THE ROLE AND REGULATION OF THE WNT/β-CATENIN PATHWAY AT THE TIME OF IMPLANTATION IN THE MOUSE

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ABSTRACT

One of the crucial events during mammalian embryogenesis is the process of implantation. Implantation enables the embryo to invade the uterine endometrium and to gain access to the maternal circulation. The attachment reaction requires a highly coordinated dialogue between the implanting blastocyst and the receptive uterus, known as the embryo-uterine cross-talk. Since the blastocyst expresses multiple Wnt genes, in this study, we have characterized the role and regulation of the Wnt/β-catenin pathway in embryo-uterine communication.

Using a transgenic mouse that reports Wnt signalling through the canonical pathway, we have demonstrated that the Wnt/β-catenin pathway was transiently activated in the regions of the uterine luminal epithelium apposed to the blastocyst at the time of implantation. Activation of this pathway within the endometrium depended on the presence of the blastocyst and required the oestrogen surge. We further demonstrated that activation of the Wnt/β-catenin pathway was essential for proper implantation to occur.

One possible mechanism that regulates the responsiveness of the luminal epithelium to Wnt is the regulation of components of the Wnt pathway. We show that there is dynamic pattern of expression of the components of the Wnt pathway at the time of implantation. Furthermore, we demonstrate that LIF signalling is required for the expression of a subset of these Wnt components in luminal epithelial cells at the time of implantation. Our results demonstrate that the LIF and Wnt signalling pathway form a network involved in coordinating the process of implantation

As Wnt/ß-catenin signalling is essential for embryo attachment, we proposed that this pathway activates downstream target genes required for implantation. Using microarray analysis, we determined what are the specific target genes of the Wnt/ß-catenin pathway in the uterus. Genes up-regulated by the Wnt/ß-catenin pathway are involved in a variety of cellular processes

such as cell differentiation, cell proliferation, cell cycle, cell adhesion and enzyme regulatory activity.

Despite the discovery of numerous molecules and signalling pathways involved in this process, the precise sequence and hierarchy of events leading to successful embryo implantation remains to be elucidated. Unravelling the hierarchy of events that initiates the attachment process is of clinical relevance to improve fertility and develop new contraceptives.

RÉSUMÉ

Le processus de l'implantation embryonnaire joue un rôle crucial lors du développement des mammifères. L'implantation de l'embryon est un processus extrêmement complexe au cours duquel l'embryon va d'abord s'apposer, puis adhérer à l'endomètre pour ensuite y pénétrer. Ultérieurement, il a été démontré que le blastocyste exprime plusieurs gènes de la famille Wnt. C'est pour cette raison que dans cette thèse, j'ai décidé caractériser le rôle et la régulation de la voie de signalisation Wnt/β-catenine lors du dialogue materno-fœtal. Nous avons utilisé une souris transgénique qui permet de contrôler la voie de signalisation Wnt/β-catenine. Ainsi, nous avons démontré que la voie de signalisation Wnt/β-catenine était activée de façon transitoire dans les cellules épithéliales du lumen de l'utérus, dans la région adjacente au blastocyte au moment de l'implantation. L'activation de cette voie de signalisation dépend de la présence du blastocyste et de la sécrétion d'oestradiol. Nous avons également démontré que l'activation de la voie de signalisation Wnt/β-catenine était essentielle au processus d'implantation embryonnaire.

Par la suite, nous avons examiné la possible interaction entre la voie de signalisation Wnt et LIF. En effet, dans les souris mutantes pour le gène LIF, la voie de signalisation Wnt/β-catenine n'est pas activée. Grâce à la technique de rt-PCR, nous avons caractérisé le profil d'expression des récepteurs Frizzled, des co-récepteurs LRP, des antagonistes sFRP et DKK et des gènes Wnt dans les souris mutantes pour le gène LIF et dans les souris de type sauvage. Nous avons démontré que les composants de la voie de signalisation Wnt sont exprimés de manière dynamique lors de l'implantation embryonnaire. De plus, nous avons démontré que la voie de signalisation LIF est nécessaire pour l'expression des composants de la voie de signalisation Wnt dans les cellules épithéliales lors du processus implantatoire.

Étant donné que la voie de signalisation Wnt est essentielle pour l'attachement de l'embryon dans l'utérus, j'ai analysé les gènes régulés par la

voie de signalisation Wnt/β-catenine dans l'utérus en utilisant la technologie de micro-puces à ADN. Les gènes régulés par cette voie de signalisation sont impliqués dans une variété de processus cellulaires comme la différentiation cellulaire, la prolifération cellulaire, le cycle cellulaire, et l'adhésion cellulaire.

Malgré la découverte, de nombreuses molécules et voies de signalisation impliquées dans l'implantation embryonnaire, peu de choses sont connues quant à la hiérarchie des événements menant à une implantation réussie. Déchiffrer le réseau complexe qui dirige l'implantation embryonnaire est d'une importance cruciale pour améliorer la fertilité et mener au développement de nouveaux contraceptifs.

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ABBREVIATIONS

AMPc: adenosine monophosphate cyclic

ART: assisted reproductive technologies

BMP: bone morphogenetic protein

CAM: cell adhesion molecules

CE: catecholoestrogen

COX: cyclooxygenase

D1: day of vaginal plug

DBD: DNA-binding domain

Dkk-1: Dickkopf 1

DNA: deoxyribonucleic acid

Dpc: day post-coitum

Dvl: Dishevelled

E: embryonic day

E_2: oestrogen

ECM: extracellular matrix

EGF: epidermal growth factor

ER: oestrogen receptor

ES: embryonic stem

FGF: fibroblast growth factor

Hh: hedgehog

HNF: hepatocyte nuclear factor

HRE: hormone response element

ICM: inner cell mass

IGF: insulin growth factor

Ihh: indian hedgehog

IL: interleukin

FGF: fibroblast growth factor

Fzd: frizzled

GE: glandular epithelium

Kb: kilobase

LBD: ligand-binding domain

LDL: low-density lipoprotein

LRP: low density lipoprotein receptor-related protein

LE: luminal epithelium

LIF: leukemia inhibitory factor

LPA: lysophosphatidic acid

MMP: matrix metalloproteinase

NK: natural killer

P: progesterone

PG: prostaglandin

PR: progesterone receptor

SP: serine protease

STAT : signal transducer and activators of transcription

TCF/LEF : T cell factor/lymphoid enhancer factor

β-TrCP : beta-transducin repeat containing protein

VEGF: vascular endothelial growth factor

PUBLICATIONS

1. **Mohamed OA, Jonnaert M, Labelle-Dumais C, Kuroda K, Clarke H, and Dufort D** (2005). Uterine Wnt/β-catenin signalling is required for implantation. *Proc. Nalt. Acad. Sci. USA* **102**, 8579-8584.

2. **Jonnaert M and Dufort D** (2007). Leukemia inhibitor factor modulates the expression of components of the Wnt signalling pathway in the uterus. *Submitted, under revision, Biology of Reproduction.*

3. **Jonnaert M, Lou Y, and Dufort D.** Identification of novel Wnt target genes essential for embryo implantation. *To be submitted*

CONTRIBUTION OF AUTHORS

1. **Mohamed OA, Jonnaert M, Labelle-Dumais C, Kuroda K, Clarke H, and Dufort D** (2005). Uterine Wnt/β-catenin signalling is required for implantation. *Proc. Nalt. Acad. Sci. USA* **102**, 8579-8584.

Othman Mohamed generated the TCF/Lef-LacZ reporter transgenic line; he demonstrated that the Wnt/β-catenin pathway was transiently activated in the regions of the uterine luminal epithelium apposed to the blastocyst at the time of implantation, and that activation of this pathway within the endometrium depended on the presence of the blastocyst and required the oestrogen surge. I further demonstrated that Wnt proteins could activate the Wnt/β-catenin pathway in the uterus and that activation of the Wnt/β-catenin pathway was essential for proper implantation to occur. I also participated in the experimental design, animal maintenance and genotyping, and to the revision of the manuscript.

2. **Jonnaert M and Dufort D** (2007). Leukemia inhibitor factor modulates the expression of components of the Wnt signalling pathway in the uterus. *Submitted, under revision, Biology of Reproduction.*

I wrote the manuscript and performed all the experiments presented in this study.

3. **Jonnaert M, Lou Y, and Dufort D**. Identification of novel Wnt target genes essential for embryo implantation. *To be submitted*

Youfei Lou participated in the experimental design, the microarray experiments, and in the analysis of the microarray data. I wrote the manuscript and performed all the other experiments.

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Merci à mes parents et mes sœurs, Alexandra et Caroline.

CHAPTER I : INTRODUCTION

I. Introduction.

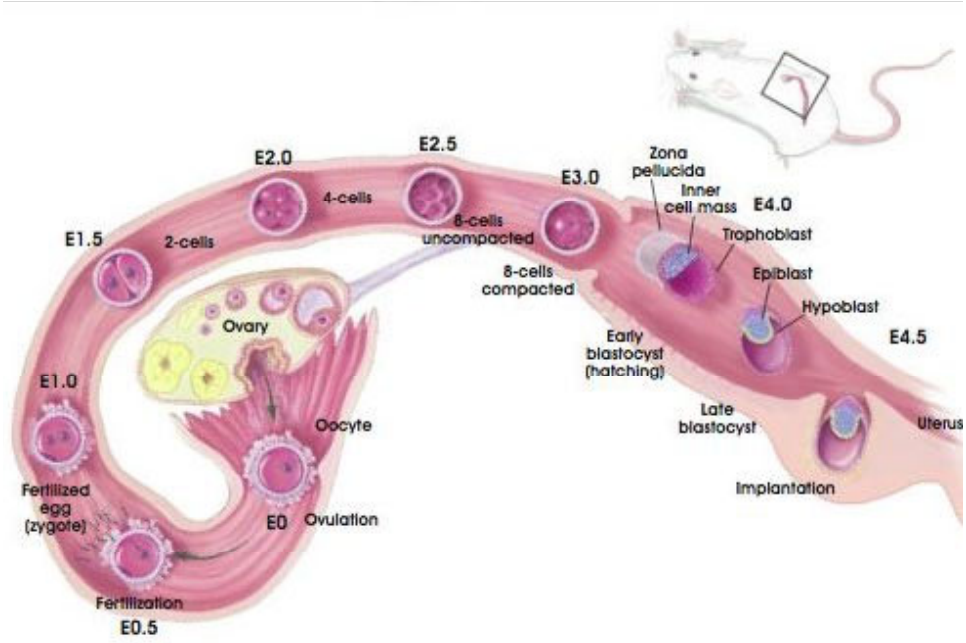
Fertility is declining in most countries of the western hemisphere and has become a worldwide social and economical concern. Infertility affects 10% to 15% of couples of reproductive age. Although Assisted Reproductive Technologies (ART) have developed rapidly, only 25% to 27% of embryo transfers result in successful pregnancy (Kalantaridou *et al.* 2007; Ombelet *et al.* 2007). Significant pregnancy loss frequently occurs before, during or immediately after implantation. Failure to complete on-time implantation can compromise pregnancy and foetal well-being. The process of embryo implantation requires a tightly regulated dialogue between the implanting blastocyst and the receptive uterus. Unravelling the complexity of this first materno-foetal cross-talk is of crucial importance to improve future treatments of infertility and develop novel contraceptives.

Embryo implantation, a steroid hormone dependent process, requires a tightly regulated dialogue between the uterus and the embryo, and the coordinated action of both oestrogen (E_2) and progesterone (P_4). Upon fertilization, the embryo undergoes a series of cleavages that leads to the formation of an implantation competent embryo, also termed the activated blastocyst. The uterus must also undergo a series of changes to become receptive to the attachment of the activated blastocyst. First, ovarian progesterone primes the uterus to a prereceptive state (Huet *et al*. 1990). Then, the oestrogen surge converts the prereceptive uterus to a receptive state that can support the attachment of the implantation competent blastocyst (Ma *et al.* 2003) (Figure 1).

Since the process of embryo implantation shares characteristics of epithelial to mesenchymal transition, it is expected that developmental genes participate in the embryo-maternal interplay (Wang *et al*. 2006). Indeed, molecular and genetic evidence indicates that FGF, BMP, Noggin, HH and Wnt have temporal and cell-specific functions in the uterus during pregnancy (Kierszenbaum *et al*. 2001; Paria *et al.* 2001; Daikoku *et al*. 2004; Mohamed

et al. 2006). Besides developmental genes, ovarian hormones, cytokines, adhesion molecules, growth factors, lipid mediators and morphogens have been shown to serve as autocrine, paracrine and/or juxtacrine factors to promote embryo implantation (Dey *et al*. 2004). Despite the discovery of numerous molecules and signalling pathways involved in this process, the precise sequence and hierarchy of events leading to successful embryo implantation remains to be elucidated. We need to understand whether these signalling pathways function independently, in parallel or converge to a common pathway to establish successful reciprocal dialogue between the embryo and the uterus. In the following sections, I will present to you the crucial steps of embryo preimplantation development, as well as the physiological, functional and molecular changes leading to uterine receptivity.

Figure 1: Chronology of embryo development until implantation in the mouse. The diagram demonstrates cell division after fertilization and through the morula stage until the blastocyst is formed. Blastocyst formation and activation are required prior to implantation of the embryo within the uterus (www. Stemcells.nih.gov).



II. Preimplantation embryo development and genomic activation.

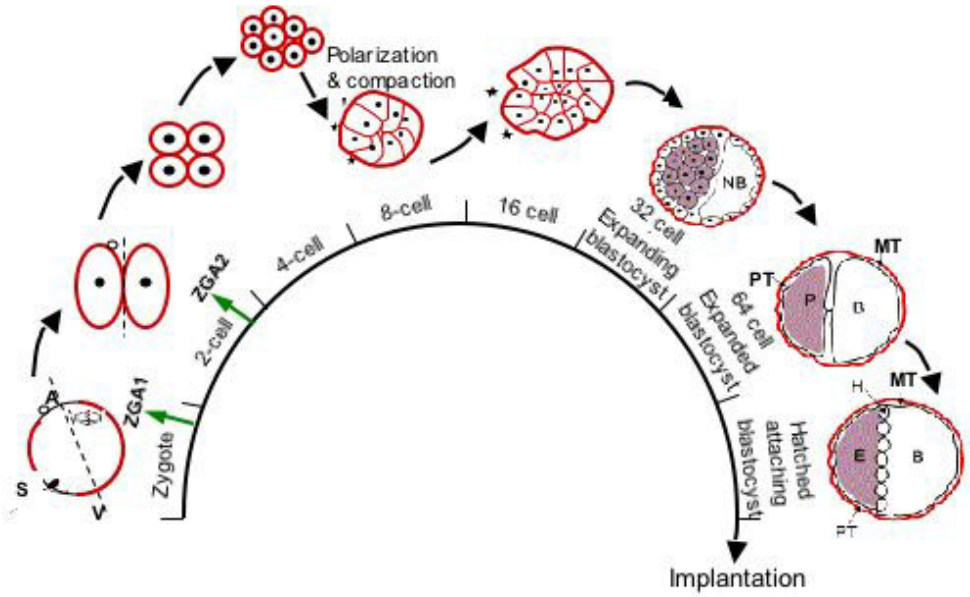
Early embryonic development comprises the period between oocyte fertilization and blastocyst implantation. Preimplantation development is marked by four major events: initiation of zygotic transcription, compaction, first lineage differentiation into inner cell mass and trophectoderm, and implantation (Figure 2). Endometrium-derived signals maintain the pace of blastocyst development, and the presence of the blastocyst mobilizes uterine factors required for subsequent signalling (Zhang *et al*. 2007). This molecular dialogue serves to synchronize embryonic and maternal tissues during the preimplantation period. Minute perturbations in this orchestration can engender cellular defects leading to implantation failure, embryo fragmentation and/or cleavage arrest, depletion of cells, aberrant lineage allocation, and chromosomal abnormalities (Hartshorne *et al.* 2001). Substantial embryo loss due to preimplantation death is common to many mammals, and is considered a selection process that leads to the survival of superior embryos.

a. Genomic activation

The first major developmental transition that occurs after fertilization is zygotic gene activation. Mammalian development is initially sustained by transcripts and polypeptides produced and stored in the oocyte during oogenesis (Hamatani *et al.* 2006). Fertilization triggers the degradation of oocyte-stored transcripts (Nothias *et al.* 1995). In mice, by the 2-cell stage, maternally inherited mRNAs are completely degraded which coincides with activation of the embryonic genome (Nothias *et al.* 1995). This maternal to zygotic transition entails a dramatic genetic reprogramming, establishing the appropriate gene expression profile that is essential for continued development (Kidder *et al.* 1992).

