic cells from the adherent blastocysts (Haimovici *et al.*, 1991). IL-1 was reported to be toxic to the embryo. The effect of this cytokine was observed on 2-cell embryos even at low concentrations (Hill *et al.*, 1987; Fakih *et al.*, 1987). Nevertheless, when the impact of IL-1 on 4-cell embryos was examined, no detectable effect of IL-1α and IL-1β on embryonic development was observed (Schneider *et al.*, 1989). Clearly other studies are needed in order to understand the role of IL-1 in implantation and embryogenesis.

*Tumour necrosis factor*-α (TNF-α). The precise role of TNF-α in implantation, and on the well-being of the fetus is a controversial issue. Some studies indicate a positive whereas others show a negative impact of TNF-α in these processes. This cytokine has been detected in the culture supernatants of preimplantation embryos (Witkin *et al.*, 1991; Zolti *et al.*, 1991). It was reported that daily injections of TNF-α during the preimplantation period reversed the high rate of embryo malformation and fetal loss that occurs spontaneously in mice in the CBA×DBA/2 mating combination (Chaouat *et al.*, 1990). In a model of pregnancy failure in mice, pregnancy and peri-implantation development was shown to be restored by TNF-α or granulocyte–macrophage colony-stimulating factor (GM–CSF) and not by TGF-$\beta_1$ (Tartakovsky and Ben Yair, 1991). Conception in CBA/J mice females using various male strains spontaneously produces a high proportion of abnormal precompaction embryos. In this model, TNF-α and to a lesser extent GM-CSF and IL-1α, promoted development of normal morulae and blastocysts (Tartakovsky and Ben Yair, 1991).

Apart from these studies, other experimental evidence has shown that either TNF-α is not essential to the implantation processes or that it may have adverse effects. Only the mRNA of the p55/60 and not the p75/80 form of TNF-α receptor was found in the mouse blastocyst (Pfeffer *et al.*, 1993).

Deletion of the p55/60 receptor in mouse, however, did not

**Table Id.** The effect of gene disruption on fertility and viability of mouse embryo and newborn; group IV genes: genes whose deletion results in postnatal lethality in homozygous mice. Effect of homozygosity on fertility and implantation unknown.

| Disrupted gene | Phenotype | Reference |
|---|---|---|
| Bcl-2 | Grey hair, polycystic kidney, fulminant lymphoid apoptosis; death occurs 10 days to 10 weeks after birth in homozygotes; normal fertility of heterozygous mice | Nakayama *et al.*, 1993; Veis *et al.*, 1993 |
| TGF-β1 | Multifocal inflammatory disease followed by a wasting syndrome; death occurs ~20 days after birth | Shull *et al.*, 1992 |
| NGF | Profound cell loss in the sensory and sympathetic ganglia in homozygous mutant mice with death within the first 3 days of life; homozygous mutant mice grow and breed normally | Crowley *et al.*, 1994 |
| c-abl | Thymic and splenic atrophy; death occurs 1–2 weeks after birth in homozygous mutant mice | Tybulewicz *et al.*, 1991 |
| Keratin 8 | Mid-gestational lethality in homozygous mutant mice | Baribault *et al.*, 1993 |

BCL = B cell lymphoma/leukaemia-2 gene; NGF = nerve growth factor; TGF = transforming growth factor.

result in reduced fertility of homozygous females or the inability of homozygous mice to reach full term (Pfeffer *et al.*, 1993; Rothe *et al.*, 1993) (Table Ia). These results suggest that TNF-α is either not essential to the process of implantation or embryo development or that its action can be duplicated by other members of the cytokine family (Pfeffer *et al.*, 1993). In fact certain data favour the concept that the action(s) of TNF-α may be directed to the detriment of the developing embryo. Addition of TNF-α to the mouse blastocyst reduced cell proliferation primarily in the inner cell mass. However, exposure to TNF-α was not associated with increased cytotoxicity in cells of the blastocysts (Pampfer *et al.*, 1994). Other studies, however, showed that TNF-α at high concentrations inhibited early development of the embryo (Hill *et al.*, 1987; Eisermann *et al.*, 1989). TNF-α had an inhibitory effect on the 2-cell mouse embryos; however, this cytokine did not have any effect on the attachment of the developing blastocyst and outgrowth of the trophoblasts (Hill *et al.*, 1987; Haimovici *et al.*, 1991).

In an in-vitro model of mouse implantation, TNF-α had no effect on the attachment of the blastocyst or trophoblastic outgrowth (Haimovici *et al.*, 1991). However, a single injection of TNF-α was sufficient to induce fetal resorption in pregnant mice (Tartakovsky and Ben-Yair, 1991). In a separate study, repeated injections of a monospecific polyclonal antibody to mouse TNF-α, to pregnant or newborn mice led to severe and transient growth retardation of the pups. The body weight reached 35% of controls at the third week with complete recovery at 8 weeks. This change was associated with marked atrophy of the thymus, spleen and lymph nodes, lymphopenia and impairment of development of T and B cell areas of lymphoid tissues (Kossodo *et al.*, 1992). However, it cannot be ruled out that the cell losses observed in this model may be due to the lytic effect of complement bound to the immobilized antibody. Thus, the question remains whether TNF-α exerts a positive or negative influence in the maintenance of pregnancy, in the implantation processes and in fetal development.

*Gamma interferon* (*IFN-γ*). In a series of in-vitro studies, IFN-γ significantly inhibited the development of 2-cell embryos and trophoblastic outgrowth of the attached blastocysts (Hill *et al.*, 1987; Haimovici *et al.*, 1991). In addition, deletion of the *IFN-γ* gene was not associated with a fertility impairment in homozygous females or the inability of homozygous mice to reach full term (Dalton *et al.*, 1993) (Table Ia). These studies show that presence of this cytokine is not essential, or is expendable, during implantation and embryogenesis.

*Transforming growth factor-β* (TGF-β). The expression patterns of TGF-β during embryogenesis and in reproductive organs have suggested that these molecules are involved in the reproductive processes and in embryo development (Paria and Dey, 1990; Paria *et al.*, 1992; Selick *et al.*, 1994). Addition of TGF-$\beta_1$ to the culture medium of mouse blastocysts that were grown alone and not in groups enhanced the percentage of embryos that developed into blastocysts (Paria and Dey, 1990). The targeted gene disruption of TGF-$\beta_1$, however, was not associated with failure of the mouse embryos to implant or to develop to term (Table Id). The homozygous mutant animals with the disrupted TGF-$\beta_1$ gene appeared phenotypically normal until ~3 weeks after birth. Then, these animals developed a severe wasting syndrome characterized by marked inflammatory infiltrates in various organs including heart, stomach, liver, lung, pancreas, salivary gland, and striated muscle. The syndrome resulted in the demise of mice about 20 days after birth (Shull *et al.*, 1992). In addition, the expected number of homozygous mutant mice in intercrosses between heterozygote female and male mice with a wild-type gene and a disrupted TGF-$\beta_1$ allele was reduced, suggesting the occurrence of some type of prenatal lethality (Shull and Doestschman, 1994). The occurrence of lethalities in homozygous mice do not allow the consequence of the TGF-$\beta_1$ gene deletion to be examined (Shull *et al.*, 1992). Furthermore, available evidence does not rule out the possibility that TGF-$\beta_1$ from maternal sources may play a role in implantation. For example, TGF-$\beta_1$ modulated the production of oncofetal fibronectin by trophoblasts *in vitro,* suggesting a mechanism through which TGF-β regulates the adhesion of trophoblasts by stimulating production of trophoblast-derived fibronectin (Feinberg *et al.*, 1994).

