The key steps of the implantation

IMPLANTATION

The life journey of a conceptus consists of many stages right from the point of conception to the point of birth which is one of nature's wonders. However, an essential stage in this journey is the process of implantation. Implantation is generally defined as an event in which an embryo becomes progressively attached to the wall of the uterus during early pregnancy. This process is pivotal to the events that occur later in pregnancy. According to Makrigiannakis (2005), implantation is an active process in which a blastocyst apposes, attaches and progressively invades into the endometrium to establish the placenta (Figure 5). From this definition, we can deduce that the embryo undergoes the process of implantation at the blastocyst stage (Figure 1). Implantation is a process that occurs in mammals and it takes places in the endometrial lining of the uterus.

IMPORTANCE OF IMPLANTATION

Implantation is a key event in the reproductive physiology of mammals as it is a pre-requisite for further embryonic development. It is the first stage in the process of placental formation which is in turn is a crucial component of fetal development as it serves as a medium for nutrient absorption, gaseous exchange and waste disposal. Physiological defects in humans and other mammals have gone further to emphasize the importance of the implantation process. Implantation defects have been associated with non-chromosomal early pregnancy loss and infertility (Makrigiannakis, 2005). Many complications that show up late in pregnancy such as pre-eclampsia and preterm labour appear to have originated early in pregnancy with abnormalities in the process of implantation and placental development

(Norwitz, 2006). Another implantation defect is a phenomenon known as ectopic pregnancy. In this case, the blastocyst implants outside the uterine cavity usually in the fallopian tubes although ectopic implantation could also occur in the cervix, abdomen and ovaries. A good understanding of the steps involved in the process of implantation and the factors controlling these steps are necessary in order to be able to influence clinical outcomes in humans such as reduction of recurrent miscarriages and improvement of implantation rates in both natural and assisted reproduction. This will also be beneficial to the use of animals in the area of research and agriculture. A greater detail of the events that take place before and during the process implantation shall be discussed herein.

PRE-IMPLANTATION DEVELOPMENT

The developmental events that take place between the fertilization of the ovum and the implantation of the blastocyst are important in order to understand the process of implantation. Following fertilization, a process known as cleavage occurs (Figure 1). Cleavage is the mitotic division of the cells of the resulting embryo without any growth. This starts from the time the embryo is at the 2-cell stage and each cell continues to divide up to the morula stage. At this point the embryo is a solid ball of 16 or more cells. In humans, this stage is normally observed at about 4 days after fertilization. In continuation of development, the morula undergoes a process known as compaction. Here, the embryonic cells begin to change shape and gap junctions start to form between adjacent cells. The inner cells of the embryo then start to differentiate from the outer cells as different genes are being expressed in the inner and the outer cells. Blastocyst formation follows

shortly after and the inner cells give rise to the inner cell mass whilst the outer cells give rise to the trophoblast cells (Figure 1).

A vast knowledge of the structure of the blastocyst is important as each of its structural components play an important role in the process of implantation. The inner cell mass of a blastocyst gives rise to the embryo proper while the trophoblast gives rise to the fetal component of the placenta (Schoenwolf et al., 2009). The process of implantation is generally known to take place a few days after fertilization and the uterine wall is ready to accept the implanting blastocyst during a limited period of time outside of which it may not optimally support the implantation of the embryo. This period of time is known as the ‘ window of implantation’ (Psychoyos et al., 1995; Klentzeris, 1997).

THE BLASTOCYST AND THE UTERUS BEFORE IMPLANTATION

In the build up to the implantation process following pre-implantation development, there are a number of necessary events that take place. First of all, there has to be a receptive and hormonally primed uterus present. The uterus is composed of 3 layers namely the endometrium, perimetrium and myometrium (Figure 2). The endometrium which is the most important uterine tissue involved in implantation consists of the luminal epithelium, the stroma and the germinal basalis (Yoon et al., 2004). The hormone progesterone, which is secreted by the corpus luteum, is actively involved here as it makes the already thickened endometrial lining of the uterus more favourable for the implantation of the blastocyst. The thickening of the endometrium is due to the effect of estrogens (Norwitz, 2006). The blastocyst is then transported to the uterus via signalling mechanisms and