Figure 2: Schematic outline of key events during pre-implantation mouse development.

S: sperm entry site; A: Animal pole; V: Vegetal pole; ZGA: Zygotic Gene Activation; (*): Polarized cells; NB: Nascent Blastocoel; B: Blastocoel; MT: Mural trophectoderm; P: Inner Pluriblast; PT: Polar Trophectoderm; E: Epiblast; H: Hypoblast (adapted from Johnson *et al.* 2004).



b. Preimplantation embryo development

Compaction, the first onset of cellular differentiation during mammalian development, arises following the third cleavage division in the mouse (Mayor *et al*. 1994). At this stage, the outer cells of the conceptus begin to polarize as a prelude to trophectoderm differentiation (Rossant *et al*. 2004; Edwards *et al.* 2005). Compaction and polarization generates an asymmetry of adhesivity in each blastomere: the basolateral domain is highly adhesive while the apical domain are not (Piotrowska *et al*. 2001). This segregation establishes the placental (trophectoderm) and embryonic (inner cell mass) progenitors that diverge at the molecular level. Transcription factors such as Oct4, Sox2, Nanog, Cdx2 and Eomesodermin are crucial for the segregation of these two cell lineages (Johnson *et al.* 2004). Trophectoderm expresses gene products that facilitate the transport and retention of blastocoelic fluid as it accumulates in the nascent blastocyst cavity (Schultz *et al.* 2005). As a consequence, the blastocyst consists of a fluid-filled cavity, the blastocoele, surrounded by the trophectoderm and the inner cell mass (ICM). A third cell lineage, the primitive endoderm, is formed on the blastocoelic surface of the ICM by the late blastocyst stage (Rossant *et al.* 1980). The primitive endoderm, which derives from the ICM, will form the parietal endoderm, and the visceral endoderm (Gardner *et al.* 1982).

At this stage, the blastocyst hatches and escapes from the zona pellucida. By virtue of its non-adhesive nature, the zona pellucida, which functions as a glycoprotein barrier, facilitates the journey of the embryo through the oviduct and from the oviduct to the uterus (Epifano *et al.* 1995). The zona limits paracrine signalling since only soluble molecules present in fluids of the oviduct can reach the embryo (Lu *et al.* 2002). Once freed of the zona, the embryo can interact directly with the luminal epithelium (LE) of the uterus. Two serine proteases (SP) have been involved in embryo hatching. SP1 is produced by the blastocyst while uterine glands secrete SP2 (O'Sullivan *et al.* 2004). After the embryo has escaped from the zona, the trophectoderm differentiates into invasive trophoblast, and the blastocyst is activated to an implantation competent state. In order to become competent for implantation,

the blastocyst must undergo structural and molecular changes, which will be discussed in details in the next section.

c. Activation of the blastocyst to an implantation-competent state.

Conversion of the blastocyst to an implantation competent state marks the final step of early embryo development, and requires 4-hydroxy-estradiol (catecholoestrogen, CE), an active metabolite of oestrogen (Philips *et al.* 2004). Catecholoestrogens are formed by aromatic hydroxylation at C-2 or C-4 of the ring A of the phenolic oestrogen or by the peroxidase system (Paria *et al.* 1998). CE, like primary oestrogen can function via classical nuclear receptors or membrane receptors, and are synthesized in various tissues including the uterus and the embryo (McLaren *et al.* 1971; Roblero *et al.* 1979; Paria *et al.* 1998). Activation of the blastocyst by catecholoestrogen is mediated by COX-2 signalling and prostaglandins (Paria *et al.* 1998). In the absence of an oestrogen surge, the blastocyst stays in a dormant state that can lead either to implantation delay or implantation failure (Lubahn *et al.* 1993).

Delayed implantation, or embryonic diapause is a reproductive strategy used by mammals, such as rodents, marsupials and mustelids, to time the birth of their offspring in favourable metabolic and environmental conditions. In embryonic diapause, the blastocyst is maintained in a state of dormancy and retains its capacity to implant in the uterus (Hamatani *et al.* 2004). Delayed implantation can be produced experimentally in rodents: shortly after fertilization, the female is ovariectomized and treated with progesterone to maintain pregnancy and embryo survival in a dormant state (Psychoyos *et al.* 1973; Yoshinaga *et al.* 1976). Blastocyst activation and implantation can then be triggered by a single injection of estradiol (Weitlauf *et al.* 1968). The experimental model of diapause provides a powerful tool to study signalling components that direct blastocyst dormancy and activation (Lopes *et al.* 2004).

Using the experimental model of diapause, the gene expression profile of dormant and activated blastocyst has been established. In this way, gene

expression analysis has identified 229 differentially regulated genes between dormant and activated blastocyst (Hamatani *et al.* 2004). These include cell cycle, cell signalling, and energy metabolic pathways. In dormant blastocyst, genes encoding integrins and G-protein-coupled cannabidoid receptor 1 are up-regulated (Paria *et al.* 1998). In contrast, integrin α2, integrin α6A, integrin α7, epidermal growth factor (EGF), COX-2, histamine type 2 and ErbB, the receptor for HB-EGF, are downregulated (Paria *et al.* 1998). The expression of these genes is resumed after injection of estradiol (Paria *et al.* 1993; Hamatani *et al.* 2004; Sutherland *et al.* 1993; Das *et al.* 1994; Das *et al.* 1997). Up-regulated transcription of these factors in activated blastocysts is thought to modulate gene expression in the surrounding uterine luminal epithelial cells in a paracrine manner (Hamatani *et al.* 2004). Indeed, several genes including *HB-EGF, epiregulin,* and *beta-cellulin* that are not expressed in ovariectomized mice in which the embryo remains in a dormant state become transcriptionally activated at implantation specifically in the region adjacent to the implanting blastocyst (Das *et al.* 1994). Thus, implantation requires factors secreted by the blastocyst that modulate gene expression in uterine cells. The expression of at least some of these factors secreted by the blastocyst is dependent on estradiol, or its metabolite, CE. Once activated, the embryo is ready to implant in the receptive uterus.

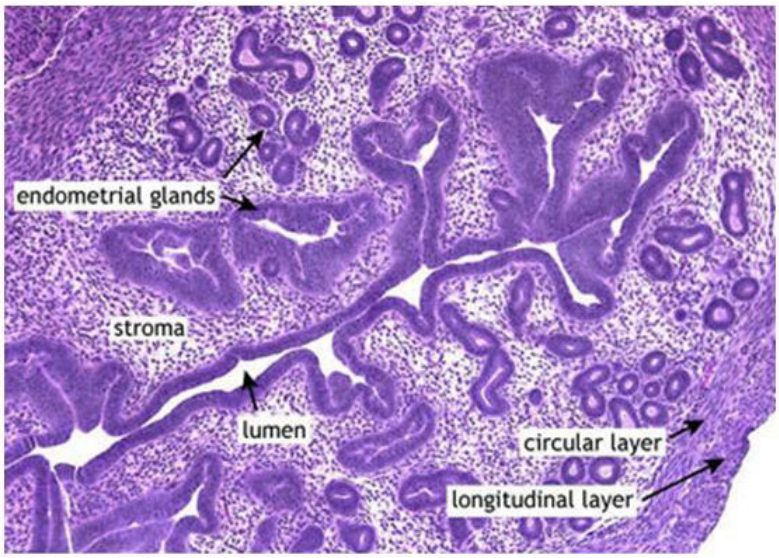
The blastocyst also undergoes structural changes to become competent for implantation. After the embryo has escaped from the zona, the trophectoderm differentiates into invasive trophoblast (Thie *et al.* 2002). Trophectoderm differentiation involves morphological and behavioural transformation that resembles an epithelial to mesenchymal transition. At the late blastocyst stage, α5β1 and α7β1 integrins relocalize to the apical domain of the trophectoderm (Rout *et al.* 2004). This change in cell polarity allows attachment of the blastocyst to the uterine LE. Adherens junctions are observed between the two cell types in mice and rats (Thie *et al.* 1996). This new cell-cell and cell-matrix interaction leads to changes in gene expression, promoting further cell differentiation to the invasive phenotype. Invading

trophoblast cells breach the uterine basement membrane to infiltrate the deciduas (Sutherland *et al.* 2003).

III. Embryo implantation

Mammalian embryos implant and develop within the uterus. This organ is specially adapted to provide a site for embryonic development. The walls of the uterus are composed of a mucosal layer, the endometrium and a fibromuscular layer, the myometrium (Li *et al.* 1994). The endometrium, which represents the mucosa that lines the uterine cavity, consists of a single layer of columnar epithelium, resting on a layer of connective tissue, which varies in thickness according to hormonal influences - the stroma (Li *et al.* 1994). Simple tubular uterine glands reach from the endometrial surface through to the base of the stroma, which also carries a rich blood supply of spiral arteries (Bourgain *et al.* 2007) (Figure 3). The endometrium is a dynamic tissue that under hormonal control undergoes constant remodelling through the menstrual cycle, and is subjected to continual cycles of proliferation, differentiation and degeneration that display many features of typical developmental processes (Carson *et al.* 2002; Cunha *et al.* 1976). Moreover, during pregnancy, the uterus undergoes a series of changes to become receptive to the attachment of the implantation competent blastocyst (Kao *et al.* 2002).

Figure 3: Histological diagram of mouse uterus.
(www.histology-world.com)



a. Uterus states of receptivity: Pre-receptive/receptive/refractory

The different states of receptivity of the uterus, pre-receptive, receptive, and refractory, are regulated directly by ovarian progesterone and oestrogen and indirectly by growth factors, cytokines and peptide hormones (Tranguch *et al.* 2006; Giudice *et al.* 2004). Most of our understanding on the hormonal requirement for the preparation of the uterus for implantation is based on studies performed in rodents, by transferring embryos in ovariectomized females under diverse conditions of exogenous steroids (Figure 4).

In mice, on D1 and D2 (D1 = day of vaginal plug), ovarian oestrogen stimulates epithelial cell proliferation. On D3, progesterone from corpus luteum initiates stromal cell proliferation, and primes the uterus to a prereceptive phase (Minas *et al.* 2005). The stromal cell proliferation is further stimulated by a small amount of ovarian oestrogen secreted on the morning of D4. During the prereceptive phase, the uterus is unable to initiate implantation, but the uterine environment is not hostile to embryo survival. Transition from pre-receptive to receptive uterus occurs on D4, after exposure to a small amount of oestrogen. The size of the oestrogen dose affects the duration of the window of receptivity of the uterus (Ma *et al.* 2003). In humans, the embryo implants between days 7 to 11 after the luteinizing hormone (LH) peak, which triggers ovulation (Kalra *et al.* 1975). In rodents, the uterus is fully receptive to blastocyst on D4. During the receptive phase, the endometrium becomes more oedematous and vacularized (Enders *et al.* 1976). Luminal closure allows apposition of the blastocyst to luminal epithelial (LE) cells, which constitute the site of receptive sensitivity. Apical-basal polarity of LE cells becomes less marked; cells flatten, lose microvilli and develop pinopods (Glasser *et al.* 1993; Murphy *et al.* 2000). Pinopods, which are large ectoplasmic projections, are markers of uterine receptivity and appear progesterone-dependant. Besides, endometrial glands display enhanced secretory activity (Bell *et al.* 1988). At the molecular levels, uterine

receptivity is specified by the production of numerous signalling molecules such as cytokines, growth factors, adhesion molecules, homoebox transcription factors, lipid mediators and morphogens, which work as autocrine, paracrine and/or juxtacrine factors (Achache *et al.* 2006).

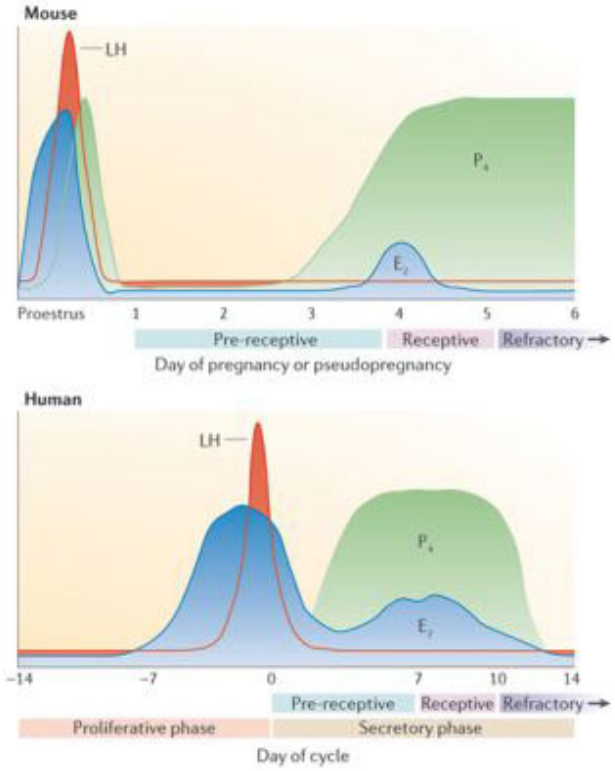
The transient state when the blastocyst and the uterus are capable of efficient two-ways communication is defined as the window of implantation. In rodents, the implantation window lasts for about 24h on D4-5 of pregnancy. Unimplanted blastocysts transferred to receptive mothers give normal pregnancies, and blastocysts transferred to pre-receptive uteri implant according to the maternal schedule (Aitken *et al.* 1977). This indicates that the receptivity is largely maternally controlled. In humans, a receptive window of implantation occurs in the uterus between postovulatory days 7 and 10 of a normal 28-day menstrual cycle and may be of at least five days' duration (McLaren *et al.* 1971). In mice, it has been demonstrated that the presence of a blastocyst is required for uterine expression of heparin-binding epidermal growth factor (HB-EGF), followed by production of betacellulin, epiregulin, neuregulin-1, and cyclooxygenase-2 (COX-2) as the blastocyst attaches to the LE (Lin *et al.* 1994; Lim *et al.* 1997; Das *et al.* 1994). HB-EGF is expressed in the LE, adjacent to the implanting blastocyst, 6-7 hours before the embryo attaches and is the earliest marker of embryo-uterine cross-talk (Lee *et al.* 2005; Isaacs *et al.* 2002; Matsumoto *et al*. 2002). In humans and rodents, the communication facilitating this dialogue includes cytokines, growth factors, angiogenic factors, adhesion molecules and transcription factors. Inherent among these factors is the fact that their effects are redundant, pleuripotent and pleiotropic (Domínguez *et al.* 2002).

Uterine receptivity for the blastocyst lasts for a limited period of time, and is followed by spontaneous progression to the non-receptive phase when the uterine milieu becomes hostile to survival of the blastocyst. On D6, the uterus enters a refractory state that can no longer support attachment of embryos, and that is unfavourable to embryo survival.

Figure 4: The window of uterine receptivity in humans and mice.

Uterine sensitivity to implantation is classified into pre-receptive, receptive and refractory phases. In mice (top diagram), the uterus is receptive on D4 of pregnancy or pseudopregnancy, whereas it is pre-receptive on days 1-3, and by the afternoon of D5 it becomes non-receptive to implantation.

In humans (bottom diagram), the uterus is classified histologically and functionally into proliferative (follicular) and secretory (luteal) phases during the average 28–30-day menstrual cycle. During the secretory phase, the uterus is considered prereceptive for the first 7 days following ovulation (day 0). The uterus then becomes receptive during the mid-secretory phase, which spans 7–10 days after ovulation; the nonreceptive (refractory) phase comprises the rest of the secretory phase. (Wang *et al.* 2006)



b. Embryo : apposition/adhesion/penetration

In the uterus, embryo implantation occurs in three different phases: apposition between the trophectoderm layer of the blastocyst with the uterine luminal epithelium, attachment of these layers and, invasion of the uterine luminal epithelial epithelium by the embryo. This general process applies to both humans and mice. In mice, the day of vaginal plug is taken as D1. The blastocyst enters the uterus on D3 of pregnancy and adheres to the endometrium on D5, to the extent that it can no longer be flushed from the uterine lumen (Dey *et al.* 2004).

Apposition consists of the first initial, unstable contact between the blastocyst and the uterine wall. A generalized stromal oedema leads to luminal closure, resulting in interdigitation of microvilli of the trophoblast and luminal epithelial cells (Dockery *et al.* 1990). In rodents, luminal closure occurs in pregnant and pseudopregnant females (Enders *et al.* 1976). Although it does not require the presence of blastocyst, progesterone priming of the uterus is essential for luminal closure to take place (Clark *et al.* 1975). During apposition, soluble proteins such as cytokines, growth factors, morphogens and others factors are produced and received in a bidirectional way between the uterus and the blastocyst and are responsible for the materno-foetal cross-talk (Yoon *et al.* 2004).