*Colony stimulating factor-1* (CSF-1). The osteopetrotic (op/op) mutant mice are deficient in several monocyte subpopulations and osteoclasts. These animals also lack CSF-1 due to an inactivating mutation in the *CSF-1* gene. Therefore, op/op mice served as a model to illustrate that CSF-1 is required in female fertility (Pollard *et al.*, 1991). Op/op males were fertile when crossed with heterozygous females. Homozygous mutant crosses between op/op males and females, however, were consistently infertile. When op/op females were crossed with

heterozygous males, fertility occurred at a rate of 46% of that observed for the +/op females and op/op males. Based on these findings it was suggested that CSF-1 is required for pregnancy. However, a maternal source of CSF-1 does not seem to be essential to pregnancy since pregnancy occurs when the male is +/op, suggesting that +/op fetus or +/op seminal fluid can provide the CSF-1 required for pregnancy. Consistent with the notion that CSF-1 plays a role in implantation, it was shown that CSF-1, when added to cultures of human trophoblasts, led to their differentiation to form a syncytium and to the production of placental lactogen (Garcia-Lloret *et al.*, 1989). CSF-1 also stimulated the outgrowth of trophoblast cells in an in-vitro implantation model in the mouse (Haimovici and Anderson, 1993). However, contrary to these findings, paradoxical effects mediated by CSF-1 were reported. Administration of CSF-1 increased resorption and reduced successful implantation (Tartakovsky, 1989; Tartakovsky and Goldstein, 1990; Tartakovsky *et al.*, 1991). CSF-1 was found to impair considerably the capacity of the mouse embryos to implant *in vivo* or to further develop *in vitro*. CSF-1 induced a low rate of pregnancy and high rate of fetal resorptions and inhibited gestation due to its ability to induce defective early development (Tartakovsky, 1989; Tartakovsky and Goldstein, 1990; Tartakovsky *et al.*, 1991). Therefore, the question whether CSF-1 is required for female fertility or may simultaneously impose adverse effects on fertility remains to be clarified.

*Epidermal growth factor (EGF)/TGF-α.* When salivary glands, the major source of EGF, were removed, administration of anti-EGF to pregnant mice increased the abortion rate. However administration of EGF to these animals improved the pregnancy outcome (Fisher and Lakshamanan, 1990). EGF also enhanced the outgrowth of trophoblasts from blastocysts in an in-vitro model of implantation (Haimovici and Anderson, 1993). Presence of EGF in the culture medium of 2-cell embryos did not increase the cell number in the blastocysts (Colver *et al.*, 1991) but produced a higher frequency of hatching from the zona pellucida (Paria and Dey, 1990). EGF bears amino acid sequence homology with TGF-α and both these cytokines bind to a receptor that shares homology with the V-*erb*-B oncogene, a protein with tyrosine kinase activity (Downward *et al.*, 1984). Homozygous mice which lacked a functional gene for TGF-α were viable, displayed waviness of the whiskers and fur and eye abnormalities, yet they were fertile (Luetteke *et al.*, 1993) (Table Ia). These findings show that the actions of TGF-α are dispensable during implantation.

As noted previously, the increased permeability of the subepithelial capillaries is the earliest discernible event that occurs in endometrium in response to an implanted blastocyst. However, more recently changes were described in the surface epithelium even before the blastocyst attached to endometrium. HB-EGF is a member of the EGF family of growth factors. It was recently demonstrated that 6–7 h before the attachment reaction that occurs at 2200–2300 h on day 4 of pregnancy, HB-EGF is expressed in the mouse uterine luminal epithelium surrounding the blastocyst (Das *et al.*, 1994). The gene expression was not detectable when the implantation was delayed by the administration of progesterone. However, this expression was readily induced in the surface epithelium in response to the activated blastocyst when the progesterone-maintained delay in implantation was interrupted by injection of oestrogen. Furthermore, HB-EGF induced autophosphorylation of the receptor for EGF, and promoted hatching of the blastocyst from zona pellucida and enhanced the growth of the blastocyst and the trophoblast. The induction of HB-EGF in the surface epithelium by free blastocyst shows that a dialogue is established between blastocyst and the endometrium even before implantation takes place.

*Granulocyte–macrophage colony-stimulating factor (GM-CSF).* GM-CSF is produced and released by murine and human decidual cells (Wegmann *et al.*, 1989). GM-CSF enhanced the attachment of mouse blastocysts to monolayers of endometrial cells (Robertson *et al.*, 1991). On the other hand, it was reported that GM-CSF inhibited the binding of blastocysts to fibronectin-coated substratum and once bound, GM-CSF had no effect on outgrowth of trophoblasts from the blastocysts (Haimovici *et al.*, 1991). Consistent with this effect of GM-CSF, this cytokine was found to inhibit the development of preimplantation mouse embryos (Hill *et al.*, 1987). Therefore, more studies are required in order to establish the role of this cytokine in implantation.

### *Specific changes in the surface epithelium and underlying endometrium*

Trophoblasts first come into contact with the surface epithelium. Therefore, considerable effort has been invested to show that changes in the apical plasma membranes of surface epithelial cells participate in endometrial receptivity. Other changes have been observed to occur in the underlying tissues during the putative 'implantation window' and in the implanted blastocysts. These changes are discussed below.

*Changes in the surface morphology of surface epithelium.* In the rat, the onset of the receptive phase is characterized by a number of changes in the endometrium. On day 5 of pregnancy or pseudopregnancy, subnuclear vacuoles in the luminal epithelium move apically and nuclei move basally (Psychoyos, 1993). Identical changes occur on the post-ovulatory day 4 in glandular epithelium of human endometrium (Blaustein, 1982). Prior to the receptive phase in the pseudopregnant rat uterus, the surface epithelium exhibits dense, long, thick microvilli and numerous droplets which may be secretory products. On day 5, the microvilli are shortened with some showing a cystic appearance which are designated as 'pinopodes'. From day 6, however, the microvilli become short and pinopodes disappear. The pinopodes do not exist on day 4 in the surface epithelium of pseudopregnant rat uterus and they appear on day 5 (Psychoyos, 1973b, 1993).