arrives there at about 5 to 7 days after fertilization (Bischof and Campana, 1996). After the blastocyst arrives in the uterus, it begins to move towards the endometrium with the inner cell mass positioned towards the endometrial lining (Bischof and Campana, 1996). Before any further interaction with the endometrium, the blastocyst must undergo a process known as hatching. This simply involves the blastocyst boring a hole through the zona pellucida with the aid of enzymes and squeezing out. It is a general school of thought that serine proteases are responsible for this process although the mechanisms behind its action are not clearly understood (O'Sullivan et al., 2002). After hatching, the blastocyst is naked of all its original investments and can interact directly with the endometrium (Schoenwolf et al., 2009). At this time, blastocystis also known to secrete moleculesthat affect the activity of the ovary, fallopian tube and the endometrium (Norwitz, 2006). Shortly before the blastocyst comes in contact with the endometrium, thetrophoblastdifferentiates intotwo different cell masses, which are the inner cytotrophoblast and the outer syncytiotrophoblast which is formed as a result of the fusion of cytotrophoblast cells.

PATTERNS OF IMPLANTATION

There are three known patterns of implantation which are centric, eccentric and interstitial (Wimsatt, 1975). Centric implantation occurs when the embryo expands and increases in size before implantation, then stays in the centre of the uterus (Lee and DeMayo, 2004). Examples of animals that undergo this pattern of implantation include rabbits, dogs, cows, pigs, sheep, horses and a number of marsupials. Eccentric implantation occurs when the

embryo is small in size and implants inside the endometrium usually taking place on the side of the uterus, opposite to the mesometrium (Lee and DeMayo, 2004). Examples of animals that show this pattern of implantation include rats, mice and hamsters. In Interstitial implantation, the embryo is also small and it invades through the endometrial epithelium into the subepithelial connective tissue (Lee and DeMayo, 2004). Examples of animals under this category include guinea pigs and humans (Wimsatt, 1975).

KEY STEPS OF THE IMPLANTATION PROCESS

There are three key steps in the process implantation namely apposition, attachment and invasion.

Apposition

This is the first major step of the implantation process following the hatching of the blastocyst. During apposition, the blastocyst comes in slight contact forming a weak bond with the uterine luminal epithelium. Microvilli on the apical surface of the cytotrophoblasts interlock with microprotrusions called pinopodes (Figure 3) which are present on the apical surface of the endometrial epithelium (Norwitz et al., 2001). This interaction involves changes in the expression of cell adhesion molecules and extracellular matrix (ECM) proteins (Nagaoka et al., 2003). Pinopodes are progesterone-dependent organelles, and they have been suggested to be indicators of endometrial receptivity (Nikas, 1999). In humans, they are usually visible on days 20-21 of the menstrual cycle prior to the time of implantation (Nikas, 1999). They could facilitate implantation by preventing the blastocyst from being swept away by uterine cilia (Stavreus-Evers, 2005). It has been

identified that high molecular weight mucin glycoproteins particularly MUC1, are dominant inhibitors of embryo apposition and attachment (Thathiah and Carson, 2002).

However, a decrease in the expression of MUC1 at the time of implantation could facilitate blastocyst apposition (Thathiah and Carson, 2002). During apposition, soluble mediators such as chemokines e. g. CX3CL1, CCL7, CCL14 and CCL4 have been found to establish a dialogue between the maternal cells and those of the blastocyst (Hannan and Salamonsen, 2007). Chemokines are a large family of chemotactic cytokines, well known for their functions in leucocyte recruitment and activation (Dominguez et al., 2003). They have a wide range of functions and have been implicated to play a role in implantation (Dominguez et al., 2003). Chemokines have been localized in areas of inflammation and they are suggested to be potential mediators of inflammation (Feng, 2000). This could be the reason why blastocysts tend to implant on scar tissue from caesarean sections which is an area of inflammation (Dominguez et al., 2005). The dialogue between the maternal and blastocyst cells has important influences on the development of the implanting blastocyst and maintenance of endometrial receptivity (Hannan and Salamonsen, 2007). It also results in the expression of a unique array of adhesion molecules on the surface of both fetal and maternal cells, promoting the attachment of the trophoblast cells to the endometrial epithelium (Hannan and Salamonsen, 2007).