Apposition is followed by the adhesion stage, when the trophectoderm anchors to the opposing surface of the LE. The first conspicuous sign of the adhesion process appears on the evening of D4 in mice and coincides with localized increase in stromal vascular permeability (Chakraborty *et al.* 1995). This process can be visualized in the uterus as discrete blue bands after an intravenous injection of a blue dye solution. During the attachment stage, the association between the trophoblast and the LE is firm enough to resist dislodging of the blastocyst by flushing the uterine lumen. The most important adhesion molecules involved in this process are: integrins, selectin, HSPGs, mucin-1, cadherins and the trophinin-tastin-bystin complex (Kimber

et al. 2000). Integrin trafficking is the rate-limiting step in establishing blastocyst adhesion to the ECM (Paria *et al.* 2000).

Embryo attachment is followed by LE apoptosis and localized stromal cell decidualization at the site of implantation (Abrahamsohn *et al.* 1993). Penetration involves invasion of the syncitiotrophoblasts through the LE and basal lamina in the stroma, to establish a vascular relationship with the mother (Cohen *et al.* 2007). At this stage, stromal-cell differentiation into decidual cells is extensive and leads to the loss of the LE at the site of the implanting blastocyst. The dynamic and overlapping expression of signalling molecules during these three stages makes it difficult to assign the contribution of specific signalling pathway to a particular stage.

IV. Decidualization

Upon attachment of the embryo to uterine luminal epithelial cells, proliferative trophoblasts differentiate into invasive trophoblasts. Placental and decidual proteins regulate the molecular regulation of trophoblast differentiation, invasion and vascularization. During human implantation the syncytiotrophoblast is formed and invades the endometrium (Hess *et al.* 2007). There is a downregulation of E-cadherin, known to maintain epithelial integrity, and a decrease in membrane bound β-catenin (Shih *et al.* 2002). During this process, matrix metalloproteineases and receptors for fibronectin and collagen, ie, integrin α5β1 and integrin α1β1 are up-regulated (Sutherland *et al.* 1993; Damsky *et al.* 1994; Alexander *et al.* 1996). These events, leading to impaired cell-cell contact, may initiate trophoblast invasion process. Endovascular invasion of trophoblast and development of the placental vascular network is essential for growth and maintenance of the developing embryo (Huppertz *et al.* 2005). Several factors are involved in the angiogenic process including VEGF, PDGF, and PAF. VEGF, which is expressed in the endometrium and in trophoblastic cells, induces angiogenesis and increases vascular permeabilization (van den Brûle *et al.* 2005).

The decidua functions as a barrier to materno immunological responses to semi-allogenic embryos (Saito *et al.* 2007; Lea *et al.* 2007). Extravillous trophoblasts invading endometrium is an essential process of decidualization and is critical for foetal growth and survival. Deregulation of this process is associated with gestational diseases such as superficial invasion of decidual tissue, incomplete transformation of spiral arteries which is a characteristic of preeclampsia, potential malignant transformation or hydatidform moles (Lockwood *et al.* 2007)

V. Steroid hormones

The major factors that specify uterine receptivity are the ovarian steroids, which orchestrate the program of cell proliferation and differentiation. In mice and rats, ovarian oestrogen and progesterone are essential to establish successful embryo implantation. In pigs, guinea pigs, rabbits, and hamsters, ovarian oestrogen is not essential, as oestrogen-synthesizing capacity has been proven for embryos of these species (Psychoyos *et al.* 1973; Heap *et al.* 1967; Harper *et al.* 1969; Kwun *et al.* 1974; McCormack *et al.* 1974; Heap *et al.* 1981). If oestrogen plays a role in implantation in these species is still debatable. In humans, it is not known whether oestrogen secreted by the ovary or the blastocyst plays a role in implantation (Yagel *et al*. 1989). On the other hand, progesterone is essential for implantation and pregnancy maintenance in all mammals studied so far (Carson *et al.* 2000). In pseudopregnant mice, secretion of ovarian progesterone and oestrogen is similar to that of pregnant females, due to the presence of the newly formed corpus lutea (Bronson *et al.* 1975). Accordingly, blastocysts transferred into the uterine lumen during the receptive phase implant normally.

The actions of oestrogen and progesterone are mediated by their nuclear receptors that are differentially expressed in the endometrium (Makrigiannakis *et al.* 2006). The uterus is comprised of heterogeneous cell types that respond differentially to oestrogen and progesterone.

a. Oestrogen

The naturally occurring oestrogens 17β oestradiol (E_2), estrone and oestradiol are C18 steroids derived from cholesterol (Basdevant *et al.* 1992). After binding to lipoprotein receptor, cholesterol is taken up by steroidogenic cells, stored and moved to the sites of steroid synthesis (Basdevant *et al.* 1992). The primary sources of oestradiol in women are granulosa and theca cells of the ovaries and the luteinized derivative cells (Armstrong *et al.* 1977).

The theca cells secrete androgens that diffuse to the granulosa cells to be aromatized to oestrogens. Aromatization is catalyzed by the P450 aromatase monooxygenase enzyme complex that is present in the smooth endoplasmic reticulum and functions as a demethylase. Oestradiol is formed from its precursor testosterone in three consecutive hydroxylating reactions. During menstrual cycles, oestradiol is produced cyclically with the highest rates and serum concentrations in the preovulatory phases, and with the lowest levels premenstrually (Gurpide *et al.* 1974; Simpson *et al.* 2002).

Growth factors secreted in response to oestrogen and progesterone contribute to the milieu of the developing uterus. In rodents, uterine growth responses to oestrogen are grouped as early and late. Hewitt SC *et al*. determined whether early and late biological responses are correlated with altered regulation of a single set of genes or distinct set of genes characteristics of early and late responses, by analyzing the uterine response of ovariectomized mice treated with vehicule or with estradioll for 2 hours (early) or 24 hours (late). Early response occurs during the first 6 h after administration of E_2 and lead to an increase in water imbibitions, vascular permeability and hyperemia, prostaglandin release, glucose metabolism, and eosinophil infiltration (Hewitt *et al.* 2003). A series of biosynthetic responses are also characteristic of the first phase and include increased RNA polymerase activity, lipid and protein synthesis, and increased RNA levels of glucose-6-phosphate dehydrogenase (Das *et al.* 1994; Das *et al.* 2000). Contrarily, the late response begins 10-16h after oestrogen administration and engenders cycles of DNA synthesis and epithelial cell mitosis (Hewitt *et al.* 2003). In human, the angiogenic activity is triggered by postmenstrual hypoxic milieu (Koga *et al.* 2001). Analogy between the role of oestrogen in mouse and human is restricted to the major growth promoting effects (Kodaman *et al.* 2004). Therefore, oestrogen is essential for both epithelial proliferation and embryo implantation in rodents. Oppositely, in humans, oestrogen is only required for endometrial growth.

i. Oestrogen Receptor.

The specific actions of oestrogen are mediated through their receptors and may involve membrane as well as nuclear receptors. Two structurally related subtypes of oestrogen receptor, known as ERα and ERβ, have been identified in mouse and human, and are each encoded by a separate gene, present on different chromosomes (Beato *et al.* 1995; Kuiper *et al.* 1996).

ERα and ERβ are members of the nuclear-hormone superfamily, which comprises approximately 150 members that share structural similarities. Oestrogen receptors are composed of multiple functional domains. The DNA-binding domain contains two zinc-fingers that are involved in receptor binding and dimerization (Fawell *et al.* 1990). The DNA-binding domain contains a P-box, which is a short motif responsible for DNA-binding specificity and is involved in dimerization of nuclear receptors (Norman *et al.* 2004). The ligand-binding domain is responsible for the binding of cognate ligand or hormone; this domain also contains a ligand-regulated transcriptional activation function necessary for recruiting transcriptional co-activators (Klinge *et al.* 2004). The N-terminal domain has a high degree of variability and normally contains a transactivation domain that may mediate differential promoter regulation in vivo (Lees *et al.* 1989; Valley *et al.* 2005).

RT-PCR and ribonuclease assays have demonstrated different tissue distribution for ERα and ERβ in the mouse. ERα mRNA is detected in the uterus, mammary gland, testis, pituitary, liver, kidney, heart, and skeletal muscle, whereas ERß transcripts are significantly expressed in the ovary and prostate (Byers *et al.* 1997; Kuiper *et al.* 1997; Couse *et al.* 1997; Brandenberger *et al.* 1998). There is some overlap in the expression pattern of ERα and ERβ in the epididymis, thyroid, adrenals, bone, and various regions of the brain (Nilsson *et al.* 2001). However, in those tissues expressing both ERs, there is a distinct expression pattern within the heterogeneous cell types composing the tissue (Rosenfeld *et al.* 1999; Sar *et al.* 1999; Hiroi *et al.* 1999).

ii. ER mutant mice

The mutant mice for each ER receptor, αERKO and ßERKO, have contributed to our current understanding of the various roles of oestrogen during the implantation process. The uteri of adult ßERKO females appear normal (Krege *et al.* 1998). Contrarily, the uteri in the αERKO are hypoplastic, the stroma show a disorganized structure and hypertrophy, with a scattered distribution of uterine glands, although epithelial cells appear healthy (Hewitt *et al.* 2003; Emmen *et al.* 2003; Lubahn *et al.* 1993).

Embryo implantation occurs in ßERKO, which are subfertile owing to a reduced ovarian efficiency. In these females early and late responses to oestrogen were indistinguishable from the wild-type (wt), which suggest that ERß is not essential for mediating the actions of oestrogen in the uterus (Wada-Hiraike *et al.* 2006). αERKO are profoundly insensitive to oestrogen and are infertile due to a complete ovarian inefficiency (Lubahn *et al.* 1993). Uteri of αERKO are unable to support implantation, but retain biological functions that allow decidualization (Paria *et al.* 1999). These results demonstrate that decidualization-associated signalling is oestrogen independent. αERKO showed only little response to oestradiol treatment, as evidence by: (1) lack of growth response and (2) lack of induction of target genes, such as lactoferrin and progesteroone receptor. These results emphasize the crucial role of ERα for mediating oestrogen actions in the mouse uterus (Lubahn *et al.* 1993). In αERKO, ERß is hardly expressed in the uterus, making it unlikely to play a compensatory role (Curtis *et al.* 1999) (Table 1, Table 2).

Oestrogen upregulates the uterine levels of epidermal growth factor and its receptor (EGF, EGF-R), transforming growth factor-α, and insulin-like growth factor-I (IGF-1) (Klotz *et al.* 2002; Hewitt *et al.* 2003). The uteri of α ERKO females possess wild-type levels of functional EGF and EGF-R. However, the mitogenic actions and induction of oestrogen-responsive genes

elicited by EGF in the wild-type uterus are absent in the αERKO, which suggest that these two signalling systems interact in mediating mitogenic action of oestrogen (Das *et al.* 1997). Tissue recombination studies have shed light on the role of oestrogen in uterine cell proliferation and differentiation (Das *et al.* 1997). It was demonstrated that ERα was neither necessary nor sufficient to mediate proliferative response to estradiol, while the stromal ERα was obligatory (Cooke *et al.* 1997). Induction of cell proliferation in the LE by E_2 directly depends on the adjacent stroma expressing the oestrogen receptor alpha. The LE responds to as yet unidentified growth factors produced by the stroma in response to E_2 (Cooke *et al.* 1997).

Aside from the proliferative effect of oestrogen and its capacity of stimulating the secretion of factors such as lactoferrin and complement component C3, it has been demonstrated that the oestradiol-ERα complex strongly regulates progesterone receptor (PR) expression (Ing *et al.* 1997; Kastner *et al.* 1990; Kraus *et al.* 1993; Liu *et al.* 1992). The lack of estradiol-induced increases in PR mRNA levels in the αERKO uterus confirms a regulatory dependence of the PR gene on ERα action (Curtis *et al.* 1999). The levels of PR protein in the αERKO uteri are reduced to approximately 60% of that in wild type (Curtis *et al.* 1999). One role of oestrogen in uterine decidualization is thought to be the induction of significant increases in PR in the endometrial stroma (Kurita *et al.* 2000). Nonetheless, the deciduomas induced in the αERKO females are neither reduced in size nor appear less complex or differentiated than those observed in the wild type (Paria *et al.* 1999). The reasons for the apparent loss of oestrogen dependence for successful decidualization in the αERKO uterus can be explained by the complexity of the decidualization process. Indeed, several mouse models, such as homozygous females for Leukemia Inhibitory Factor (LIF), prostaglandin synthase-2 and Hoxa10, lack the ability to induce decidualization (Stewart *et al.* 1992; Lim *et al.* 1997; Song *et al.* 2000; Benson *et al.*1996)

Table 1: Genes critical to uterine physiology in adult life; results from knockout mice

Genes	Molecule encoded	Knockout phenotype in females	References
Adamts1	A disintingrin-like and metalloprotease with thrombospondin-type-1 motif-1 (enzyme, tissue remodelling)	Impaired follicular development and fertilization, subfertility.	Shindo T. *et al*, 2000.
Bteb1	Basic transcription-element-binding protein-1 (TF)	Uterine hypoplasia, compromised P_4 function; impaired implantation; subfertility	Simmen R. *et al,* 2004
Cenpb	Centromere protein B (centromere assembly)	Disrupted luminal and glandular uterine epithelia ; subfertility	Fowler K. *et al.,* 2000
Cyp27b1	25-hydroxyvitamin D 1α-hydroxylase enzyme (Vitamin D metabolism)	Uterine hypoplasia ; no corpus lutea ; infertility	Panda D.K. *et al.,* 2001.
Esr1	Oestrogen receptor-α (NR,TF)	Ovarian cysts, uterine hypoplasia ; infertility	Lubahn D.B. *et al*., 1993.
Igf1	Insulin-like growth factor-1 (IGF-1)	Ovulation failure, uterine myometrial hypoplasia; infertility	Baker J. *et al.* 1996.
Pgr	Progesterone receptor (NR, TF)	Unopposed oestrogen action, uterine hyperplasia ; infertility	Lydon J.P. *et al*., 1995.
Ube3a	Ubiquitin-protein ligase E3A (protein modification, proteolysis and peptidolysis)	Impaired follicular development and uterine hypoplasia; subfertility	Smith C.L. *et al*., 2002.
Vdr	Vitamin D receptor (R, TF)	Uterine hypoplasia with impaired folliculogenesis; infertility	Yoshisawa T. *et al.*, 1997.

Table 2: Genes critical to uterine preparation for initiating implantation; results from knockout mice.

Genes	Molecule encoded	Knockout phenotype in females	References
Bsg	Basigin (Immunoglobulin)	Defective fertilization; no implantation	Igakura *et al.* 1998; Kuno *et al*. 1998
Esr1	Oestrogen receptor-α (NR,TF)	No uterine attachment, but uterine responsiveness to decidualization persist with P_4 priming	Lubahn *et al.* 1993; Curtis *et al*. 1999
Fkbp52	FK506-binding protein-4 (immunophilin co-chaperone for steroid hormone NRs)	Compromised P_4 function; no uterine receptivity	Tranguch *et al*. 2005
Gp130/ Stat	GP130/Signal transducer and activator of transcription (cytokine-receptor signalling)	No implantation	Ernst *et al*. 2001
Hmx3	H6 homeobox-3 (TF)	No implantation	Wang *et al.* 1998
LpA3	Lysophosphatidic acid receptor-3 (LPA signalling)	Deferred, on-time implantation; aberrant embryo spacing; postimplantation defects; small litter size	Ye *et al.* 2005
Lif	Leukemia inhibitory factor (cytokine)	No implantation	Stewart *et al*. 1992
Pgr	Progesterone receptor (NR, TF)	No implantation or decidualization	Lydon *et al.* 1995
Pla2g4a	Phospholipase A2, group IVA (arachidonic acid releasing enzyme)	Deferred, on-time implantation; aberrant embryo spacing; postimplantation defects; small litter size	Song *et al*. 2002

Genes	Molecule encoded	Knockout phenotype in females	References
Ppard	Peroxysome proliferator-activated receptor-δ (NT, TF)	Delay in initiating embryo attachment; placental defects; subfertility	Lim *et al*. 1999; Barak *et al.* 2002
Ptgs2	Prostaglandin-endoperoxide synthase-2 (prostaglandin synthesis)	Multiple reproductive failure; including defective attachment reaction	Lim *et al*. 1997; Wang *et al.* 2004.

b. Progesterone.