The appearance of pinopodes seems to be strictly controlled by progesterone. On the other hand oestradiol, when given along with progesterone at high doses producing a plasma concentration of ~300 pmol/l, inhibits the formation of pinopodes. Low doses of oestradiol, however, do not interfere with the development of the pinopodes until the fourth day of the treatment (Martel *et al.*, 1991). In the rabbit and human, around the time of endometrial receptivity, slender cytoplasmic processes extend into the basal lamina from the surface

epithelial cells (Roberts *et al.*, 1988). Endometrial receptivity in human endometrium is also associated with the appearance of pinopodes. A study showed that in normal menstrual cycles, the pinopodes appeared on post-ovulatory days 5–6. About 78% of bioposies showed presence of pinopodes during this period. The pinopodes regressed on post-ovulatory day 8 and cells were covered with dense, short microvilli (Psychoyos, 1993). Pinopodes have also been observed in the artificially-induced and stimulated cycles (Martel *et al.*, 1987; Massai *et al.*, 1993). Based on these studies it has been suggested that the timing of the opening of the implantation window depends on the length of progesterone treatment (Psychoyos, 1993).

*Changes in the glycocalyx and protein distribution in surface epithelium.* The apical plasma membrane of surface epithelial cells seems to be non-adhesive unless specifically altered. This non-adhesive nature may be attributed to some extent to the layer of glycocalyx that coats the apical plasma membrane of the surface epithelial cells. A decrease in the thickness of glycocalyx and the cell surface charges of the surface epithelial cells during the receptive period have been demonstrated in several species (Enders and Schalfke, 1977; Morris and Potter 1984, 1990; Anderson *et al.*, 1986, 1990; Morris *et al.*, 1988; Potter and Morris, 1990). Therefore, it seems that loss of apically disposed glycoconjugate moieties may be required for development of a receptive phenotype by surface epithelial cells. It has been demonstrated that in mice the bulk of the apical glycoconjugates belong to the integral transmembrane mucin-type glycoproteins (Valdizan *et al.*, 1992). A glycoprotein designated as Muc-1 is expressed on the surface epithelium both in mouse (Valdizan *et al.*, 1992; Braga and Gendler, 1993) and human (Hey *et al.*, 1994). Muc-1 at both the protein and mRNA levels was found in the proestrous and oestrous phases in epithelial cells in the mouse uterus. The expression of both mRNA and protein in the luminal epithelium was found to decrease during diestrus and to become undetectable by day 4 of pregnancy prior to blastocyst attachment. Therefore, it was suggested that the loss of Muc-1 contributes to acquisition of endometrial receptivity (Valdizan *et al.*, 1992). Paradoxical findings were reported in humans. It was reported that the expression of Muc-1 mRNA and protein was regulated during the menstrual cycle, with maximal expression of this glycoprotein being attained during the implantation phase (Hey *et al.*, 1994). Therefore, further studies are required in order to clarify the role of Muc-1 in implantation.

A number of studies show that endometrial receptivity is associated with changes in the apical plasma membrane of surface epithelium. As shown by freeze-fracture studies, the density of intramembranous protein particles increases during the period of endometrial receptivity in the apical plasma membrane (Murphy *et al.* 1982a; Winterhager *et al.*, 1990). Perhaps, the most convincing evidence for the alterations that make the surface epithelium receptive is the observation of Carson *et al.* (1990). These investigators showed that receptors for heparin sulphate proteoglycans were expressed during the receptive phase in the apical membrane of surface endometrial epithelium in mice. These receptors were used by trophoblasts for attachment. Endometrial receptivity has been shown to be positively associated with the expression of glycoconjugates with a terminal galactose (Chavez and Anderson 1985; Anderson 1986, 1990). However, there is a decrease in lectin binding, including those that recognize galactose in the endometrial epithelium around the blastocyst, casting doubt on the importance of these residues in implantation (Bukers *et al.*, 1990).

The expression of both heparin sulphate proteoglycan (HSPG) and laminin on mouse trophectoderm is confined to specific phases of implantation. Both HSPG and laminin were reported to be expressed at the exterior surface of the trophectoderm during the attachment phase of implantation *in vivo*. However, in-vitro experiments demonstrated that the immunoreactivity for these moieties had diminished by the outgrowth stage of development (Carson *et al.*, 1993).

In mice, the expression of tenascin in endometrium is both spatially and temporally confined to specific phases of implantation (Julian *et al.*, 1994). During the oestrous cycle and on days 1–4 of pregnancy, the expression of tenascin in extracellular matrix of endometrial stroma was found to be low. However, once the blastocyst was attached to surface epithelium (day 4.5), an intense deposition of tenascin fibrils was observed in the extracellular matrix of endometrial stroma immediately beneath the site of blastocyst attachment. Subsequently, this expression was diminished on day 5.5. Tenascin expression in the endometrial stroma could also be induced artificially *in vivo* and by conditioned medium of endometrial epithelial cells *in vitro*. Based on these findings, it has been suggested that the expression of this protein may be used as a marker for endometrial receptivity (Julian *et al.*, 1994). In addition, purified tenascin and a recombinant fragment consisting of alternately spliced fibronectin type III repeats interfered with the adhesion of endometrial epithelial cells to Matrigel® whereas other recombinant fragments of tenascin and fibronectin were ineffective. Therefore, it was suggested that the function of tenascin at the implantation site is to interfere with epithelial cell adhesion, facilitating penetration of the endometrium by the embryo (Julian *et al.*, 1994). Despite these findings, mice lacking tenascin were found to have a normal phenotype. Histological abnormalities could not be found in these animals. Furthermore both the homozygous male and female mice were found to be fertile, suggesting that the action of tenascin during development is dispensable (Saga *et al.*, 1992) (Table Ia).

*Changes in junctions and adhesion molecules.* Tight, gap, intermediate (adherens) and desmosomal junctions tightly bind cells together and are responsible for the maintenance of tissue integrity (Rhodin, 1974). Alterations within some of these junctions or their molecular constituents have been reported to occur in the surface epithelium of receptive endometrium. During the receptive phase, the apical plasma membrane acquires the ability to form reflexive gap junctions (Murphy *et al.*, 1982b, 1982d) and under some experimental conditions, hemidesmosome-like junctions (Denker, 1977). During this phase, the gap junctions extend to the lateral plasma membranes and finally towards the basal membrane (Murphy *et al.* 1982c). The closely related molecules desmoplakin-I and -II, are the most abundant proteins of the cytoplasmic portion of the desmosome which consists of an electron dense, trilaminar

membrane (Mueller and Franke, 1983; Kapprell *et al.*, 1988). These proteins are thought to be important in the adhesive action of the desmosomal plaque (Skerrow and Matoltsy, 1974; Mueller and Franke, 1983; Miller *et al.*, 1987; Steinberg *et al.*, 1987). It was reported that during the receptive phase, desmoplakin-I translocated into the lateral borders of the endometrial epithelium in the rabbit (Classen-Linke and Denker 1990).