Attachment

Following apposition, the next step in the process of implantation is known as attachment or adhesion. This is characterized by increased physical

contact between the blastocyst and the uterine epithelium (Norwitz et al., 2001). At this point the blastocyst can no longer be dislodged. A ligand carbohydrate known as trophinin has been identified as an adhesion molecule that mediates the initial step of attachment of the blastocyst to the endometrial epithelium (Fukuda and Sugihara, 2008). Trophinin mediates cell adhesion by homophilic Trophinin-Trophinin binding (Fukuda and Sugihara, 2008). A carbohydrate-binding protein known as L-selectin which is expressed in the blastocyst, has also been discovered to play a role in human embryo attachment (Genbacev et al., 2003). Interaction between L-selectin on the blastocyst and L-selectin ligands on the endometrial surface allows for loose attachment and rolling of blastocyst to its implantation site ((Fukuda and Sugihara, 2008). The human Chorionic Gonadotrophin (HCG) produced by the blastocyst up regulates trophinin expression on pinopodes and down regulates MUC1 expression (Fukuda and Sugihara, 2008). The blastocyst then adheres to the pinopodes by trophinin-trophinin interaction (Figure 4). A substance known as Heparin Binding Epidermal Growth Factor (HB EGF) has also been implicated in blastocyst attachment (Lim and Dey, 2008). This growth factor is expressed by the endometrium whilst its receptors are present on the blastocyst. This interaction also helps in facilitating the attachment of the blastocyst.

Figure 4: Proposed role of L-selectin and trophinin in human embryo implantation. Source: Fukuda and Sugihara, 2008. (a) A human blastocyst entering the uterine cavity is prevented from attaching to the endometrial epithelia by MUC1, except for epithelia that express the L-selectin ligand (T). The human blastocyst expresses L-selectin (L), and ‘ rolls’ on the surface of

the endometrium covered by glycocalyx. (b) The blastocyst feebly interacts with the glycocalyx. Here, human chorionic gonadotropin (hCG) which is secreted from the blastocyst, acts locally on endometrial epithelia to induce trophinin expression. (c) Trophinin expressed by endometrial epithelia is enriched in the pinopodes, the structure extended above the glycocalyx. MUC1, which carries the L-selectin ligand, is down-regulated from the endometrial epithelia underneath the blastocyst, allowing direct contact and attachment of blastocyst trophectoderm cells and pinopodes via trophinin-trophinin binding.

Invasion

The next step which is critical to the implantation of the blastocyst is known as invasion. As the term implies, this involves the infiltration of the endometrium by the cytotrophoblast cells of the blastocyst. This starts with the progression of the trophoblast cells between the adjacent endometrial epithelial cells to reach the underlying basement membrane. This membrane is destroyed, allowing the trophoblast cells to reach the stromal compartment (Bischof and Campana, 1996). The syncytiotrophoblast undergoes proliferation and invades the endometrial stroma. The progressive invasion of the blastocyst into the endometrium continues until the blastocyst is completely embedded in subepithelial stromal tissue and the uterine epithelium grows to cover the implantation site (Norwitz, 2006).

The syncytiotrophoblast cells continue to develop quickly and surround the blastocyst until it has completely embedded itself in the endometrial stroma. In the syncytiotrophoblast, fluid-filled spaces known as lacunae are formed as a result of the fusion of syncytiotrophoblast cells. The lacunae are

separated by trabeculae and they transform the syncytiotrophoblast into a sponge-like material (Bischof and Campana, 1996). The trabeculae are arranged radially, and cytotrophoblastic cells divide within the trabeculae, leading to the formation of primary chorionic villi (Bischof and Campana, 1996). Following this event, the primary villi grow and branch into secondary and tertiary villi (Bischof and Campana, 1996). This process is known as placentation. A wide range of factors e. g. cytokines, integrins, matrix metalloproteinases (MMPs), Leukaemia Inhibiting Factor (LIF) e. t. c have been found to play a role in the invasion process (Makrigiannakis, 2005; Norwitz, 2006). The role of these components in the process of implantation shall be discussed shortly under the factors that regulate implantation.