Progesterone, a C21 steroid derived from cholesterol, is produced directly from pregnenolone. After follicle rupture and release of the ovum, granulosa cells mature to form the corpus luteum, which is responsible for secretion of progesterone (Spencer *et al.* 2002). In humans, if fertilization does not occur within 1 to 2 days, the corpus luteum will continue to enlarge for 10–12 days followed by regression of the gland and concomitant cessation of progesterone release (Fujita *et al.* 1981). If fertilization occurs, the corpus luteum will continue to grow and function for the first 2 to 3 months of pregnancy. After this time it will slowly regress as the placenta assumes the role of hormonal biosynthesis for the maintenance of pregnancy (Diaz *et al.* 2002). In humans, progesterone receptor antagonist administrated before seven weeks of gestation induce abortion. Similarly, surgical removal of the corpus lutea results in pregnancy lost (Franz *et al.* 1988).

Progesterone primes the endometrium for embryo implantation and maintains pregnancy through effects on both the uterus and the developing blastocyst. Indeed, progesterone facilitates embryo hatching by stimulating the synthesis by uterine glandular epithelium of serine protease 2 (SP2), responsible for lysis of the zona pellucida (O'Sullivan *et al.*, 2004). On D3, progesterone induces luminal closure of the endometrium, and stimulates stromal cell proliferation (Tranguch *et al.* 2006). Growth factors, homeobox transcription factors and cytokines, which are under progesterone control, mediate this stromal cell proliferation (Lydon *et al.* 1995). Moreover, progesterone is crucial for decidualization that involves differentiation of stromal cells into decidual cells. These decidual cells are morphologically different from stromal cells, have unique secretory and biosynthetic properties and secrete prolactin, IL-15 and IGFBP1 (Tang *et al.* 2005; Okada *et al.* 2000; Kutsukake *et al.* 2007). Following the oestrogen surge, this differentiation is induced by progesterone, and is a prerequisite for successful implantation and maintenance of pregnancy (Slayden *et al.* 2006).

i. Progesterone receptors

Physiological effects of progesterone are mediated by its interaction with specific intracellular progesterone receptors (PRs) (Evans *et al.* 1988). The PR exists in two isoforms, PR_A and PR_B that arise from the same gene and are also members of the nuclear receptor superfamily of transcription factors (Kastner *et al.* 1990; Conneely *et al.* 1989). The expression of PR (PR_A and PR_B) during the pre-implantation period in the endometrium is cell- and stage-specific. On D1 and D2, PR is barely detected in the luminal epithelium and stroma. The PR expression level increases on D3, and is detected in both epithelium and stroma. On D4, PR amount of protein decreases in the luminal epithelial cells. Contrarily, the stroma shows increasing levels of PR (Tan *et al.* 1999). This high level of expression in stromal cells continues after embryo implantation (Conneely *et al.* 2002).

Progesterone receptors have several functional domains (Tsai *et al.* 1994). The amino terminal or A/B region of PRs is the most hypervariable region, and contains transactivation domains (AF-1 and AF-3) that recruit coactivator proteins to the receptor to modulate the level and promoter specificity of target gene activation (Giangrande *et al.* 1997). An inhibitory domain (ID) responsible for recruitment of transcriptional corepressor proteins is also present in the A/B region. The DNA binding domain (DBD), which is the most conserved region (C), is composed of two type II zinc finger structures (Tsai *et al.* 1988). The carboxy terminal side of the DBD contains a highly conserved ligand binding domain (LBD), and an additional transactivation domain (AF-2) required for hormone-dependent coactivator recruitment, sequences important for interaction of inactive receptors with heat shock proteins and for receptor dimerization (DIM) (Smith *et al.* 1990; Savouret *et al.* 1991). PR_A and PR_B isoforms differ in that the PR_B protein contains an additional sequence of amino acids at its amino terminus that encodes a third transactivation function (AF3) (Sartorius *et al.* 1994). It has been demonstrated that AF3 allows binding of a subset of coactivators to PR_B that is not efficiently recruited by progesterone-bound PR_A (Kazmi *et al.*

1993). The modular protein structure of the PR allows it to bind steroidal ligand, to dimerize liganded receptors, to interact with hormone-responsive DNA elements, and with co-regulator proteins required for bridging receptors to the transcriptional apparatus (Tung *et al.* 2001; Luisi *et al.* 1991; Freedman *et al.* 1992). The ability of PRs to interact with a variety of coactivator and co-repressor proteins, illustrates a key role of these proteins in mediating different tissue-specific responses (Tung *et al.* 2006). Strikingly, progesterone receptors can be activated in the absence of steroidal ligand by phosphorylation pathways that modulate their interactions with co-regulator proteins (Bain *et al.* 2007).

Through its interaction with PR, progesterone induces the expression of a wide array of genes in the endometrium during early pregnancy. These include the genes encoding the growth factor amphiregulin, the homeobox transcription factors Hoxa10 and Hoxa11, peptide hormones clacitonin and proenkephalin, and the enzyme histidine decarboxylase (Lim *et al.* 1999; Das *et al.* 1995; Paria *et al.* 1998; Zhu *et al.* 1998). The current challenge is to link these molecules and their pathways to previously well characterized morphological, physiological, and biochemical events that are associated with the process of implantation and decidualization (Conneely *et al.* 2003).

ii. PR mutant mice.

The receptor-knockout (PRKO) models, in which both PR isoforms are not expressed, are infertile due to multiple reproductive abnormalities (Lydon *et al.* 1995). The homozygous females for PR show altered sexual behavior and neuroendocrine gonadotropin regulation, anovulation, uterine dysfunction and impaired pregnancy-associated mammary gland morphogenesis (Mani *et al.* 1996; Chappell *et al.* 1999; Tibbetts *et al.* 1999). PRKO mouse has demonstrated that the decidualization process is dependent on progesterone action. Indeed, the uterus of PRKO mouse fails to support implantation after embryo transfer and is unresponsive to artificial decidual stimulus (Lydon *et al.* 1995). Moreover, uterine epithelial cells of PRKO mice become hyperplastic due to an absence of downregulation of the proliferative

oestrogen action (Jeong *et al.* 2005). Gene expression studies have demonstrated that the absence of embryo implantation observed in PRKO mice are associated with inhibition of expression of several epithelial markers of uterine receptivity and at least one essential stromal mediator of decidualization, Hoxa-10 (Conneely *et al.* 2001; Jeong *et al.* 2005).

The analysis of the functional differences between PR_A and PR_B isoforms has been hampered by a lack of information on the specific cell types that express each isoform *in vivo*. Indeed, in order to analyse the distinct roles of PR_A and PR_B, mouse models lacking either PR_A or PR_B have been generated using the CRE-LoxP system to introduce a point mutation at either of the two ATG translation initiation codons (Conneely *et al.* 1987; Giangrande *et al.* 1997). PRBKO mice are fertile and show no defect in ovarian and uterine functions, which suggest that expression of PR_A is necessary and sufficient to mediate both the antiproliferative- and implantation-associated responses to progesterone. On the contrary, PRAKO are infertile due to severe abnormalities in ovarian and uterine functions (Mulac-Jericevic *et al.* 2000 and 2003). Actually, in PRAKO mice, progesterone-induced differentiation of endometrial stromal cells to a decidual phenotype is inhibited. Selective activation of PR_B in the uterus of PRAKO mice results in an abnormal induction of epithelial cell proliferation (Mulac-Jericevic *et al.* 2000). This gain of PR_B-dependent proliferative activity upon removal of PR_A suggests that PR_A is required to inhibit oestrogen-induced hyperplasia of the uterus but also to limit potentially adverse proliferative effects of the PR_B protein (Lydon *et al.* 1995 and 1996; Kurita *et al*. 2000) (Tables 3).

Table 3: Genes critical uterine decidualization; results from knockout mice.

Genes	Molecule encoded	Knockout phenotype in females	References
Fkbp52	FK506-binding protein-4 (immunophilin co-chaperone for steroid hormone NRs)	Compromised P_4 function; defective decidualization	Tranguch S. *et al*., 2005
Hoxa10	Homeobox A10 (TF)	Defective decidualization; reduced fertility	Benson G.V. *et al*., 1996; Lim H. *et al*., 1999; Satokata I. *et al*., 1995.
Hoxa11	Homeobox A11 (TF)	Defective decidualization and implantation; infertility	Hsieh-Li H.M. *et al*., 1995.
Il11ra1	Interleukin-11 receptor-α1 (cytokine signalling)	Impaired decidualization; infertility	Robb L. *et al*. 1998; Bilinski P. *et al*., 1998.
Pgr	Progesterone receptor (NR, TF)	Lack of decidual response even after P_4 priming	Lydon J.P. *et al*., 1995.
Ptgs2	Prostaglandin-endoperoxide synthase-2 (prostaglandin synthesis)	Defective decidualization; reduced angiogenic response	Lim H. *et al*., 1997; Matsumoto H. *et al*., 2002.

VI. Embryo uterine signalling pathways in implantation.

a. Signalling via adhesion molecules.

Endometrial receptivity consists in the acquisition of adhesion ligands together with the loss of inhibitory components that may act as a barrier to an implanting embryo. The cell adhesion molecules (CAM) family is composed of four majors groups: integrins, cadherins, selectins and immunoglobulins. Their functions include maintenance of tissue integration, wound healing, morphogenic movements, cellular migration and tumour metastasis (Ruggeri *et al.* 2007; Naik *et al.* 2008). Cell binding to the extracellular matrix influences behaviour pattern of migration and differentiation. Many glycoproteins and carbohydrate ligands and their receptors are expressed in the uterine luminal epithelium and blastocyst cell surfaces during embryo implantation (Foulk *et al.* 2007; Sugihara *et al.*; Skrypczak *et a*l. 2001; Wang *et al.* 2002). The most relevant molecules involved in trophoblast-uterine adhesion are selectins, galectins, heparan sulfate proteoglycans, Muc-1, integrins, cadherins, and the trophinin-tastin-bystin complex (Lessey *et al*. 2002; Witz *et al*. 2003). Amongst these adhesion factors, integrins have been studied more extensively in the human endometrium because of their cycle-dependent changes and the potential role in uterine receptivity (Thiery *et al*. 2003).

i. Integrins

Integrins comprise a large family of cation-dependent heterodimeric transmembrane receptor. Integrins are obligate heterodimers made from α-subunits and β-subunits (Hynes *et al*. 2002). To date, nineteen α and eight β subunits have been identified in mammals. Each subunit has a large N-terminal extracellular domain, a transmembrane domain, and a short C-terminal cytoplasmic domain (Hynes *et al.* 2002). The extracellular domains bind a wide variety of ligands, whereas the intracellular cytoplasmic domains

anchor to cytoskeletal proteins. In this manner, the exterior and interior of the cell are linked, which allows for bidirectional transmission of signals across the plasma membrane (Gilmore *et al*. 1996). The ligand specificity for each heterodimer is determined by the distinct combination of α and ß subunits. Several integrins, such as αvβ3, serve as receptors for components of the ECM that have exposed arginine-glycine-aspartic (RGD) tripeptide sequence. These include vitronectin, fibronectin, fibrinogen, laminin, collagen, Von Willibrand's factor, osteoponin, and adenovirus particles (Giancotti *et al*., 1999**)**.

Invasive mouse trophoblasts adhere, spread, and migrate on ECM substrates and penetrate three-dimensional ECM structures. Fibronectin, laminin, and collagen type IV, which are ECM components expressed in the periimplantation endometrium, support trophoblast outgrowth *in vitro* (Wang *et al.* 2007). Trophoblast interactions with the ECM are mediated primarily by integrins. It has been demonstrated that hexapeptides containing the RGD sequence could block trophoblast outgrowth on fibronectin, collagen type II and IV, entactin, and vitronectin (Coutifaris *et al*. 2005). The endometrium and the developing blastocyst express a multitude of α and β integrin subunits (Goldman-Wohl *et al*., 2002).

Among many subunits, integrins α5β1, integrin α6β1, and integrin αvβ3 are expressed in the mouse embryo from the fertilized egg stage through the peri- and post-implantation periods (Lu *et al.* 2002). Implantation of the blastocyst to luminal epithelial cells of the receptive uterus depends on the attachment of trophoblast cells with extracellular matrix (ECM) (Sutherland *et al*.1993). Adhesion competent blastocyst displays a strong fibronectin (FN) binding activity (Illera *et al*. 2000). Reinforcement of FN-binding activity only occurs after exposure to FN and involves trafficking of integrins α5β1, α7β1 from basal to apical plasma membrane, and thus a change in cell polarity. In humans, preimplantation embryos have also been shown to express a number of integrin subunits such as α3, αv, β1, β3, β4 and β5 (Kabir-Salmani *et al*. 2004). Little is known about their regulation, their

ligands, and their association partners. Noteworthy, at the blastocyst stage, the embryo expresses numerous ligands for integrins, including fibronectin, laminin and type IV collagen (Lessey *et al.*, 1992; Tabibzadeh *et al*. 1992). These results suggest that integrins and their ligands play a role in the attachment of the blastocyst to the uterus.

The role of endometrial and embryonic integrins during implantation remains unresolved although work in animal models and in humans supports their involvement in this process. Temporal and spatial distribution of the αvβ3 integrin on both embryo and endometrium in women and mice coincides with the time of initial attachment during implantation. This suggests that αvβ3 may play a critical role in the cascade of events leading to successful implantation (Lessey, 1994).

ii. Mucins

The apical surface of most epithelial cells is decorated with a thick glycocalyx that is believed to protect the cell surface from pathologic processes. This protective barrier is mostly composed of mucins, which make the cell surface enzymatically resistant, limit access to receptors, severely impair cell-cell and extracellular matrix adhesion, and may, in addition, protect the cell from the host immune system (Aplin *et al.*, 2001).

Mucins are high molecular weight type-I transmembrane glycoproteins that are composed of three domains: short cytoplasmic and transmembrane domains that are highly conserved among species, and a large extracellular domain. The extracellular domain, which contains a variable number of tandem repeat (VNTR) sequences of twenty amino acids and is highly O-glycosylated, is likely to be responsible for the anti-adhesive properties of mucins. Among the fourteen cloned human mucins, only Mucin-1 (MUC-1) has been found in human endometrium. The MUC1 is expressed on the apical surface of most simple epithelia, including mammary gland, female reproductive tract, lung, kidney, stomach, gall bladder, and pancreas as well as

some non-epithelial cell types. In uterine epithelium, mucins are abundant and concentrated at the apical surface, where they presumably provide a barrier to trophoblast invasiveness by controlling accessibility of integrin receptors to their ligands. Their unmasking at the implantation site correlates with increased blastocyst adhesiveness to the uterus (Carson *et al*., 1998).

In mouse and human, MUC1 is highly expressed in the uterine luminal epithelial cells, and is down-regulated in the surface epithelium during embryo implantation. In mouse, down regulation of MUC1 in the LE defines the "window of implantation" (Aplin *et al.,* 1995; Carson *et al*. 1998). During the implantation process, progesterone combined with oestrogen upregulate MUC1 expression during the apposition phase, when the blastocyst must be stationary in position. Local depletion of MUC1 at the site of embryo implantation is triggered by paracrine signals emanating from the activated blastocyst. Clinical studies have shown a reduced expression level of MUC1 core protein in uterine flushings and in midsecretory endometrium from women suffering from recurrent miscarriage. Moreover, it is believed that unexplained infertility may be associated with a polymorphism in the MUC1 VNTR, resulting in a protein with a reduction in the number of O-glycosylation sites, thereby causing implantation failure (Alameda *et al*. 2007).

Collectively, these findings suggest that Muc1 acts as an antiadhesive molecule that must be removed from the site of implantation, generating a small zone of the luminal epithelium adhesive.

b. Signalling by vasoactive factors.

The process of implantation is considered analoguous to a proinflammatory reaction and is accompanied by an increased endometrial vascular permeability at the site of blastocyst attachment. Accordingly, it is expected that vasoactive agents, such as prostaglandins (PGs), histamine, platelet-activating factor, and vascular endothelial factor, are crucial for implantation. PGs are arachidonate-derived mediators that belong to the

eicosanoid class. They possess vasoactive, mitogenic, and differentiating properties. Based on studies utilizing inhibitors of prostaglandin (PG) synthesis and transgenic mice, considerable evidence has accumulated indicating that PGs have an important role in the early events of implantation and artificially induced decidualization.