Cadherin molecules are present within the adherens junctions and along with a complex of proteins composed of catenins and actin molecules contribute to the cell–cell binding (Nagafuchi and Takeichi, 1989; Ozawa *et al.*, 1989; Suzuki *et al.*, 1991; Takeichi, 1991; Geiger and Ayalon, 1992; Luna and Hitt, 1992; Mareel *et al.*, 1993). In the rabbit, E-cadherin is distributed in the subapical region in the surface epithelium during the pre-receptive phase. However, around the blastocyst, the expression is more evenly distributed in the lateral plasma membranes. On days 8–9 of gestation, the expression of this molecule translocates to the basal regions of the plasma membrane of surface epithelial cells in the small cytoplasmic processes that extend into the basal lamina (Denker, 1993). Some evidence suggests that cadherins are expressed at the interface of trophoectoderm and uterine epithelium (Kadokawa *et al.*, 1989). However, these findings have not been extensively supported and other investigators have not been able to find similar accumulation of cadherin proteins (Vestweber *et al.*, 1987). Presence of E-cadherin is essential to the maintenance of function of the epithelial cells in the embryo and to the process of compaction. It was recently demonstrated that although compaction occurred normally, lack of E-cadherin in the null mutant mice embryos resulted in their death (Larue *et al.*, 1994). At the blastocyst stage, E-cadherin negative embryos failed to form trophectoderm epithelium and the blastocyst cavity (Larue *et al.*, 1994) (Table Ic).

Expression of CAM-105, another adhesion molecule, in the endometrial epithelial cells and blastocysts is lost before adhesion, a finding that is inconsistent with the role of this protein in the embryo attachment (Svalander *et al.*, 1987, 1990).

*Involvement of integrins.* In mice, the first sign that the trophoblasts have developed the phenotype appropriate for implantation appears about 10–15 h after the embryo hatches from the zona pellucida. This property is evident from conversion of the quiescent non-adherent trophoblasts to cells that exhibit the ability to penetrate the epithelium and to bind to the underlying basal lamina and extracellular matrix components. Therefore, the developing trophoblasts simultaneously exhibit two distinct properties. On one hand, they become competent to show cell–cell and cell–matrix binding. On the other hand, consistent with an invasive behaviour, they exhibit the ability to interpose themselves among other cells by separating them and degrading the components of the extracellular matrix.

Many basement membrane and extracellular matrix components such as fibronectin, laminin, entactin, collagen type IV, hyaluronic acid and heparin sulphate proteoglycan, promote the attachment and outgrowth of the blastocyst *in vitro* (Armant, 1986; Carson *et al.*, 1993). Some of the proteins that have been found to enhance blastocyst attachment are part of the

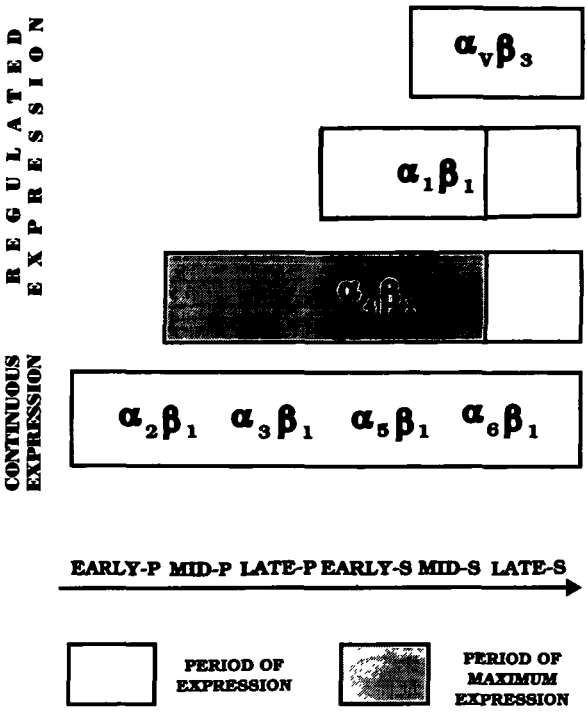


**Figure 5.** Patterns of integrin protein expression during the menstrual cycle in human endometrial glands. The immunoreactivity of some integrins is continuously seen in endometrial glands throughout the entire menstrual cycle. However, other immunoreactive proteins are observed during defined periods. P = proliferative, S = secretory (Tabibzadeh, 1992; Lessey *et al.*, 1992, 1994).

basal lamina of the surface epithelium of endometrium, whereas others are found in the supporting meshwork of underlying stroma. Recent data suggest that many adhesive proteins share the tripeptide arginine–glycine–aspartic acid (RGD) sequence (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Ruoslahti, 1991). The RGD sequences of these ligand proteins are recognized by one or several members of the family of structurally related receptors that are collectively called integrins (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Ruoslahti, 1991). These molecules are involved in cell–cell and cell–matrix interactions and have been recognized to contribute to diverse functions of cell migration, organization of the cytoskeleton, and transduction of differentiation signals (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Ruoslahti, 1991). The integrins are heterodimeric glycoproteins composed of at least one of 11 α and one of six β chains (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Ruoslahti, 1991). The $\beta_1$ subfamily, consists of association of the $\beta_1$ with one of six different α chains. These molecules serve as receptors for collagen ($\alpha_{1-3}\beta_1$), fibronectin ($\alpha_{3-5}\beta_1$), or laminin ($\alpha_{1-3}\beta_1$, $\alpha_6\beta_1$). The immunoreactivity of two members of this family, $\alpha_4\beta_1$ and $\alpha_v\beta_3$ was found during the putative implantation window in human endometrium (Tabibzadeh, 1992; Lessey *et al.*, 1992, 1994). Immunoreactive $\alpha_1\beta_1$ which was not present in glandular epithelial cells in the proliferative phase. However, intense immunoreactivity was found in these cells post-ovulation, with the staining reaction diminishing in the late secretory phase (Figure 5). This molecule was not present in the stromal cells; however pre-decidual cells were character-

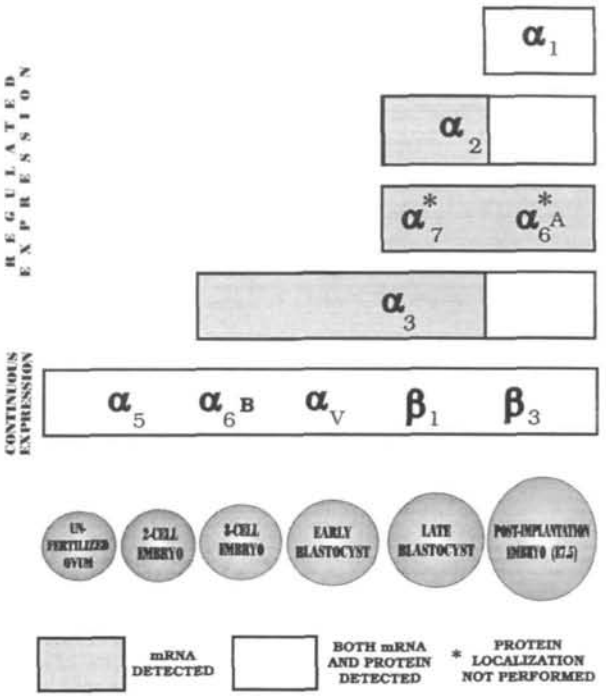


**Figure 6.** Patterns of integrin subunit expression during the peri-implantation period in mouse embryo. The expression of some integrins is continuously seen in embryonic cells during the initial period of development. However, the expression of other integrins is regulated (Sutherland *et al.*, 1993).