Figure 5: Implantation of the human blastocyst step by step. Source: Bischof and Campana, 1996. (1): Transport. The blastocyst arrives in the uterus after fertilization. (2) Orientation: The inner cell mass is positioned towards the endometrial lining. (3) Hatching: The zona pellucida is perforated making way for the release of the blastocyst. (4) Apposition: The blastocyst is now in close contact with the endometrial lining but no connections have been established. (5) Adhesion: Connections are established between the embryo and the endometrial epithelium. (6) Invasion: Thin folds of trophectodermal cells intrude between the endometrial epithelial cells. (7) Syncytialization: Some trophectodermal cells fuse to form syncytia which proliferate and invade the endometrial stroma. (8) Villous formation: The cytotrophoblastic cells migrate between the syncytia followed by the fetal stroma. This will lead to the formation of the placental villi.

The cells of the endometrial stroma react to the presence of the blastocyst and the secretion of progesterone by differentiating into metabolically active, secretory cells called decidual cells (Schoenwolf et al., 2009). This response is known as the decidual reaction or decidualization. In humans, this begins in the secretory phase of the menstrual cycle. The decidua is also known as the maternal portion of the placenta (Gilbert, 2006) and it is believed to provide an element of control of trophoblast invasion during implantation (Loke and King, 1995). The decidualized stroma cells have been found to secrete prolactin and Insulin-like Growth Factor Binding Protein-1(IGFBPI) which are held to function in complex gene networks that function in the regulation of trophoblast invasion as well as many other endocrine and paracrine factors (Bazer et al., 2010). This regulatory function is required for the optimal implantation of the blastocyst as the invasion of the cytotrophoblast to the proper depth is a major factor in determining pregnancy outcome (Norwitz, 2006). Excessive invasion resulting from the inability of the decidua to control the invading cytotrophoblast cells could lead to an unusually strong attachment of the placenta to the myometrium (placenta accreta), extension into the myometrium (placenta increta), or invasion through the myometrium into adjacent organs also known as placenta percreta (Norwitz, 2006). Insufficient cytotrophoblast invasion has also been associated with pre-eclampsia (Lyall, 2006; Lee et al., 2010) which is a medical complication that presents itself late in pregnancy. During implantation, a process known as angiogenesis has been identified to be important (Sherer and Abulafia, 2001). Angiogenesis is the growth of new capillaries from pre-existing blood vessels.

In this case, it occurs in the endometrium and takes place throughout the implantation period. Endometrial angiogenesis starts with the degradation of the capillary vessel membrane, creating a means through which migrating endothelial cells proliferate to create a new lumen and further vessel maturation (Sherer and Abulafia, 2001). This vascularization functions to maintain endometrial structure and receptivity. Angiogenesis is known to be mediated by some factors present in the endometrium such as fibroblast growth factor, vascular endothelial growth factor and platelet activating factors (Norwitz 2006; Sherer and Abulafia, 2001).

FACTORS THAT REGULATE IMPLANTATION

The regulation of implantation and early development is dependent on a wide range of factors. Although the molecular and cellular mechanisms behind implantation are not well understood, it is apparent that multiple factors (including maternal and fetal) are needed to synchronize blastocyst maturation and uterine receptivity up to the point of initiation of implantation and through the process of implantation (Norwitz, 2006). A closer look will now be taken at some of the important factors associated with implantation and early pregnancy maintenance.

Maternal factors

Starting with the uterine (maternal) side, there are a lot of components to consider. Cytokines and growth factors have been shown by different studies to be important to the maternal role in implantation. These include interleukin-1 (Sheth et al., 1991; Simon et al., 1996; Stewart and Cullinan, 1997; Huang et al., 1998), Interleukin-2 (Stewart and Cullinan, 1997), Insulin-like growth factor I and II (Stewart and Cullinan, 1997; Giudice and Irwin,

1999), transforming growth factor a and (Slowey et al., 1994; Stewart and Cullinan, 1997; Godkin and Dore, 1998), vascular endothelial growth factor (Athanassiades et al., 1998) and leukemia inhibitory factor (Cullinan et al., 1996; Stewart and Cullinan, 1997). The mode of function of the leukemia inhibitory factor is not well understood but has been established as a critical factor in the process of implantation (Stewart et al., 1992; Cheng et al. 2002). These cytokines and growth factors all work towards facilitating the communication between the blastocyst and the uterus whilst promoting endometrial proliferation and differentiation (Norwitz, 2006). They have also been found to regulate endometrial angiogenesis and vascular permeability (Norwitz, 2006).