PGs are generated from arachidonic acid by phospholipase A2 (PLA2s) followed by cyclooxygenases (COX). COX mediates the conversion of arachidonic acid into prostaglandin H2, which is then converted to various PGs by specific synthases. COX exists in two isoforms, COX-1 and COX-2, and is the rate-limiting enzyme in the biosynthesis of PGs. The COX isoforms, encoded by Ptgs1 and Ptgs2, share similar structure and kinetics properties, and show distinct cell-specific expression, subcellular localization and regulation. COX-1 is considered to be a constitutive enzyme that mediates housekeeping functions. In contrast, COX-2 is highly inducible by diverse stimuli including cytokines, growth factors, mitogens, and tumor promoters, and regulates inflammation, differentiation, mitogenesis, and angiogenesis (Simmons *et al*. 2004).

PGs exert their functions by interacting with cell surface G protein-coupled receptors. Receptors for PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2, and thromboxanes have been named as EP_1–EP_4, FP, DP, IP, and TP, respectively. However, PGs can also function as ligands for nuclear peroxisome proliferator-activated receptors (PPARs). PPARs, initially cloned as a family of orphan receptors, are known for their ligand promiscuity. The ligands range from free fatty acids and their derivatives produced by the cyclooxygenase or lipoxygenase pathway to certain hypolipidemic drugs (Ding *et al*., 2003).

cPLA2 is expressed in the uterine epithelium on D4 of pregnancy. By the time of embryo attachment, late D4 to D5, cPLA2 is expressed in stromal cells surrounding the implanting blastocyst. These results suggest that cPLA2 is available as an arachidonic acid provider for uterine PG biosynthesis during implantation. COX genes expression is regulated in a spatio-temporal manner

in the uterus during pregnancy. Cox-1 and Cox-2 genes show a unique expression pattern in the periimplantation mouse uterus, which further suggest that PGs play important roles in these processes. Cox-1 transcripts are first detected on the morning of D4 in uterine luminal and glandular epithelial cells. Once the attachment reaction has occurred, Cox-1 is no longer expressed in the luminal epithelium. Contrarily, Cox-2 is expressed in the luminal epithelium and underlying stromal cells solely at the site of blastocyst attachment, at the anti-mesometrial pole on D4 and D5. From D6 onward, COX-2 expression switches to the mesometrial pole of the uterus. It is believed that HB-EGF expressed in the LE induces expression of COX-2. Using the delayed implantation model, it has been demonstrated that the expression of COX-2 in the receptive uterus is dependent on the presence of an active blastocyst. COX-2 derived PGI_2 is the first PG produced at the site of embryo implantation. PGI_2 participates in implantation via the activation of PPARδ, the expression of which overlaps that of COX-2 at the site of implantation (Wang and Dey, 2005; Shah and Catt 2005). Blockade of PGs synthesis before or during the implantation process causes complete inhibition, a delay in implantation or a reduction in the number of implantation sites with diminished decidual tissue.

COX-2 is expressed either in the uterus, blastocyst, or both during implantation in a variety of species with different modes of implantation, including sheep, mink, skunk, baboon, and pig. These results suggest a conserved function of COX-2 in implantation in various species. In humans, concentrations of PG in the deciduas in early pregnancy are lower than those in the endometrium at any stage of menstrual cycle, primarily because of a decrease in PG synthesis. Elevated levels of COX-2 are associated with endometriosis and preterm birth, while decreased expression of COX-2 is associated with some cases of preeclampsia. Noteworthy, an administration of exogenous PG induces an inflammatory response leading to abortion in all species and at any stage of gestation (Wang and Dey 2005).

c. Signalling by growth factors.

i. Vascular endothelial growth factor (VEGF)

Vasculogenesis and angiogenesis are two consecutive processes during blood vessel development in the placenta and are essential for the growth and maintenance of the developing embryo. While vasculogenesis is the formation of first blood cells, angiogenesis involves the proliferation and migration of endothelial cells from pre-existing blood vessels. Endothelial cells sprout out from these vessels and recruit other cells to become capillaries and smooth muscle cells for larger vessels. VEGF and its cognate receptors are involved in this angiogenic process (Torry *et al*. 2007).

VEGF is a heparin-binding, secreted homodimeric glycoprotein of 30-46 kDa, whose expression is under the control of oestrogen in the uterus. The protein-coding regions of VEGF are arranged in eight exons. Alternate splicing of these exons results in five isoforms. VEGF is a potent mitogen for vascular endothelium, and plays a critical role in the development of blood vessels. Its angiogenic action may, in part, result from the enhancement of permeability, which invariably precedes new blood vessel growth, and which is a marker of endometrial receptivity. VEGF acts via two tyrosine kinase family receptors: VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1) (Dvorak *et al.* 1995). The close temporal and spatial correlation between VEGF expression and increased vascular permeability in the uterus suggests that VEGF is the factor responsible for this phenomenon. Indeed, in the uteri of pregnant mice, VEGF mRNA accumulation primarily occurs in epithelial cells on D1 and D2. On D3 and D4, the subepithelial stroma in addition to epithelial cells exhibit accumulation of VEGF transcripts. On D5, after the initial attachment reaction, luminal epithelial and stromal cells immediately surrounding the blastocyst exhibited high expression of VEGF (Halder *et al*. 2000; Chung *et al.* 2000; Smith 2000). These results emphasize the role of VEGF in the process of decidualization and placentation.

In the human endometrium, VEGF expression is both cell- and cycle-specific. VEGF is detected in the luminal and glandular epithelium during the proliferative phase of the cycle, that is under the control of oestrogen. The VEGF receptors Flt-1 and Flk-1 are also expressed in the human endometrium at all stages of the menstrual cycle. These results indicate that as for the mouse, VEGF is under the control of oestrogen (Plaisier *et al.* 2007; Sugino *et al.* 2002).

ii. Epidermal Growth Factor family (EGF)

Growth factors act as intercellular signalling molecules, which control cellular functions during development, embryo implantation, and in response to injury or infection. Spatiotemporal expression patterns of EGF gene family members and their cognate receptors in the uterus during the periimplantation period suggest a role in the process of embryo implantation. The epidermal growth factor (EGF) family is an expanding group of structurally related polypeptides that regulate cell proliferation, migration and differentiation, via tyrosine kinase receptors on target cells. This family includes EGF, TGF-α, HB-EGF, amphiregulin, betacellulin, epiregulin, and neuregulins. These molecules are synthesized as transmembrane proteins that are proteolytically processed to release the mature forms. Both the transmembrane and the mature forms are biologically active. The EGF-like growth factors interact with the receptor subtypes of the erbB gene family, which is comprised of four receptor tyrosine kinases: ErbB1 (EGF-R), ErbB2, ErbB3, and ErbB4. They share common structural features but differ in their ligand specificity and kinase activity. Dimerization between co-expressed receptors upon ligand binding constitutes the classical mechanism of action of EGF-like ligands (Leahy *et al.* 2004).

HB-EGF is the earliest marker of uterine receptivity, and is expressed in the luminal epithelium, at the prospective site of embryo implantation, six to seven hours before the attachment reaction in mice. Expression of HB-EGF is dependent on the oestrogen surge and the presence of the blastocyst. ErbB1

(EGF-R), ErbB2, and ErbB4, the receptor subtypes for the EGF family of growth factors, are expressed in the mouse blastocyst. Moreover, *in vitro* experiments have demonstrated that HB-EGF can stimulate proliferation, zona-hatching and outgrowth of trophoblasts. Indeed, HB-EGF is thought to mediate the embryo uterine cross-talk in a paracrine, juxtacrine and autocrine manner, and to regulate endometrial proliferation, differentiation and secretion. The induction of HB-EGF expression is followed by the expression of betacellulin, epiregulin, neuregulin-1, and Cox-2 around the time of the attachment reaction. On D4, amphiregulin, which is a progesterone responsive gene, is expressed throughout the uterine epithelium. On late D4, at the time of embryo implantation, amphiregulin is expressed in the luminal epithelium only in the region surrounding the implanting blastocyst. By D5, amphiregulin is no longer detected in the endometrium. The spatio-temporal regulation of EGF family members shows up their crucial role for endometrial receptivity and embryo implantation (Paria *et al*. 1999; Yoo *et al.* 1997; Iwamoto *et al.* 2000).

d. Signalling by cytokines.

Cytokines are small glycoproteins with multiple biological activities and are involved in many physiological processes. Cytokines are involved in the regulation of growth, development, and activation of immune system cells and the mediation of the inflammatory response. Both pleiotropy and redundancy characterize cytokines. They act as intercellular (paracrine) and/or intracellular (autocrine) signals in local tissues and sometimes also as endocrine mediators. Embryo implantation is considered as an inflammatory type response. Indeed, the expression of various cytokines and their receptors in the uterus and embryo during early pregnancy suggests their roles in various aspects of implantation (Chaouat *et al*. 2007; Dimitriadis *et al.* 2005; Salamonsen *et al.* 2000).

The family members of IL-1 include three polypeptides: IL-1α, IL-1β, and a natural inhibitor, IL-1 receptor antagonist (IL-1ra). IL-1α, IL-1β, which

are produced by a variety of host cells, mainly macrophages, bind to the same receptors, IL-1R type I and IL-1R type II. IL-1 has an effect on many cell types and has crucial roles in haematopoiesis acute-phase protein expression and kidney function (Boraschi and Tagliabue 2006; Daun and Fenton 2000). In mice, trophoblastic cells and decidualized stromal cells produce IL-1, while IL-1R is present in endometrial epithelial cells as well as in trophoblasts (Laird *et al*. 2006; Karmakar and Das 2002). Although, homozygous females for TNFα, IL-1ß, IL-1 receptor antagonist, IL-1 receptor type 1, IL-6, and granulocyte/macrophage-colony stimulating factor do not manifest overt reproductive defects; blocking the IL-1 system in mice endometriums, either by gene deletion or by treatment with IL-1ra, leads to a drastic decrease in the number of implanted blastocysts. In the endometrium, IL-1 was shown to increase endometrial secretion of prostaglandin E2, LIF and integrin-beta3 subunit expression. Moreover, IL-1 induces trophoblast invasion by stimulating MMP9 activity in trophoblasts and expression in endometrial stromal cells. In humans, IL-1 is present at the feto-maternal interface (Das *et al.* 2002). Recently, low levels of IL-1β transcripts in non-pregnant endometrium were associated with recurrent miscarriage. Besides, decreased endometrial expression of IL-1α and IL-1β were found in small proportion of women with unexplained infertility (Laird *et al*. 2006).

IL-11 is a member of the IL-6 family of cytokines. Gene targeting experiments have demonstrated that homozygous females for IL-11 are infertile due to a defect in decidualization, but not for attachment reaction (Dimitriadis *et al*. 2007; Robb *et al*. 1998) (Table 3). Another member of the IL-6 family of cytokines, LIF has been demonstrated to be essential for embryo implantation and will be further discussed in the next chapter (Robb *et al.* 2002).

e. Homeobox genes in implantation.

While morphogenesis of most organs is completed by adulthood, the uterus undergoes a remarkable program of cellular proliferation and

differentiation during early pregnancy. Homeobox (Hox) genes are regulators of tissue differentiation in the embryo. Consequently, recent evidence has demonstrated the importance of Hox genes for endometrial development and receptivity (Vitiello *et al*. 2007).

Hox genes are highly evolutionarily conserved transcription factors that play a crucial role in determining tissue identity along the antero-posterior axis during embryonic development of all metazoans. This family of transcription factors shares a highly conserved sequence element called the *homeobox* that encodes a 61-amino acid helix-turn-helix DNA-binding domain. In mice and humans, Hox genes are arranged in four clusters (A, B, C, and D) on four different chromosomes. Their linear arrangement parallels their order of expression along the anterior-posterior body axis. Spatial co-linearity maps suggest that paralogous groups of Hox genes in the caudal region are important in the uterus (Krumlauf *et al.* 1993).

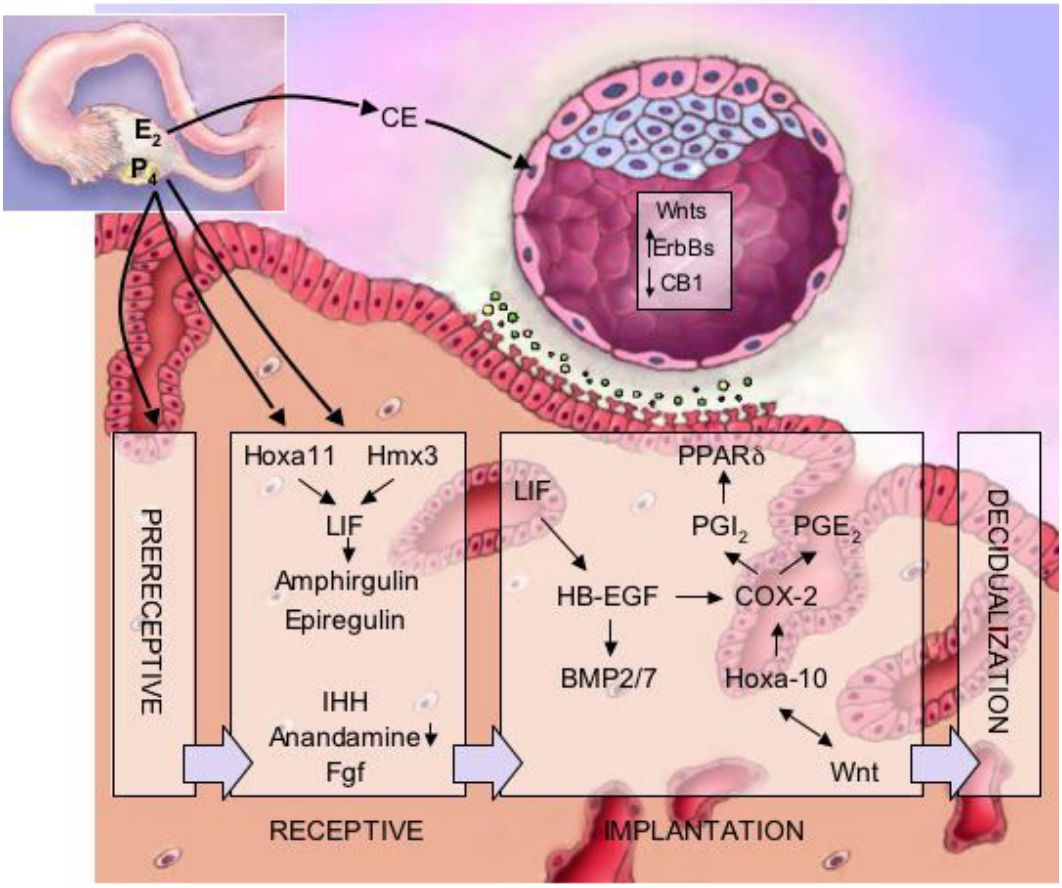
Hoxa-10 and Hoxa-11 are highly expressed in developing genito-urinary tracts and the adult female reproductive tract, suggesting roles in reproductive functions (Taylor *et al.* 2000). In the uteri of pregnant mice, Hoxa-10 and Hoxa-11 are expressed in the stromal cells starting from D2, in response to rising levels of progesterone. Both Hoxa-10 and Hoxa-11 are expressed in stromal cells during the receptive phase of implantation on D4. Mice deficient for Hoxa-10 and Hoxa-11 have been produced by homologous recombination, and show homeotic transformation of uterine structures with abnormal glands and stroma (Taylor *et al.* 1998; Cermik *et al.* 2001). Homozygous females for Hoxa-10 and Hoxa-11 show failure in embryo implantation and decidualization, and severely reduced litter sizes, unrelated to the oviductal transformation. Hoxa-$10^{-/-}$ females, in which initial uterine attachment of blastocyst occurs normally, are infertile due to reduced stromal cell proliferation in response to progesterone, and consequent failure of decidualization. Defective proliferation of stromal cells suggests that Hoxa-10 is involved in the regulation of cell cycle molecules (Bagot *et al*. 2002).

There is a homeobox gene family, unrelated to other larger classes of homeobox genes, called the Hmx family of transcription factors. A member of this family of homeobox transcription factors, Hmx-3, is present in the myometrium during early pregnancy. Strikingly, homozygous null females for Hmx-3 show normal fertilization and preimplantation embryo development, but are infertile due to a failure of blastocyst implantation. The reasons for this implantation failure are still unexplained. Although, it has been demonstrated that Hmx-$3^{-/-}$ females do not express LIF in response to the oestrogen surge.