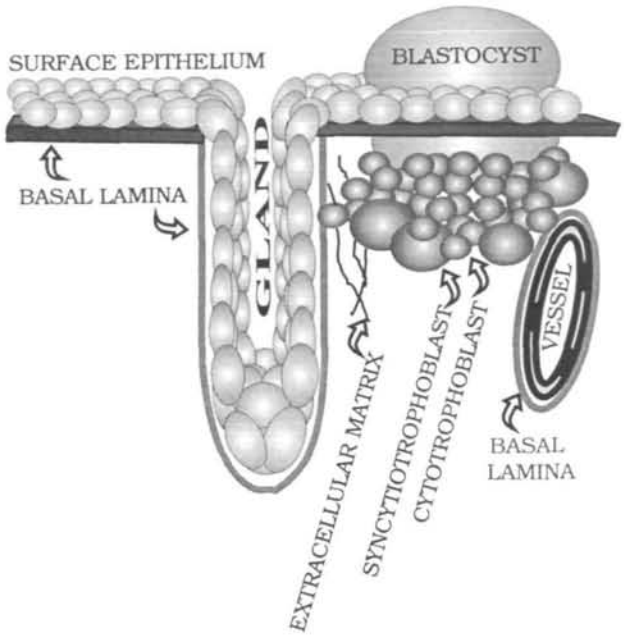


**Figure 7.** Trophoblast invasiveness. The trophoblasts in defined periods gain the ability to degrade the basement membrane of the surface epithelial cells and the extracellular matrices in the underlying stroma. In some instances, the cytotrophoblasts violate the integrity of the basement membranes of the adjacent glands and endometrial vessels.

istically immunoreactive for this molecule in the late secretory phase. Intense immunoreactivty for $\alpha_4\beta_1$ was present in the glandular epithelial cells in the mid-proliferative to mid-secretory phases (Figure 5). The immunoreactivity for $\alpha_v\beta_3$ in the endometrial epithelium was maximal in the mid-secretory phase (Figure 5). The distinct cycle-specific distribution of the integrin molecules suggests that they may be involved in the process of implantation. Clinical observation of lack of $\alpha_v\beta_3$ during the implantation window in patients with documented luteal phase deficiency, certain patients with minimal or mild endometriosis and infertility are consistent with this viewpoint (Lessey *et al.*, 1992; Klentzeris *et al.*, 1994).

Integrins are also expressed in the blastocyst and, consistent with the notion that they are implicated in the implantation process, the expression of integrins in the embryo is developmentally regulated (Sutherland *et al.*, 1993). $\alpha_5\beta_1$, $\alpha_6\beta_1$ and $\alpha_v\beta_3$ mRNA and proteins were expressed on the blastocyst continuously whereas the expression of five other integrin subunits was developmentally regulated (Figure 6). The mRNA of $\alpha_2$, $\alpha_6$A and $\alpha_7$, which all bind laminin, were first detected in the blastocyst during endodermal differentiation and the outset of attachment competence (Figure 6). The expression of these integrins was confined to the differentiating trophoblasts (ectoplacental cone). The mRNA for $\alpha_1$ was undetectable until outgrowth of trophoblasts had begun (Sutherland *et al.*, 1993). Thus, site-specific and temporal appearance of integrins both in endometrium and in the blastocyst provide support for the concept that these proteins are essential to the process of implantation.

## Proteins implicated in trophoblast invasion and its control

Subsequent to the receptive phase, the endometrium can act as a barrier to the implantation process. The fact that the endometrium possesses the ability to inhibit the implantation is evident by successful implantation of mouse blastocysts in the uterus where endometrium is removed (Cowell, 1969). Furthermore, as alluded to previously, implantation of the trophoblasts can be delayed by administration of progesterone. However, when removed from the uterus of the animal treated with progesterone, blastocysts exhibit the ability to proliferate, to attach and to assume an invasive behaviour similar to their normal counterparts. The delay in implantation induced by progesterone can be overcome by injection of oestrogen or actinomycin D (Finn and Bredl, 1973). Therefore, endometrial epithelium possesses the ability to regulate or to prevent the implantation process. The signals and the molecular pathways that underlie this controlling mechanism are unknown. However, it has been suggested that Muc-1, an integral membrane protein, may serve as one such barrier mechanism (Braga and Gendler, 1993). The intrusive behaviour of trophoblasts is sequentially coordinated to displace endometrial epithelium in some species, to penetrate the basal lamina underlying the surface epithelium, to displace the underlying stromal cells and to degrade the components of the extracellular matrix (Figure 7). Undoubtedly, controlling mechanisms exist that curtail the excessive penetration of the endometrial stroma by the invasive blastocyst. Excessive penetration of the endometrium (placenta accreta) to the point that trophoblasts reach the underlying myometrium or their inability to adequately penetrate endometrium (preeclampsia) are conditions where

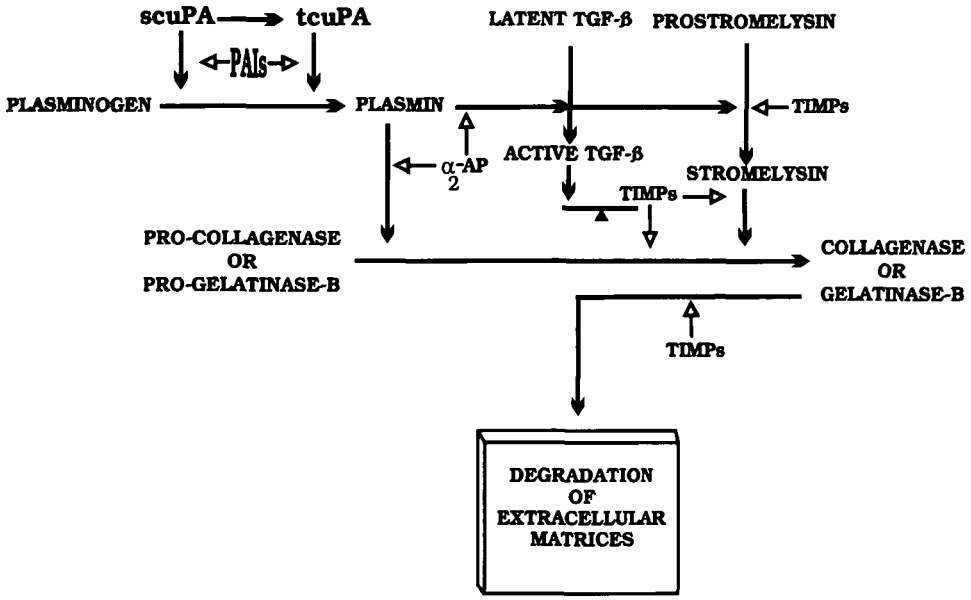


**Figure 8.** Molecular pathways involved in the degradation of extracellular matrices. The stimulation or activation pathways are shown by solid arrows and the inhibitory pathways by open arrows. TGF-β increases the expression of TIMP; Sc = single chain; tc = two chain; uPA = urokinase-type plasminogen activator; TGF-β = transforming growth factor-β; TIMP = tissue inhibitor of metalloproteinase; PAI = inhibitor of plasminogen activator; $\alpha_2$–AP = $\alpha_2$, antiplasma.

these controlling mechanisms have failed. Control of the invasive behaviour of trophoblasts is seen in different species. In some animals such as pig, the trophoblasts remain in apposition with the surface epithelium without exhibiting an invasive potential (epitheliochorial type of placentation). In ruminants, only some trophoblasts fuse with the cells of the surface epithelium (synepitheliochorial type of placentation). In primates and rodents, the trophoblasts are invasive, and eventually form contacts with the maternal vasculature (haemochorial type of placentation).