As mentioned earlier, some steroid hormones such as Progesterone (Peyron et al., 1993) and Oestradiol-17 (Miller, 1988) have also been found to be important. They function in the proliferation of uterine epithelial cells and endometrial stromal cells (Norwitz, 2006). Some changes in the uterine luminal epithelium such as the expression of pinopodes (Nikas, 1999) and MUC 1 (Thathiah and Carson, 2002) have been suggested to be important for blastocyst recognition and attachment. Transcription factors such as the peroxisome proliferator activated receptor-s (Lim et al., 1999; Barak et al., 1999) have been identified to function in defining the molecular mechanisms by which the regulatory factors exert their effects at a cellular level (Norwitz, 2006). Studies have shown the relevance of some other components such as homeobox genes Hoxa-10 and 11 which have been found to regulate the responsiveness of stromal cells to progesterone (Benson et al., 1996; Taylor et al., 1997; Lim et al., 1999), Cyclooxy-genase-2 which regulates

prostaglandin production (Norwitz and Wilson 2000) and oxygen tension (Genbacev et al., 1997) which has been found to promote trophoblast vascular mimicry by initiating integrin expression. Also, proteins such as Rac1 and RhoA which are found in stromal cells have been implicated in trophoblast invasion (Grewal et al., 2008).

Fetal factors

Evaluations of the blastocyst (fetal) factors also reveal that present here, are some of the groups of factors present on the maternal side of implantation as they possess some overlapping functions. Cytokines and growth factors, in addition to facilitating communication between the blastocyst and uterus, could also enhance trophoblast differentiation and invasion. These include Interleukin-1, Interleukin-6 (Stewart and Cullinan, 1997), leukemia inhibiting factor, transforming growth factor a and , insulin-like growth factor II and colony stimulating factor-1 (Stewart and Cullinan, 1997, Cohen et al., 1997). Some trophoblast proteinases and inhibitors such as the matrix metalloproteinases (Makrigiannakis, 2005) and cathepsin B and L (Afonso et al., 1997) have also been found to regulate trophoblast invasion. The expression of some adhesion molecules e. g. integrins have been identified in the enhancement of trophoblast invasion. Some of them include integrin a64, integrin a11 and E-cadherin (Lessey, 1998; Lessey and Arnold, 1998; Damsky and Fisher, 1998). Some other important factors include prostaglandin E2 which aids the process of endometrial apoptosis and platelet-activating factor which stimulates uterine prostaglandin production (Norwitz, 2006).

Immunological factors

The immunological interactions between the blastocyst and the uterine decidua are essential in the regulation of the implantation process. Implantation is of immunological significance because the blastocyst contains half of its paternal genes hence it is immunologically foreign to its mother. Despite this fact, blastocyst implantation still turns out to be a successful process and the blastocyst is not rejected by the maternal immune system during normal implantation. This is down to the action of immunological factors. These factors are a combination of elements expressed by both the blastocyst and the uterus and they act together to ensure fetal survival. On the maternal side such factors include Interleukin-10 (Roth et al., 1996; Roth and fisher, 1999) which plays an important role in immunosuppression thus reducing the activity of the maternal immune system against the foetus (Norwitz, 2006). Another factor is 2, 3-dioxygenase (Kamimura et al., 1991; Munn et al., 1998) which has been found to be responsible for macrophage action during implantation (Norwitz, 2006). Uterine natural killer cells found in the stroma also play a role here as they produce cytokine, chemokines and angiogenic factors which all promote and regulate trophoblast invasion (King and Loke, 1997). On the fetal side, factors such as histocompatibility antigen, class I, G (HLA-G) have been found to be involved in preventing the maternal immune rejection of the semi-allogenic foetus (Lanier, 1999; Norwitz, 2006).

Coordination of the regulatory factors within the window of implantation

The window of implantation as defined earlier is characterized by the perfect synchrony of all the components that play a role in the implantation process. These include the endometrium, the blastocyst and the factors regulating

the process of implantation. Figure 6 below illustrates how all these factors are coordinated during the implantation window. This shows that within the window of implantation, the endometrium is highly influenced by steroid hormones (oestrogen and progesterone) and the interaction between the blastocyst and the endometrium is as a result of stage-specific actions of different implantation factors.