Msx-1 is an important homeobox gene involved in several developmental processes including cranio-facial and tooth development. Msx-1 is expressed in uterine luminal epithelial cells on D4, which coincides with epithelial differentiation in preparation for implantation. In the evening of D4, during the embryo attachment period, Msx-1 expression is down regulated. Moreover, it has been demonstrated that LIF null homozygous females show a sustained expression of Msx-1 in the evening of D4. In humans, there is a down regulation of MSX1 in receptive human endometrium. Altogether, these results strongly suggest a role for Msx-1 in uterine receptivity and implantation. $Msx1^{-/-}$ females die shortly after birth due to cranio-facial defects. We need conditional KO of Msx-1 to further study its role in embryo implantation (Pavlova *et al*. 1994; Daikoku *et al*. 2004) (Figure 5).

Figure 5: Molecular signalling during implantation in the mouse.

The prereceptive mouse uterus is unresponsive to blastocysts. Ovarian oestrogen (E_2) and progesterone (P_4) transform the prereceptive uterus to a receptive state via a number of uterine factors, whereas uterine-derived catecholoestrogen (CE) activates the blastocyst to an implantation-competent state. During the attachment phase, sequential signalling events within the uterus lead to blastocyst implantation. Stromal cell decidualization follows the attachment phase. (Adapted from Dey *et al*. 2005).



VII. Leukemia inhibitory factor.

Growth factors and cytokines that act in a paracrine or autocrine fashion have been shown to regulate hormone secretion, hormone action, and metabolic homeostasis. One of these cytokines, LIF, has recently been shown to exert striking control of endocrine systems, and to be crucial for uterine preparation and implantation. Although LIF signalling is known to be essential for implantation, its exact role during this process remains elusive.

a. Introduction

Leukemia inhibitory factor (LIF) is a member of the IL-6 family of cytokines that was first identified for its ability to induce differentiation of myeloid leukemia cells into macrophage-like cells (Tomida *et al*. 1984; Moreau *et al*. 1988). LIF is a glycoprotein of 180aa, with a molecular weight ranging from 38 to 67kDa depending on its state of glycosylation (Tomida *et al*. 1984; Moreau *et al*. 1988). LIF is transcribed from a single copy gene, which shares a high degree of sequence similarity between species. A wide range of hormones, cytokines and growth factors are known to induce its expression (Arici *et al*. 1999; Gollner *et al*. 1999). This pleiotropic cytokine can act on multiple tissues and cell types, and exerts a vast variety of functions such as induction of acute-phase protein synthesis, suppression of differentiation in normal embryonic stem cells, stimulation of calcium-release from bones, and establishment of cholinergic phenotypes in rat sympathelic neurons (Kordula *et al*. 1991; Boeuf *et al*. 1997; Palmqvist *et al*. 2002; Kondera-Anasz *et al*. 2004; Davey *et al*. 2007).

In the endometrium, major cell types producing LIF are T cells, uterine natural killer cells (NK cells), epithelial cells and stromal cells (Gearing *et al.* 1987; Kojima *et al*. 1994; Lee *et al*. 2005). Nonetheless, gene targeting, reciprocal embryo transfer, and expression studies have shown the essential role of maternal LIF in uterine preparation and blastocyst attachment in mice (Stewart *et al*. 1992). Moreover, there is evidence that LIF might be one of

the factors that play a key role in human reproduction (Auernhammer and Melmed 2000).

b. LIF signalling.

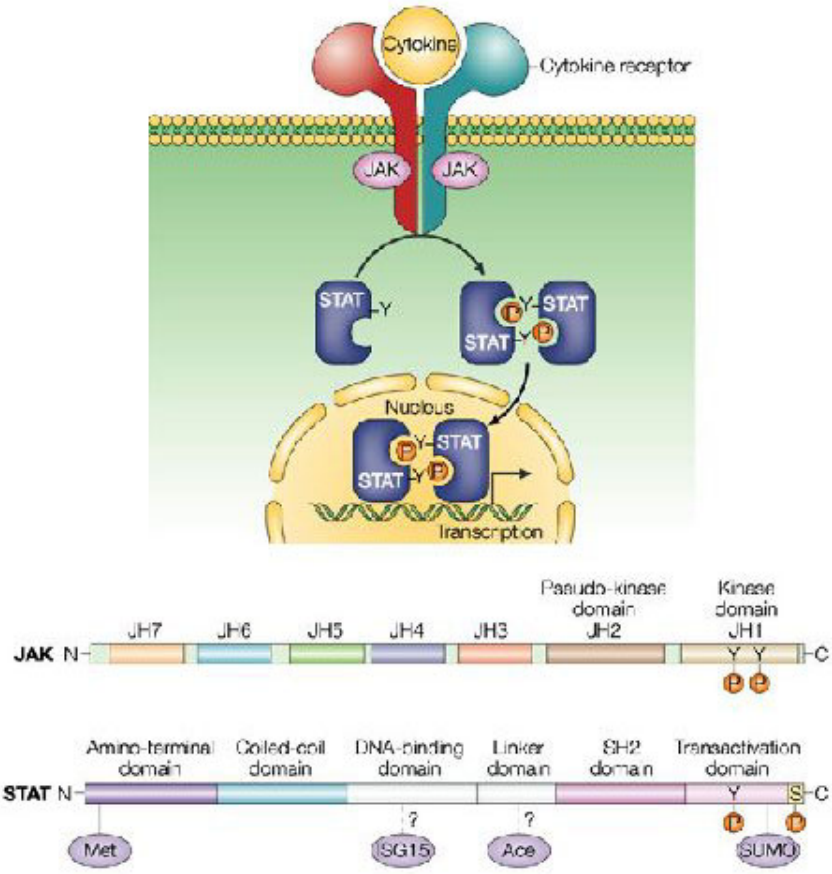
LIF acts through the LIF heterodimeric transmembrane complex consisting of the LIF receptor (LIFR) and the gp130 receptor subunit (Gearing *et al*. 1992; Zhang *et al*. 1994; Smith *et al*. 1998). The LIFR chain binds LIF specifically, but with relatively low affinity. A high affinity receptor complex is formed and activated only when the LIF-LIFR complex recruits the gp130 subunit (Smith *et al*. 1998). In the absence of LIF, Janus kinases 1 and 2 (Jaks), associate with the cytoplasmic domains of gp130 and LIFR. LIF binding to LIFR causes conformational changes and dimerization with gp130, followed by activation of cytoplasmic Jak1, Jak2 and Tyk2 kinase, and LIFR and gp130 phosphorylation (Heinrich *et al*. 2003). Phosphorylated residues on LIFR and gp130 provide specific docking for the SH2-domains of signal transducer and activators of transcription (Stat) proteins, causing receptor association and subsequent phosphorylation of Stat1, Stat3 or Stat5a (Cheng *et al*. 2001). Upon phosphorylation, Stats dimerize and translocate to the nucleus where they act to regulate gene transcription (Pellegrini *et al*. 1997). Suppressors of cytokine signalling or protein inhibitors of activated Stat (PIAS) proteins can inhibit LIF signalling. The Jak-STAT signalling cascade is a common signalling pathway, shared by several types I and type II cytokines receptors (Heinrich *et al*. 2003; Catalano *et al*. 2005) (Figure 6).

The LIF-R and the common gp130 receptor subunit belong to the class I cytokine receptor superfamily (Gearing *et al*. 1991). This family of receptors is characterized by structural and sequence similarities in their extracellular regions containing cytokine-binding domains (CBD), a single transmembrane domain, and an intracellular domain of variable length, lacking endogenous kinase activity (Robinson *et al*. 1994). LIFR and gp130 have been shown to serve as receptors for other cytokines such as Oncostatin M (OSM), Ciliary neurotropic factor (CNTF) and Cardiotrophin-1 (CT-1), while IL-6 receptor requires gp130 (Gearing *et al*. 1992; Ip *et al*. 1992; Liu *et al*. 1992). The

shared activities of the LIFR and gp130 suggest that other members of the class I cytokine receptor superfamily may also be important in regulating blastocyst development and implantation (Boulanger *et al.*, Garcia *et al.* 2004; Tomida *et al.* 2000).

Figure 6: A schematic representation of the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway.

The activation of JAKs after cytokine stimulation results in the phosphorylation of STATs, which then dimerize and translocate to the nucleus to activate gene transcription. The domain structure of JAKs. The domain structure of STATs. (Shuai *et al.* 2003).



c. Uterine LIF, LIFR and gp130 expression

In adult mice, expression of LIF by uterine endometrial glands is regulated throughout the reproductive cycle by oestrogen (Lee DS 2005). In this way, LIF expression coincides with physiologically elevated levels of oestrogens (Chen *et al.,* 2000). During periimplantation in pregnant mice. LIF is first expressed on D1. Then, at D2 and D3, there is a decrease in LIF expression with a peak in endometrial LIF expression occuring on D4, leading to blastocyst attachment (Shen *et al*. 1992; Bhatt *et al*. 1991; Chen *et al*. 2000; Foulida-Nashta *et al*. 2004). After implantation at D5, uterine LIF levels drastically decline. In mammals undergoing embryonic diapause or delayed implantation, such as the mouse, western spotted skunk and mink; uterine LIF transcripts are low to undetectable (Bhatt *et al*. 1991; Arici *et al*. 1995). In the absence of LIF, as occurred in LIF knockout mice, mouse blastocysts enter by default into a state of diapause (Stewart *et al*. 1992).

There is a close link between the oestrogen surge required for implantation and the burst of LIF expression (Ni *et al*. 2002). Injection of oestradiol-17β in females undergoing either naturally or experimentally induced delayed implantation induces LIF expression (Chen *et al*. 2000). Strikingly, LIF can replace the nidatory effect of E_2 in ovariectomized female mice, inducing implantation and decidualization (Chen *et al*. 2000). In fact, at the time of embryo attachment, the principal function of nidatory oestrogen is to induce LIF expression, which in turn initiates the cascade of events associated with the onset of implantation (Chen *et al*. 2000). Gene targeting experiments have demonstrated that homozygous null females for Hmx3 and homozygous null females for Hoxa11 are infertile due to a failure of embryo implantation (Wang *et al*. 1998; Gendron *et al*. 1997). Null mice for these transcription factors fail to upregulate LIF in the glandular epithelium (GE) at the appropriate time during pregnancy (Wang *et al*. 1998; Gendron et al. 1997). This suggests that Hmx3 and Hoxa11 may be involved in the increase

of LIF expression at implantation (Yang *et al*. 1996; Chen *et al*. 2000; Daikoku *et al*. 2004; Lim *et al*. 1999; Hu *et al*. 2007).

The rise in uterine LIF expression occurs early, preceding or coinciding with the time of blastocyst attachment, in several species including mouse, rat, rabbit, pig, mink, and western spotted skunk (Yang *et al*. 1996; Anegon *et al*. 1994; Song *et al*. 1998; Hirzel *et al*. 1999). Thus, the peak in endometrial LIF expression at the time of implantation may be a constant feature of eutherians, since steroid hormone requirements at implantation differ between species (Kimber *et al*. 2005).

In humans, luminal and glandular epithelial cells are the major contributors of endometrial LIF mRNA and protein expression. In normal cycling women, the expression of LIF is cycle-dependent and peaks in the mid and late secretory phases (days 18-28) (Charnock-Jones *et al.* 1994; Chen *et al*. 1995; Arici *et al*. 1995). Mid and late secretory phases correspond to the time-point when blastocyst implantation occurs. LIF expression is maximal when the endometrium is under the control of progesterone (Hambartsoumian *et al*. 1998; Piccinni *et al*. 1998). It has been demonstrated that treatment with the progesterone receptor antagonist mifepristone (RU486) immediately post-ovulation reduces endometrial glandular immunolocalization of LIF (Danielsson *et al*. 1997). This pattern of expression suggests an important role for LIF in human reproduction and that its expression is under the influence of progesterone. Contrarily, in mice, LIF is under the control of oestrogen. In humans, LIF is also secreted by NK cells and Th2 cells, and may be one of the factors that modulate immune tolerance at feto-maternal interface (Piccinni *et al.* 1998). During pregnancy, decidual cells as well as cytotrophoblasts express LIF mRNA and protein (Sharkey *et al.* 1999; Ren *et al*. 1997; King *et al.* 1995). High levels of LIF are encountered during the first trimester and at term, but lower LIF secretion occurs during the second trimester (Sharkey *et al.* 1999). In pregnant women, serum levels of LIF are lower in comparison to non-pregnant women (Aghajanova et al. 2004; Laird et al. 2006; Tuckerman *et al.* 2004).

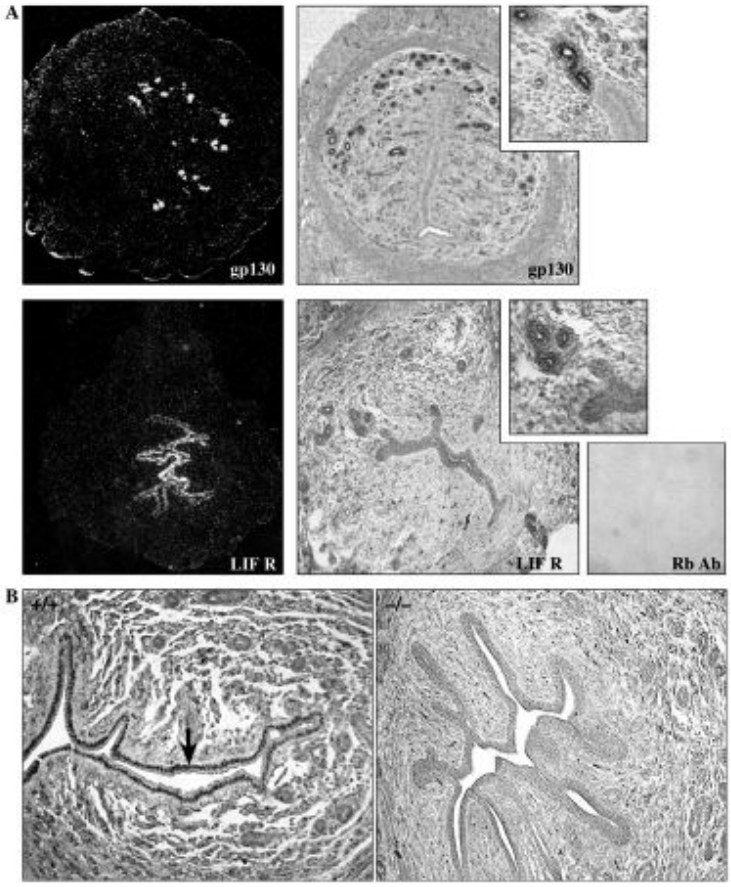
In mice, LIFR and gp130 transcripts are expressed in the LE but not in the stroma. LIFR and gp130 were detected by *in situ* hybridization in decidual tissue, with highest expression evident in deciduas directly surrounding the invading embryo (Chen *et al*. 2001; Cheng *et al*. 2002). gp130 expression in LE is stimulated by oestrogen and progesterone (Ni *et al*. 2002; Song *et al*. 2006) (Figure 7). Concordantly, LIF binding to LIFR and gp130 peaks on D4 of pregnancy, right before blastocyst implantation (Song *et al*. 2006).

In normal cycling women, LIFR and gp130 are expressed in glandular and luminal epithelium only in the mid-secretory phase and not in the proliferative and secretory phases (Gonzalez *et al*. 2003, 2004). In both mice and humans, LIFR transcripts are detected in the stroma surrounding the embryo after decidualization (Yue *et al*. 2002).

Figure 7: Localization of LIFR, gp130, and STAT3 to the luminal (LE) and glandular epithelium (GE).

(A) Left panels: detection of LIFR and gp130 by *in situ* hybridization. Right panels: detection of LIFR and gp130 by immunohistochemistry with antibodies specific to the two receptors components.

(B) Detection of STAT3 by immunohistochemistry. Left panel: Uteri of a wild-type (+/+) pregnant mice on D4. Right panel: Uteri of a $LIF^{-/-}$ pregnant mice on D4 (Cheng *et al.* 2002).