In the haemochorial type of placentation, the first evidence of the invasive nature of trophoblasts is exhibited by penetration of the basal lamina of the surface epithelium (Figure 7). Subsequently, trophoblasts degrade the extracellular matrices in order to gain access to endometrial stroma (Figure 7). Some trophoblast cells also penetrate through the basal lamina of maternal vessels and glandular epithelium (Figure 7). Once the villi have formed, some cytotrophoblast cells acquire a highly invasive phenotype. These cells extend through the syncytiotrophoblast layer and invade the decidua. These so-called intermediate or extravillous trophoblasts join and form the cytotrophoblastic shell that anchors the placenta to the decidua (Boyd and Hamilton, 1967). These cells occasionally penetrate as deeply as the underlying myometrium. Some of these cells penetrate the basal lamina of uterine arterioles and line these vasculatures to form the endovascular trophoblasts, assuring a continuous blood supply to the placenta (Loke, 1990).

From the diverse attributes that characterize invasive trophoblasts, the best characterized are the molecules involved is the degradation of the extracellular matrix. This degradation takes place by the cooperative interaction of members of the matrix metalloproteinase (MMP) family, plasminogen activators that belong to the serine proteinase family and plasmin. The action of these molecules is controlled respectively by tissue inhibitors of metalloproteinases (TIMP) and by inhibitors of plasminogen activation (Figure 8).

### *Role of plasminogen, plasminogen activators and inhibitors in implantation*

At the turn of century, Graf von Spee introduced the concept that trophoblasts act as invasive cells (see Denker, 1977). The fact that uterus-derived proteins exist that inhibit the degradative activity of proteases during development of placenta was consistent with this hypothesis (see Denker, 1977). It was later shown that, in the mouse, the process of blastocyst attachment required a trypsin-like activity and the trophoblast outgrowth required both the trypsin-like and plasminogen activator activities (Kubo *et al.*, 1981).

Plasminogen and its activators and inhibitors participate in the implantation process. The plasminogen activators (PA) belong to a group of proteases that require a serine residue for their catalytic activity. The activation of plasmin is controlled by two urokinase-type and tissue-type PA that are designated respectively as uPA and tPA (Figure 8). These substrate-specific serine–proteases cleave an Arg-Val peptide bond from plasminogen and convert it to plasmin (Robbins *et al.*, 1967; Dano *et al.*, 1985). Plasmin mediates fibrinolysis and activates MMP, and therefore leads to degradation of the extracellular matrix. uPA binds to a receptor that is located on the cell surface. This binding alters the activity of uPA and its susceptibility to the action of inhibitors, and localizes the site of plasmin activation to the pericellular regions. The generated plasmin may itself become bound and resistant to inhibition. This cell surface-associated event confines the action of MMP to precisely controlled locations (Murphy *et al.*, 1992). The activity of these activators is controlled by specific inhibitors called $PAI_1$ and $PAI_2$ which bind PA with a high affinity (Loskutoff *et al.*, 1989; Kruithof, 1990). Furthermore, the

activity of the plasmin in activating the MMP is inhibited by the action of $\alpha_2$-AP (Figure 8).

Trophoblastic cells of mouse blastocysts cultured *in vitro* produced PA during the period corresponding to the in-vivo invasion of the endometrium (Strickland *et al.*, 1976). Cytotrophoblasts isolated from the term human placentas also synthesized a urokinase-type PA (Queenan *et al.*, 1987). Human trophoblast cells express the uPA receptor (Zini *et al.*, 1992; Multhaupt *et al.*, 1994) and therefore by binding the active uPA are able to exert the proteolysis in precisely controlled sites (Estreicher *et al.*, 1990; Roldan *et al.*, 1990).

Consistent with the importance of PA in implantation, mutation in $t^{w73}$ which resulted in reduced PA in the homozygous mouse embryos was associated with implantation failure (Axelrod, 1985). The lack of functional low-density lipoprotein (LDL) receptor-related protein (LPR), which is required for various protease–inhibitor complexes such as PA–inhibitor complexes (Nykjaer *et al.*, 1992; Orth *et al.*, 1992) and $\alpha_2$-macroglobulin–protease complexes (Strickland *et al.*, 1990; Kristensen *et al.*, 1990), was associated with a normal phenotype until the blastocyst stage (Herz *et al.*, 1992). Careful examination of the embryos for homozyosity showed that LPR-deficient blastocysts could implant. At 9.5 and 10 days post-coitum, a delay or arrest in development was found in some but not all the homozygous early postimplantation embryos (Herz *et al.*, 1992). However, by day 13.5 post-coitum, all 10 homozygous embryos were in the process of being resorbed, indicating that lack of LPR is inconsistent with development to term (Herz *et al.*, 1992). Separate lack of functional uPA or tPA in the embryo was not associated with loss of ability to implant normally (Carmeliet *et al.*, 1994). However, mice deficient in both uPA and tPA were found to be subfertile, an effect which may be due to the role of PA at sites other than the endometrium (Carmeliet *et al.*, 1994). Despite these studies other available evidence has not confirmed a conclusive role of PA in implantation. For example, epsilon-aminocaproic acid which inhibits the activation of plasminogen, did not prevent the attachment of rabbit blastocysts (Denker, 1977; Denker and Fritz, 1979) and nor did it inhibit the degradation of the matrix by mouse trophoblasts grown *in vitro* (Glass *et al.*, 1983). Therefore, further studies are needed to clarify the role that PA and plasmin play in implantation.