Figure 6: Events that take place within the implantation window. Source: Achache and Revel, 2006 (A) Endometrium proliferates under the enhancement of estrogen. (B) Progesterone from follicles that have been luteinized leads to endometrial differentiation. (C) The blastocyst makes its way to the uterus via the ostia and rolls freely over the endometrium with the aid of L-selectin signals. (D) MUC-1 repels the blastocyst and prevents it from adhering to areas on the endometrium with poor chances of implantation. (E) Cytokines and chemokines attract the blastocyst to the optimal implantation spot. (F) Adhesion molecules (e. g. integrins and cadherins) attach the blastocyst firmly to the endometrial pinopodes to ensure successful implantation.

The functions of all the factors mentioned earlier further emphasize the importance of these components to the process of implantation. A lot of studies have also been carried out on some of these molecules to further establish their importance. A notable one amongst these molecules is the leukemia inhibitory factor. As mentioned earlier, this molecule has been identified to be critical to the process of implantation. Studies revealed that implantation did not occur in female transgenic mice that were homozygous for the deficiency of the gene responsible for the leukemia inhibitory factor

(Aghajanova, 2004). It was further proven that the lack of implantation was not caused by faults in the embryo because the implantation of the same embryos occurred when they were transferred to pseudopregnant recipients. The addition of exogenous LIF to the females with this defective gene throughout the period of normal implantation restored implantation sites and allowed proper attachments of the blastocysts (Aghajanova, 2004). LIF and LIF mRNA has also been shown to be expressed throughout the menstrual cycle of women with proven fertility (Arici et al., 1995; Charnock-Jones et al., 1994).

This was associated with peaks in the mid and late secretory phase, and in early pregnancy (Aghajanova, 2004). Leukemia inhibitory factor has also been found to be present in uterine flushings within the implantation window in fertile women. This characterized by gradually increasing concentrations from 7 days to 12 days after the LH surge (Laird et al., 1997). In future, the evidence and information obtained from similar studies may be applied clinically through a well regimented administration of LIF in a pharmaceutical form to improve implantation rates in both natural and assisted reproduction.

IMPLANTATION AND ASSISTED REPRODUCTIVE TECHNOLOGY (ART)

Successful pregnancy outcomes from assisted reproductive techniques such as in vitro fertilization (IVF) have largely depended on the process of implantation. The advances in ART techniques have not had a significant effect on implantation rates (Donaghay and Lessey, 2007) hence a lot of measures have been taken by ART units over the years in an attempt to

optimize implantation rates. Assisted hatching (Cohen, 1991) is a well known procedure carried out in this regard. This involves the manual perforation of a blastocyst's zona pellucida in order to separate the blastocyst from the zona pellucida just as it would have occurred during natural hatching. This may increase implantation and pregnancy rates in IVF cycles (Chao et al., 1997). The function of regulatory factors in the process of implantation indicates that a number of biomarkers can be adapted from these to determine the ideal period of endometrial receptivity which can be traced and monitored during IVF cycles (Haouzi et al., 2009). These may include the detection and monitoring of some of the molecules and steroid hormones involved in implantation by making use of samples obtained from IVF treatment cycles such as follicular fluids and embryo culture supernatants. Fluorescent markers can also be used to highlight proteins and any other key component in the blastocyst involved in implantation. Continuous studies are being performed with the aim of discovering techniques that would improve implantation rates during ART treatments.

CONCLUSION

Over the years, the research carried out on implantation and the factors that control implantation has been done making use of mostly animal models with the exception of some that have employed the use of in vitro human systems. The use of animals in the study of implantation has many benefits as many factors and regulatory mechanisms are being discovered. However, it is difficult to precisely extrapolate results obtained from animal data into human cases. This is one of the problems being encountered by implantation studies as the difficulty and ethical significance associated with research

using humans make scientists resort to the use of animal models. The process of implantation occurs with the uterus and the blastocyst in synchrony. It has been clearly shown that a lot of factors are responsible but the complete framework of the regulation of implantation has not yet been understood. With continuous research and more human-based studies, a better understanding of the process and regulation of implantation may be achieved in the future.

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