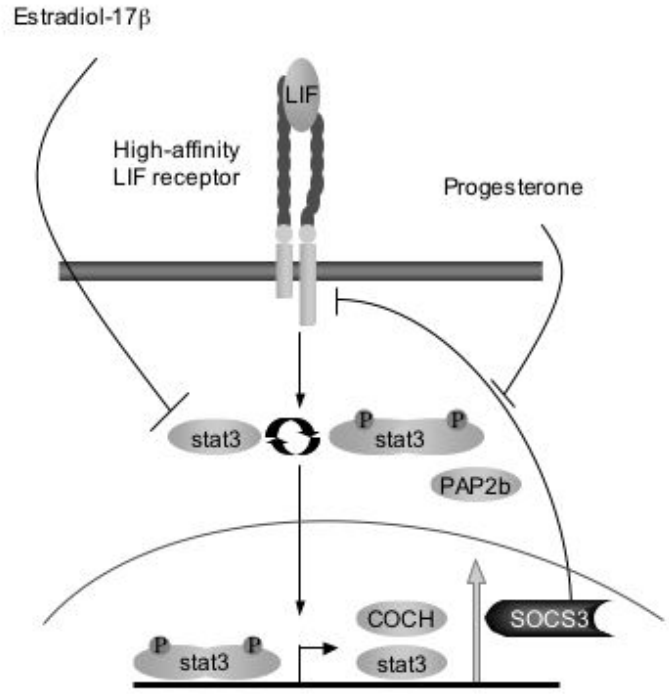
d. Regulation of LIF receptor activity is crucial for controlling uterine receptivity.

Uterine receptivity is controlled by the onset of LIF expression and activation of LIF receptor function. Indeed, responsiveness of the LE to LIF is restricted to D4, despite constant receptor expression throughout the preimplantation period. Moreover, the LE from mice on the different days of pregnancy binds LIF with the same high affinity. Thus, the temporal variation in responsiveness of the LE to LIF is not caused by changes in receptor binding or expression level (Davey *et al*. 2007).

Unphosphorylated Stat3 is present at all times in the LE, suggesting that it is the activation/phosphorylation of Stat3 that is regulated. Thus, activation of Stat3 may regulate the responsiveness of the LE to LIF. The mechanisms regulating Stat activation are many. The suppressor of cytokine activity (SOCS) inhibits the Jak-Stat pathway and is highly expressed on D4 and D5 in the LE. Besides, phosphorylated Stat3 (P-Stat3) may interact with other nuclear factors, such as the oestrogen or progesterone receptors whose expression changes in the LE during implantation and which could modulate Stat3 activity (Ernst *et al.* 2001) (Figure 8).

The LIF signalling pathway can also be regulated by natural antagonists of the receptor soluble LIFR (sLIFR) and soluble gp130 (sgp130). They act as antagonists and prevent the cytokine/receptor complex from initiating signalling and blocking the biological activity of LIF (Hui *et al*. 1998; Zhang *et al*. 1998). Sgp130 and sLIFR are produced by proteolytic cleavage or alternative splicing (Owczarek *et al*. 1996). However, the expression and function of sLIFR and sGp130 during preimplantation remain to be studied (Heinrich *et al*. 2003).

Figure 8: The regulation of LIF signalling the uterine luminal epithelial cells. A current model that summarizes the regulation of LIF and LIFR activity in the uterine luminal epithelial cells. LIF is induced by E_2 and binds to the high affinity LIF receptor. A functional STAT3 dimer enters the nucleus and initiates the expression of target genes and of negative regulators of LIF signalling, such as SOCS3. E_2 can also, by some unknown mechanism, inhibit STAT signalling. This inhibition can be suppressed by the action of P_4 (Cheng *et al*. 2002)



e. Genes regulated by LIF in the luminal epithelium.

LIF has been demonstrated to induce the expression of various factors that are associated with successful implantation, such as IGFBP-3, chorionic gonadotrophin, EGF, COX-2, urokinase type-2 plasminogen activator, and MMP9 (Sherwin *et al*. 2004). These factors regulate blastocyst adhesion, trophoblast invasion and stromal decidualization (Song et al. 2000; Sherwin *et al.* 2004; Harvey *et al*. 1995).

LIF that is locally produced by decidual cells can increase endometrial cell proliferation and differentiation in an autocrine and paracrine manner. It has been demonstrated that LIF has a stimulatory effect on the expression of vasodilator-stimulated phosphoprotein (VASP), and on HLA-G production (Kayisli *et al*. 2002; Bamberger *et al*. 2000). Moreover, endometrial LIF markedly influences the secretion and activation of MMP2 and MMP9, which are secreted by cytotrophoblasts (Inagaki *et al.* 2003; Cork *et al*. 2002). These factors are associated with trophoblastic cell motility and invasiveness (Hondo *et al*. 2005; Cork *et al.* 2005). LIF may act in synergistic manner with other cytokines to regulate the expression and activation of these factors involved in embryo implantation and decidualization.

f. LIF is essential for implantation.

The LIF knockout mouse is infertile due to a failure of implantation. On MF-1 background, the mendelian ratio is not respected and there is a substantial loss of $LIF^{-/-}$ embryos (Stewart *et al*. 1992). Indeed, the number of null embryos was 54-67% that expected (Stewart *et al*. 1992). The multifunctionnality of LIF on different cell types may account for the reduced number of $LIF^{-/-}$ offspring. LIF has a dosage effect on the survival and/or proliferation of stem cells, as $LIF^{-/-}$ mice have dramatically decreased number of stem cells in spleen and bone marrow, while heterozygous animals are intermediate in phenotype (Escary *et al*. 1993; Boeuf *et al.* 1997; Duval *et al*. 2000). In LIF deficient females, up to the onset of implantation, uterine cell

proliferation, hormone levels, gene expression, and embryo development does not differ from those observed in WT females (Fouladi-Nashta *et al.* 2005).

$LIF^{-/-}$ blastocysts are viable but unable to implant unless transplanted into a wild type or $LIF^{+/-}$ pseudo-pregnant recipient (Stewart *et a*l. 1992). Contrarily, wild type or $LIF^{+/-}$ embryos failed to implant in $LIF^{-/-}$ pseudo-pregnant females. Treatment of $LIF^{-/-}$ mutant females with recombinant LIF enabled successful implantation (Stewart *et al*. 1992). These results demonstrate that the failure of embryo implantation in $LIF^{-/-}$ females comes from the maternal side. $LIF^{-/-}$ blastocysts in $LIF^{-/-}$ uteri at D5-7 of pregnancy were appropriately located in the antimesometrial side of the uterine lumen and in tight apposition to the LE, identical to the situation in normal pregnancy (Stewart *et al*. 1992). However, in D7 $LIF^{-/-}$ uteri, there was no morphological sign that implantation had initiated, and blastocysts could be flushed (Figure 9). In wild type pregnant mice, prior to implantation, there is a change in LE cell morphology. LE cells become more cuboidal, polarity becomes less marked, and microvilli are replaced by pinopods (Benty-Ley *et al.* 1999; Salehnia *et al.* 2005). Pinopods are associated with uterine receptivity and carry potential adhesion molecules (Nikas *et al.* 1999). Both Hoxa10 and LIF appear to drive pinopod formation in the LE since after Hoxa10-antisense treatment pinopods do not appear on the LE at the start of the period of receptivity (Bagot *et al*. 2001; Daftary *et al*. 2004). Thus, failure of apical maturation and pinopods formation by LE cells is a major reason for the inability of embryos to firmly and irreversibly interact with the uterus in $LIF^{-/-}$ uteri (Kabir-Salmani *et al*. 2005; Fouladi-Nashta *et al*. 2005). The underlying stroma also retained a fibroblastic morphology and showed no overt signs of decidualizing. Moreover, $LIF^{-/-}$ uteri were not responsive to decidua inducing signals, such as oil injection (Gendron *et al*. 1997). Thus, $LIF^{-/-}$ females are infertile due to a defect in embryonic implantation and endometrial decidualization (Stewart *et al*. 1992; Fouladi-Nashta *et al*. 2005). The requirement of LIF for implantation seems to be similar for other mammals, as passive immunization of ewes and cows against LIF results in a reduced pregnancy rate (Stewart *et al*. 1992; Song *et al*. 2000) (Table 2).

Molecular abnormalities associated with implantation and decidualization failures may be secondary to deficient signalling between the blastocyst and LE. The lack of LIF expression has been shown to disrupt the expression of a number of molecules known to play a crucial role in decidualization, including the secreted protein cochlin, the insulin-growth factor binding protein (IGFBP3), immune response gene 1 (IRG1), amphiregulin, HB-EGF and secreted Frizzled receptor 4 (sFRP4) (Song *et al.* 2000; Sherwin *et al*. 2004; Daikoku *et al.* 2004). Contrarily, COX-2 has been shown to be aberrantly expressed around the blastocyst in $LIF^{-/-}$ uteri (Song *et al*. 2000). Instead of being expressed in the luminal epithelium and the underlying stroma at the site of embryo attachment, COX-2 expression is exclusively detected in the luminal epithelium (Song *et al.* 2000). It is not clear yet whether this deregulation is the effect of implantation failure or if the implantation failure leads to aberrant expression of these genes (Song *et al*. 2000). Decidual markers, such as BMP-2, BMP-4 and tenascin, are not up-regulated in $LIF^{-/-}$ stromal cells (Fouladi-Nashta *et al.* 2005; Sherwin *et al.* 2004). One important phenotype in $LIF^{-/-}$ uteri is the complete absence of OncostatinM (OsM) expression, another member of the IL-6 family of cytokines (Sherwin *et al*. 2004). In human, OsM is reported to reduce proliferation and induce differentiation of utrine stromal cells in the secretory phase (Ogata *et al*. 2000; Ohata *et al.* 2001).

Targeted disruption of Jak1 gene, but not the Jak2 gene, abrogates gp130-mediated signalling and abolishes LIF responsiveness (Rodig *et al.* 1998). These data enlighten the essential role of Jak1 for LIF signalling (Rodig *et al*. 1998). Besides, the group of Sharkey has demonstrated the importance of Stat3 phosphorylation and activation in the process of implantation (Dimitriadis *et al*. 2007; Catalano *et al*. 2005). Injection into the uterine lumen before blastocyst attachment of a Stat3 inhibitor leads to a decrease by 70% of embryo implantation (Catalano *et al*. 2005).

The LIF receptor ß chain (LIF-$R\beta^{-/-}$) mutant mice show normal embryo implantation, but have abnormal and disrupted placentation, which induces poor intrauterine nutrition (Ware *et al.* 1995). Moreover, degeneration of

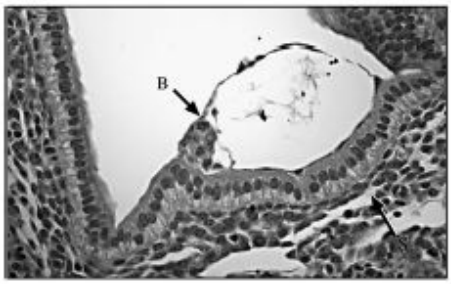
motor neurons and astocytes in LIF-Rß$^{-/-}$ mice leads to foetal death within 24 hours after birth (Ware *et al.* 1995). Depending on the genetic background, gp130$^{-/-}$ mice die *in utero* or shortly after birth due to myocardial hypoplasia and reduced hematopoiesis in the foetal liver (Ernst *et al*. 2001). In LIF-Rß$^{-/-}$ and gp130$^{-/-}$ homozygous females, the cause of perinatal lethality is suspected to be a combination of events due to the pleiotropic nature of the defects (Ware *et al*. 1995; Ernst *et al.* 2001).

Normal implantation of LIFR$^{-/-}$ and gp130$^{-/-}$ embryos in mice has been attributed to partial functional redundancy and overlapping expression patterns between members of the class I cytokine receptor superfamily (Hibi *et al*. 1996). The multiple and tissue-specific activities of cytokines are explained, in part, by activation of multiple signalling cascades. While complete inactivation of LIFR and gp130 result in phenotypes reflecting repression of all associated signalling pathways, the selective deletion of one particular domain of the receptor provides the opportunity to associate a specific signalling cascade with a particular physiological or pathological process (Table 2).

In order to identify the biological responses to LIF specifically mediated by Stats, a mouse carrying a C-terminal mutation in gp130 (gp130$^{\Delta STAT/\Delta STAT}$), which deleted all Stat binding, was generated (Ernst *et al.* 2001). The phenotype of the gp130$^{\Delta STAT/\Delta STAT}$ mutant mice is dramatically different from the phenotype observed in gp130$^{-/-}$ mice. Similar to LIF$^{-/-}$ mice, embryos failed to implant in gp130$^{\Delta STAT/\Delta STAT}$. These results suggest a pivotal role for the STAT signalling associated with the COOH-terminal region of gp130 during blastocyst implantation and decidualization (Ware et al. 1995; Ernst *et al.* 2001).

Figure 9: A $LIF^{-/-}$ blastocyst (B) in apposition to the luminal epithelium in a D7 pregnant $LIF^{-/-}$ uterus.

The luminal epithelium is intact and not undergoing apoptosis with no overt indication of decidualization of the underlying stroma (S) (Cheng *et al.* 2002).



g. LIF in human reproduction.

It is generally assumed that approximately two thirds of the implantation failures are imputable due to inadequate uterine receptivity or to defects in the essential embryo-endometrium dialogue (Horne *et al.* 2005; Lédée-Bataille *et al*. 2005; Daftary *et al*. 2001; Lessey *et al*. 1996). LIF is a key factor in uterine receptivity, indeed, clinical studies have linked some cases of unexplained infertility to an altered pattern of endometrial LIF expression or LIF gene mutation that probably leads to decreased availability or biological activity of LIF in the uterus (Giess *et al*. 1999; Steck *et al*. 2004; Hambartsoumian *et al.* 1998).

Two independent groups have demonstrated that infertile women, in comparison to fertile women had lower or undetectable levels of LIF during the secretory phase and increased LIF production during the proliferative phase (Laird *et al*. 1997; von Wolff *et al.* 2000). High concentrations of LIF during the proliferative phase are associated with disturbed endometrial proliferation in the uterine environments, and are a major cause of reproductive failure (Jasper *et al*. 2007).

Heterozygous point mutations were discovered in infertile women. One mutation was found in the start codon of exon 1 of the LIF gene (Giess *et al*. 1999). Two other mutations were identified in exon 3 positions that correspond to regions of the LIF protein, which are thought to be highly important for the interaction with the LIFR (Steck *et al*. 2004). It is suspected that heterozygosity for LIF mutations, leading to either decreased availability or decreased specific biological activity of the LIF protein, respectively, may be a cause for either failure or decreased efficacy of implantation or thus be responsible for infertility (Giess *et al.* 1999; Steck *et al*. 2004). Infertile women have abnormal level and pattern of cytokine production through the menstrual cycle (Laird *et al*. 2006). For these reasons, recombinant human Lif (r-hLIF) is currently under clinical and preclinical investigation for fertility treatment (Laird *et al*. 2006).

VIII. WNT pathway.

Since the process of embryo implantation shares characteristics of epithelial to mesenchymal transition, it is expected that developmental genes such as FGF, BMP, Nogging, HH and Wnt participate in the embryo-maternal interplay. Indeed, it has been demonstrated that the blastocyst expresses a multitude of Wnt genes at the time of implantation (Mohamed *et al.,* 2006). Moreover, genetic studies in mice have established the crucial role of Wnt signalling at the time of embryo implantation (Nusse et al., 2005). In the following section, I will described the Wnt signalling pathway and its involvment during embryo pre-implantation development.

The Wnt extracellular signalling pathway is an evolutionarily-conserved signal transduction pathways used extensively during animal development, from Hydra to humans. Wnts act as short-range ligands to control multiple aspects of development, including the proliferation, cell fate specification, polarity, and migration of cells (Zecca *et al*. 1996; Olson *et al*. 1994; Goldstein *et al*. 2006; Neth *et al*. 2006). The first Wnt gene, mouse Wnt-1, was discovered in 1982 as a proto-oncogene activated by integration of mouse mammary tumour virus in mammary tumours (Nusse *et al.* 1982). Since then, the functions of the Wnt signalling pathway have been studied in *Drosophila melanogaster*, mouse, zebrafish and *Caenorhabditis elegans*, *Xenopus laevis*, sea urchin, chicken embryos and mammalian cultured cells (Cabrera *et al*. 1987; Rijsewijk *et al*. 1987; McMahon *et al*. 1990; Nusse *et al.* 1992). Wnt signals are extremely pleiotropic in their activity; indeed Wnt malfunction is implicated in various forms of disease, including cancer and degenerative diseases (Cadigan *et al*. 1997).