### *Role of matrix metalloproteinases in implantation*

MMP belong to the family of metalloendopeptidases (Woessner, 1991). This family includes matrixin, thermolysin, astacin, serratia, and snake venom metalloproteinases (Jiang and Bond, 1992). A characteristic feature of most metalloendopeptidases is that they contain zinc as the metal ion essential to their catalytic activity (Jiang and Bond, 1992). At least three distinct subsets of enzymes within the MMP family have been recognized which include the collagenases, gelatinases and stromelysins (Table II). The enzymes within the matrixin family possess the ability to degrade the extracellular matrix components including collagens, gelatins, fibronectin, laminin, and proteoglycan. Some members also possess the additional ability to activate the latent forms of other MMP. For example, MMP-3 activates the latent form of MMP-1 and MMP-9 (Woessner, 1991; Ogata *et al.*, 1992). Members of the multigene family of zinc metalloproteinase include at least nine proteins (Table II). Comparison of the amino acid sequences predicted from cDNA of MMP reveals distinct domains that are conserved among various members of the family. In addition, there is a high degree of conservation of sequences of MMP across several mammalian species (Murphy and Docherty, 1992). Various domains have been identified within the MMP family which include a signal peptide, a propeptide, a catalytic domain, a fibronectin-like domain, a collagen-like domain, and a haemopexin-like domain. Signal peptide domain accounts for the direction of the translational product to the endoplasmic reticulum and ultimately its secretion. The loss of propeptide domain is associated with activation of the enzyme. The catalytic domain exhibits the ability to degrade the constituents of the extracellular matrix after activation. The haemopexin-like domain mediates the interaction of MMP with collagen fibres. This domain also interacts weakly with TIMP-2 when the enzyme is in inactive form and may play a role in the binding of both TIMP-1 and TIMP-2 when the enzyme becomes active (Murphy and Docherty, 1992). However, not all MMPs contain all these domains. The gelatinases are characterized by the presence of signal peptide and the propeptide, catalytic, haemopexin-like and fibronectin-like domains. Gelatinase-B (MMP-9) is differentiated from gelatinase-A (MMP-2) by the presence of a collagen-like domain. The collagenases and stromelysins contain the signal peptide and the propeptide, catalytic, and haemopexin-like domains and lack the fibronectin-like and collagen-like domains. The matrilysin (MMP-7) contains only the signal peptide and the propeptide, and catalytic domains.

Both rodent and human trophoblasts secrete proteins such as gelatinase-B that degrade extracellular matrix. It has been demonstrated that the invasiveness exhibited *in vitro* by the rodent and human trophoblasts depends on the production of gelatinase-B (Librach *et al.*, 1991; Berhrendtsen *et al.*, 1992). The expression of this protein also coincides with the maximal invasive potential of the trophoblasts *in vivo*, which is exhibited in mice on day 7.5 of gestation (Berhrendtsen *et al.*, 1992) and in humans during the first trimester (Librach *et al.*, 1991; Bass *et al.*, 1994; Shimonovitz *et al.*, 1994). Both the gelatinase A and B were secreted by human trophoblasts isolated from first trimester placentas. However, trophoblasts from third trimester placentas secreted primarily gelatinase B, and gelatinase A was secreted in minimal amounts (Shimonovitz *et al.*, 1994). These findings provide a basis for understanding the different attributes described for trophoblasts from different trimesters. For example, it was reported that human trophoblasts isolated from the first trimester placenta and not the second or third trimester degraded the components of the extracellular matrix (Fisher *et al.*, 1989). Similarly, the trophoblast cells from first trimester and not term placentas were able to degrade Matrigel (Librach *et al.*, 1991). The invasive behaviour of trophoblasts could be blocked by the inhibitors of metalloproteinases (1,10-phenanthroline and recombinant TIMP-1) or inhibitors of serine proteases (aprotinin, epsilon aminocaproic acid, and anti-plasminogen antibody), indicating

**Table II.** The matrix metalloproteinase (MMP; matrixin) family

| Group | MMP no. | Common names | Molecular weights (kDa)[a] | Extracellular matrix protein substrates | Cell sources |
|---|---|---|---|---|---|
| **Collagenases** | | | | | |
| | MMP-1 | Interstitial collagenase EC 3.4.24.7 | 57 (42) | Collagens I,II,III VII, VIII,X Gelatins PG core protein | Stroma of endometrium; Connective tissue cells |
| | MMP-8 | PMN collagenase; EC 3.4.24.34 | 85 (65) | Same as MMP-1 | Neutrophils |
| **Gelatinases** | | | | | |
| | MMP-2 | Gelatinase A; 72 kDa gelatinase; Type IV collagenase; EC 3.4.24.24 | 72 (66) | Collagens IV,V,VII X, XI; gelatin; PG core protein; fibronectin | Most cells stroma of endometrium |
| | MMP-9 | Gelatinase B; 92 kDa gelatinase; Type IV collagenase; EC 3.4.24.35 | 92 (82/84) | Collagens IV,V; gelatin; PG core protein | Stroma of endometrium; connective tissue cells; monocytes; tumour cells |
| | MMP-7 | Punctuated MP (PUMP); Matrilysin; Uterine MP; EC 3.4.24.23 | 28 (21/19 | Collagen IV; gelatin; fibronectin; proteoglycan; procollagenase | Immature monocytes; mesangial cells; tumour cells (not fully defined) |
| **Stromelysins** | | | | | |
| | MMP-3 | Stromelysin-1; transin (rat); procollagenase activator EC 3.4.24.17 | 60/57 (50/48) | Collagens IV,V,IX, X; fibronectin; laminin; casein (in zymograms); Pro-MMP-1 | Stroma of endometrium; macrophages; connective tissue cells |
| | MMP-10 | Stromelysin-2; transin-2 (rat); EC 3.4.24.22 | 53 (47) | Same as MMP-3 | Stroma of endometrium |
| | MMP-11 | Stromelysin-3 | 51 | Unknown | Epithelium of endometrium; stromal cells in tumours |
| **Others** | | | | | |
| | MMP-12 | Metallo-elastase (mouse) | 57 | Fibronectin; elastin | Macrophages |

References: Woessner, 1991, Murphy and Docherty, 1992.
MMP = matrix metalloproteinase; MP = metalloproteinase; PG = proteoglycan; PMN = polymorphonuclear cells.
[a]The molecular weights shown are the secreted latent form of the enzyme. The molecular weights of the active molecules are shown in parentheses.

that both groups of enzymes were essential for invasion (Lala *et al.*, 1989).

Control of extracellular proteolysis is critical to the organism in general and is essential in controlling the invasion of endometrium by trophoblasts. Therefore, several different mechanisms exist in order to exert stringent control over the degradation of the extracellular matrix by metalloproteinases. This includes control over transcription, translation, and secretion of metalloproteinases (Angel *et al.*, 1987; Woessner, 1991; Murphy and Docherty, 1992; Gordon *et al.*, 1993; Denhardt *et al.*, 1993; McDonnell *et al.*, 1994; Uria *et al.*, 1994). Metalloproteinases are secreted in inactive form; therefore the degradation of extracellular matrix is not initiated unless these enzymes become activated by a select group of factors. Finally, even after these enzymes are activated, their action can be inhibited by serum constituents or specific tissue inhibitors designated as TIMPs.

MMPs are regulated at the transcriptional level by a variety of growth factors, cytokines, oncogenes and tumour promoters. Furthermore, MMPs are not stored in most cell types, rather these proteins are synthesized and secreted only after the appropriate signal has been received by the cell. Therefore, loss of the stimulating signal acts as an additional means for controlling the amount of MMPs. MMPs are secreted in a latent form. The latency is attributable to a conserved cysteine that binds to zinc at the active centre of the molecule (Woessner, 1991; Kleiner *et al.*, 1993). The potential physiological activators of the MMP family include plasmin, plasma kallikrein, and cathepsins such as cathepsin B and cathepsin G, and neutrophil elastase (Murphy and Docherty, 1992). A number of studies are supportive of the concept that plasmin is involved in the generation of an active MMP enzyme (Murphy and Docherty, 1992, Murphy *et al.*, 1992). Cleavage of a given MMP by plasmin is followed by autolytic cleavage by the activated metalloproteinase. This autolytic cleavage results in separation of the propeptide and thus confers permanent activity to the enzyme (Woessner, 1991).

Stromelysin-1 (MMP-3) initially cleaves the pro-gelatinase

B (pro-MMP-9). This cleavage makes the molecule susceptible to a second cleavage by MMP-3 resulting in a 82 kDa form of the enzyme that is proteolytically active (Ogata *et al.*, 1992). Stromelysin which is sequestered on the collagenous matrix is particularly susceptible to plasmin activation (Figure 8). Once activated, stromelysin induces the activation of collagenase and gelatinase-B (Figure 8) (Murphy and Docherty, 1992). On the other hand, gelatinase-A does not have any apparent cleavage site susceptible to the action of plasmin or other proteinases. The action of this enzyme seems to depend on a self-cleavage of the pro-peptide and consequently activation of the enzyme (Nagase *et al.*, 1991; Ward *et al.*, 1991b). An alternative mechanism for production of the active form of gelatinase-B consists of activation by a membrane activator shown to be present in fibroblasts and tumour cells (Ward *et al.*, 1991a). This activator, which is inducible by TGF-β, does not activate other MMPs. It is not known whether the activator is a proteinase or whether it initiates the autocatalytic cleavage of the MMPs (Ward *et al.*, 1991a).

Several studies indicate that gestational decidualized endometrium possesses the activity to control the invasive property of trophoblasts. The extent and duration of invasion of mouse trophoblasts was greater in the extrauterine sites such as kidney, testes, spleen, liver, brain, and the non-gestational and non-decidualized endometrium than in the pregnant or non-pregnant decidualized endometrium (Kirby, 1960, 1963a, 1963b, 1965). In addition, in humans, in conditions that were associated with poor decidualization such as placenta accreta and ectopic pregnancy, a higher degree of trophoblastic invasion was observed. Some studies indicate that at least to some extent the ability to control trophoblast invasion is manifested by the decidual cells and that this control is exerted by two molecules, TGF-β and TIMP. In support of this concept it was demonstrated that conditioned media of decidual cells from the first trimester inhibited the invasiveness of trophoblasts (Repesh, 1989; Graham and Lala, 1992). This anti-invasive property could be inhibited by neutralizing antibodies and was simulated by pure exogenous TGF-$\beta_1$ (Repesh, 1989; Graham and Lala, 1992). It has been suggested that the mechanism through which TGF-$\beta_1$ exhibits this property is induction of TIMP-1 in both decidua and trophoblasts (Graham and Lala, 1991, 1992; Graham *et al.*, 1992). TGF-β resulted in an increase in the mRNA expression of TIMP-1 (Graham and Lala, 1991; Graham *et al.*, 1992) as well as a decrease in the collagenase type IV activity exhibited by the first trimester trophoblasts (Graham and Lala, 1991). TGF-βs regulate metalloproteinases since they were shown to induce the expression of TIMP and PA inhibitor in some invasive cells (Laiho and Keski-Oja, 1989). TGF-βs are produced both by decidua and by trophoblasts. Some evidence suggests that the trophoblast-derived TGF-β is secreted mainly in a latent form whereas the decidual TGF-β is released as an active molecule (Graham and Lala, 1991). The latent TGF-β can be activated by plasmin generated by the cell surface u-PA or by cathepsin D (Lyons *et al.*, 1988; Sato and Rifkin, 1989) by removal of an N-terminal amino acid sequence (Massague, 1990). In addition, TGF-$\beta_1$ can induce the differentiation of human cytotrophoblast cells into non-invasive syncytiotrophoblasts (Graham *et al.*, 1992).

Several proteins secreted by the endometrium or by the trophoblasts themselves can inhibit the activity of proteins that are implicated in the degradation of the extracellular matrix. They include broad spectrum proteinase inhibitors such as $\alpha_2$-macroglobulin and TIMP whose activity is narrowly confined to inhibition of the action of the metalloproteinases (Figure 8). For example, TIMP-1, TIMP-2 and TIMP-3 are expressed in rodent decidua (Waterhouse *et al.*, 1993) and the expression of TIMP-1 and TIMP-2 in human endometrium has been described (Hampton *et al.*, 1994; Rodgers *et al.*, 1994). TIMPs inhibit the invasiveness of trophoblasts in culture (Librach *et al.*, 1991; Berhrendtsen *et al.*, 1992). When production of gelatinase-B by human cytotrophoblasts is up-regulated *in vitro*, TIMP-3 is also up-regulated suggesting that these cells have developed pathways to regulate their own invasion (Cross *et al.*, 1994). The deletion of *TIMP-1* gene was not associated with abnormal fertility suggesting that the action of this protein is not essential or that it may be replaced by other members of this family of proteins (Cross *et al.*, 1994). Taken together, these findings show that various mechanisms are in place in order to curtail the excessive invasion of the endometrium by trophoblasts.

## Perspective

The process of implantation requires that the invasive trophoblasts encounter an endometrium during a defined period, the so-called 'implantation window'. The receptive endometrium provides the appropriate signals that on one hand are essential to the implantation process and on the other hand control excessive trophoblastic invasion. In addition, beyond the confines of the receptive period, the endometrium acts as a barrier to implantation. The signals and molecular pathways that are involved in conferring these unique qualities to the endometrium are currently poorly defined. Trophoblasts during specific periods gain the ability to adhere, and in some species to separate the surface epithelial cells, and to degrade the underlying basal lamina and extracellular matrix of the endometrial stroma. Steroid hormones act as systemic signals while the cytokines are involved in regulation of the local interactions which take place among trophoblasts and their microenvironment. Integrins are likely to be involved in cell–cell and cell–matrix interactions while the plasmin, PA and metalloproteinases are involved in the degradation of extracellular matrices. Further studies are needed to identify the molecular basis of endometrial receptivity and to unravel the specific function of a host of molecules that have been implicated in implantation. We have just begun to take a glimpse at molecular pathways that confer endometrial receptivity and that participate in the implantation process. Recent identification by two-dimensional gel electrophoresis of specific proteins that are expressed during specific phases of the menstrual cycle is the first step toward identification of molecules involved in endometrial receptivity (Byrjalsen *et al.*, 1995). The hypothesis that a given protein is involved in endometrial receptivity, or in any steps of the implantation process including placentation,

can now be clearly addressed by the gene-targeting technique. Such studies will reveal whether the function of a given protein is dispensable or is essential to fertility or to successful implantation.

## Acknowledgement

This work was supported by Public Health Research Grant CA 56866.

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*Received on February 20, 1995; accepted on March 25, 1995*