Wnt proteins constitute a large family of cystein-rich secreted ligands. To date, nineteen Wnt genes have been identified in mammals; they are similar in size, ranging from 39 kDa to 46 kDa (Fradkin *et al*. 1995; Willert *et al*. 2003). All Wnts have a signal sequence followed by a highly conserved distribution of cysteine residues, the spacing of which is highly conserved, suggesting that

Wnt protein folding may depend on the formation of multiple intramolecular disulfide bonds (Mason *et al*. 1992; Zakin *et al.* 1998). Although Wnts are secreted proteins, they are highly insoluble and have therefore been difficult to be purified (Fradkin *et al*. 1995; Schulte *et al*. 2005). As a consequence, very little is known about the structure and biochemical properties of Wnt proteins. Wnt proteins mediate their signal through the interaction with the seven transmembrane Frizzled (Fzd) receptor and the co-receptor LDL-receptor-related protein (LRP) (Yang-Snyder *et al*. 1996; Moon *et al.* 2004; Mikels *et al.* 2006). The Wnt family members can be divided in two distinct classes depending on their ability to induce transformation of the mouse mammary epithelial cell line C57MG (Wong *et al.* 1994). The highly transforming members include Wnt-1, Wnt-3, Wnt-3a, and Wnt-7a. The intermediately transforming or non-transforming members include Wnt-2, Wnt-4, Wnt-5a, Wnt-5b, Wnt-6, Wnt-7b, and Wnt-11 (Shimizu *et al*. 1997). These two groups of Wnts signal via different intracellular pathways that trigger different developmental outcomes (Wong *et al*. 1994). In mammals, Wnt can activate the canonical Wnt/β-catenin pathway, the Calcium pathway or the planar cell polarity pathway. The canonical pathway makes use of β-catenin as a transcription factor. Noncanonical Wnt signalling pathways are less well understood, but appear to function in a β-catenin independent manner to regulate processes such as convergent extension during vertebrate gastrulation, and planar polarity (Strutt *et al*. 1997; Boutros *et al*. 1999; Habas *et al.* 2001; Winter *et al*. 2001; Fanto *et al*. 2004). These non canonical pathways have also been termed the Wnt/Calcium and Wnt/JNK pathways (planar cell polarity pathway) in vertebrates (Figure 10).

Figure 10 : Diversifications of the Wnt signalling pathway.

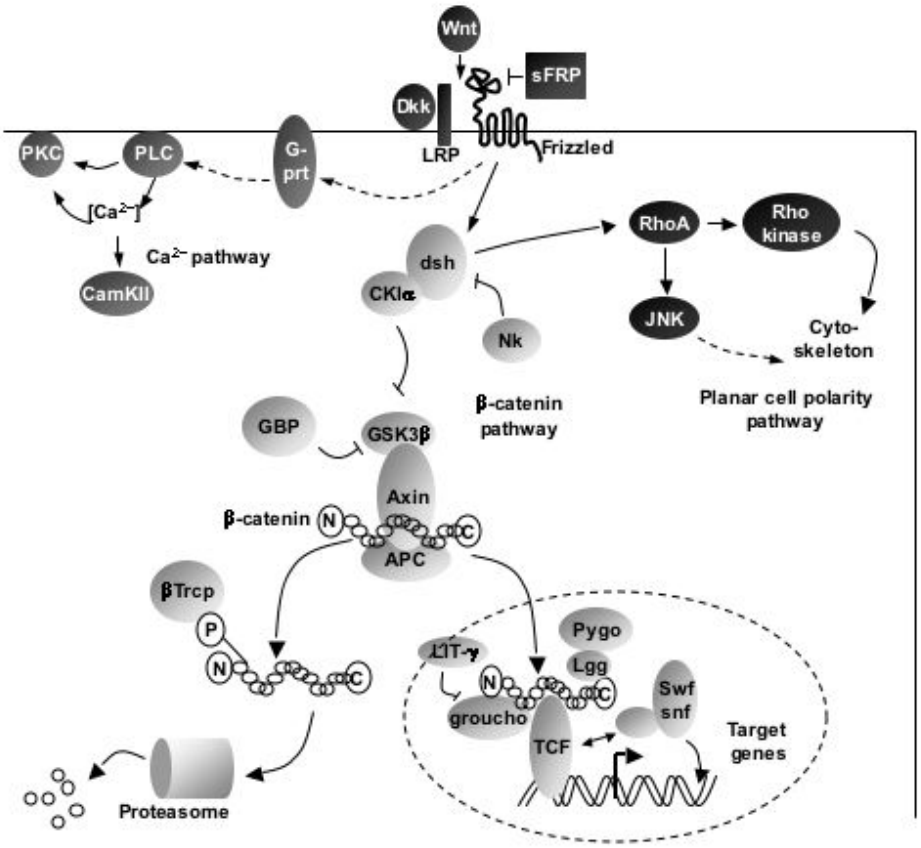
The Canonical Wnt pathway leads to activation of target genes in the nucleus. In this pathway, β-catenin is stabilized by preventing its degradation in proteasomes. Activated Frizzled receptors signal through a conserved mechanism to Dischevelled (Dvl). Casein Kinase Ie (CKIα) is a positive regulator that acts downstream Dvl and regulates β-catenin stability. Axin, in cooperation with the tumor suppressor gene product APC, promotes β-catenin degradation. This involves serine-threonine phosphorylation of the amino-terminus of β-catenin by GSK3β and subsequent ubiquitination. Stabilized β-catenin accumulates in the cytoplasm and is translocated to the nucleus, where it interacts with members of the LEF/TCF family of transcription factors and activates gene expression.

In the planar cell polarity pathway Wnt signalling activates Jun-N-terminal kinase (JNK) and directs asymmetric cytoskeletal organization and coordinated polarization of cell morphology within the plane of epithelial sheets. This pathway branches at the level of Dvl and involves downstream components like the small guanosine triphosphatase Rho and a kinase cascade including JNK kinase and JNK.

The Wnt/calcium pathway leads to the release of intracellular calcium, possibly via a G-protein mediated process. This pathway involves activation of Phospholipase C (PLC), protein kinase C (PKC) and calmodulin-dependent kinase II (CamKII).

(Adapted from Hendriks *et al*. 2002)

Figure 10 (suite): Diversifications of the Wnt signalling pathway.



a. Wnt signalling cascade.

i. Canonical or Wnt/β-catenin pathway.

Signalling through the Wnt/β-catenin pathways alters the stabilization of β-catenin. In the absence of Wnt, β-catenin is targeted for degradation by a multi-protein destruction complex composed of the tumor suppressor protein APC, the *adenomatous polyposis coli* gene product, axin, and glycogen synthase kinase 3β (GSK3β) (Miller *et al*. 1996; Willert *et al.* 1998). This process is triggered by phosphorylation of β-catenin by the serine/threonine kinases, GSK3β and Casein Kinase (Rubinfeld *et al.* 1996). The interaction between these kinases and β-catenin is facilited by the scaffolding proteins Axin and APC (Rubinfeld *et al*. 1993; Su *et al*. 1993; Yost *et al.* 1998; Zeng *et al.* 1997). Phosphorylated β-catenin is recognized by β-transducin repeat containing protein (β-TrCP) and targeted for ubiquitination, and degraded proteasomes (Yost *et al.* 1996; Aberle *et al*. 1997). Binding of Wnt to its receptors Frizzled and its co-receptor LRP5/6, inhibits the kinase activity of the destruction complex, and leads to the stabilization of unphosphorylated β-catenin (Morin *et al*. 1997; Rubinfeld *et al.* 1997). This mechanisms is not completely understood and involves either the recruitment of Axin to the plasma membrane after the phosphorylation of LRP5/6, and/or the action of an axin-binding molecule, Dishevelled (Dvl) (Wharton *et al.* 2003; Itoh *et al*. 2005). The stabilized unphosphorylated β-catenin then accumulates in the cytoplasm and enters the nucleus, where it interacts with the N-terminus of the DNA-binding proteins of the T-cell factor/Lymphoid enhanced factor (Tcf/Lef) family (Behrens *et al*. 1996; Molenaar *et al*. 1996; van de Wetering *et al.* 1997). This transient interaction with β-catenin converts Tcf/Lef factors into transcriptional activators. In the absence of Wnt, Tcf/Lef proteins repress target genes through a direct association with co-repressors such as Groucho (Brannon *et al*. 1999; Willert *et al*. 1998; Nelson *et al.* 2004).

ii. Calcium pathway.

Activation of the Wnt/Calcium pathway involves Wnt binding to a Frizzled receptor, leading to release of intracellular calcium and the activation of enzymes such as CamKII and PKC (Veeman *et al*. 2003; Wang *et al.* 2003; Khul *et al*. 2004). In the Wnt/Calcium pathway, Frizzled appears to act through G-protein and to activate phospholipase C (PLC) and phosphodiesterase (PDE), which lead to increased concentrations of free intracellular calcium and to decreased cyclic guanosine monophosphate (cGMP) (Slusarski *et al*. 1997; Nusse *et al*. 1999; Salinas *et al*. 1999; Khul *et al.* 2000). Elevated free intracellular calcium can activate the phosphatase calcineurin, which leads to dephosphorylation of the transcription factor NF-AT and its accumulation in the nucleus (Kohn *et al*. 2005).

iii. Planar cell polarity pathway.

Among the three signalling pathways activated by Wnt in mammals, the planar cell polarity pathway is the least understood. The planar cell polarity pathway regulates the polarity of cells through regulation of their cytoskeleton (Habas *et al*. 2001; Winter *et al*. 2001). At the biochemical level, the events in the planar cell polarity pathway have not yet been fully characterized. Indeed, it has been demonstrated that in vertebrates, Wnt/polarity pathway signals through the JNK pathway. The Wnt/JNK pathway involves jun N-terminal kinase (JNK) and cytoskeletal rearrangements. Upon binding of Wnt to its receptor Frizzled, there is activation of Dishevelled, JNK and Rho family GTPases, which direct asymmetric cytoskeletal organization and coordinated polarization of cells within the plane of epithelial sheets (Strutt *et al*. 1997; Kato *et al*. 2005; Fanto *et al*. 2004).

b. Components of the Wnt pathway.

i. Frizzled receptors and LRP co-receptors.

Frizzled (Fzd) genes encode seven-pass transmembrane proteins which act as receptors for secreted Wnts (Bhanot *et al*. 1996; Bhat *et al*. 1998). To date nine frizzled proteins have been identified in mouse and humans, and all share the following structural similarities: a signal peptide sequence at the amino terminus, a conserved region of 120 amino acids in the extracellular domain containing 10 invariantly spaced cysteines (called the cysteine rich domain CRD), a seven-pass transmembrane region, in which the transmembrane segments are well conserved, and a cytoplasmic domain with little homology among members of the family (Hsieh *et al*. 1999; Chen *et al*. 2004; Zhang *et al.* 1998). The CRD domain has been shown to be necessary and sufficient for Wnt ligand binding to the surface expressing cells. Fzd are coupled with trimeric G proteins, and the Wnt stimulated pathway is sensitive to inactivation by pertussis toxin. Specificity of the Wnt-Fzd interaction remains largely unknown, particularly in vertebrates, because of the large numbers of Wnts and Fzds (Rulifson *et al*. 2000; Wu *et al*. 2002; Cadigan *et al.* 2006).

Low-density receptor-related protein 5 and 6 (LRP-5 and LRP-6), which are highly homologous, are members of the low-density-lipoprotein (LDL) family of receptors (He *et al*. 2004; Tamai *et al*. 2000). LRP functions as a coreceptor for Wnt signalling leading to the activation of the Wnt/β-catenin pathway. LRP-5 and LRP-6 are type I single-span transmembrane proteins (Kato *et al*. 2002; Liu *et al*. 2003; Cong *et al*. 2004). LRP5/6, which binds to Axin through its intracellular domain, is a key signalling receptor for the β-catenin pathway (Pinson *et al.* 2000; Wehrli *et al*. 2000). The binding between LRP5/6 is directly linked to the stabilization of β-catenin (He *et al.* 2004).

ii. Secreted modulators of the Wnt pathway.

The extracellular antagonists of the Wnt signalling pathway can be divided into two broad classes (Jones *et al*. 2002; Glinka *et al*. 1998; Krupnik *et al.* 1999). The first class, which includes the secreted Frizzled receptor (sFRP), primarily binds to Wnt proteins; the second class comprises the Dickkopf (Dkk) family, which binds to the LRP subunit of the Wnt receptor complex. Both classes of molecules prevent ligand-receptor interactions by different mechanisms (Kawano *et al*. 2003).

Several proteins share homology with the cysteine-rich domain of Frizzled proteins, but lack the transmembrane domain (Rattner *et al*. 1997; Melkonyan *et al.* 1997). These molecules are termed secreted Fzd-related proteins (sFRPs). sFRPs inhibit Wnt signalling by competing with Fzd for Wnt ligands or in a dominant negative fashion by forming a non-signalling complex with Wnt (Uren *et al*. 2000). There are presently five known members of the family in mouse, sFRP1 to sFRP5. sFRP are composed of a cysteine rich domain (CRD) that lie in the N-terminal half of the protein, and of a domain that shares weak sequence similarity with the axon guidance protein netrin (NTR) in the C-terminus (Lin *et al.* 1997; Banyai *et al*. 1999). The CRD shares 30-50% sequence similarity with those of Fzd proteins and includes 10 conserved cysteine residues. Six cysteine residues define this NTR module and several conserved segments of hydrophobic residues (Rattner *et al*. 1997; Bafico *et al*. 1999). It remains unclear whether sFRPs antagonise Wnt signalling by interacting with Wnt ligands through the CRD or the C-terminal domain, which lies outside the C-terminal domain (Kawano *et al.* 2003).

The four members of the Dickkopf (Dkk) family (Dkk-1 to Dkk-4), inhibit Wnt signalling by binding to the LRP5/6 components of the receptor complex (Bafico *et al*. 2001; Glinka *et al*. 1998). Dkks contain two characteristic cysteine-rich domains (Cys-1 and Cys-2) separated by a linker region of variable length. Cys-2, in particular, is highly conserved among all

members of the family and contains 10 conserved cysteine residues (Krupnik *et al*. 1999; Kawano *et al*. 2003).

iii. Dishevelled.

Dishevelled (Dvl) is a phosphoprotein essential for the transduction of the Wnt signalling pathway. In all organisms, the Dvl family comprises three Dvl proteins (Dvl-1, Dvl-2, and Dvl-3). The structure of Dvl family members consist of three highly conserved domains: an amino-terminal DIX domain, a central PDZ domain, and a carboxy DEP domain (Wharton *et al*. 2003). Dvl act as a key transducer of the Wnt signal and act at the plasma membrane or in the cytoplasm. Dvl is differentially targeted to participate in either Wnt/β-catenin or PCP signalling (Logan *et al*. 2004; Veeman *et al*. 2003; Miller *et al.* 1999). Activation of a specific pathway through Dvl depends on its subcellular localization and activation of specific modulator downstream. During the Wnt/β-catenin pathway, once Wnt binds to the Fzd transmembrane recptor and the co-receptor LRP5/6, this is followed by the recruitment of Dvl to the Fzd/LRP complex. Dvl is phosphorylated by casein kinase Iε to form a complex with Frat1 and inhibit GSK3β activity, which lead to stabilization of cytoplasmic β-catenin.

The Planar cell polarity pathway makes use of Dvl to modify the actin cytoskeleton (Wallingford *et al*. 2002; Marlow *et al*. 2002). At the level of Dvl, an independent and parallel pathway leads to the activation of the small GTPases Rho (Habas *et al*. 2001). Rho signalling occurs through the molecule Dishevelled associated activator of morphogenesis 1 (DAAM1) (Habas *et al*. 2001). The Rho pathway leads to the activation of Rho associated kinase, which mediates cytoskeletal reorganization (Kim *et al*. 2005; Marlow *et al*. 2002; Veeman *et al*. 2003)

Table 4: Wnt responsive genes.

(Adapted from www.standford.edu/~rnusse/wmtwindow.htm)

Gene	Organism	Up/Down	Ref
c-Myc	Human	Up	He *et al*. 1998
Cyclin D	Human	Up	Tetsu *et al.* 1999
T cell factor-1	Human	Up	Roose *et al.* 1999
Lymphoid enhancer factor-1	Human	Up	Hovanes *et al.* 2001
Peroxysome proliferators-activated receptor-δ	Human	Up	He *et al.* 1999
c-jun	Human	Up	Mann *et al.* 1999
Fra-1	Human	Up	Mann *et al.* 1999
Urokinase-type plasminogen activator receptor	Human	Up	Mann *et al.* 1999
Matrix metalloproteinase-7	Human	Up	Brabletz *et al.* 1999
Axin-2	Human	Up	Yan *et al.* 2001
Neuronal cell adhesion molecule	Human	Up	Conacci-Sorrell *et al.* 2002
Immunoglobulin transcription factor-2	Human	Up	Kolligs *et al.* 2002
Gastrin	Human	Up	Koh *et al.* 2000
CD44	Human	Up	Wielenga *et al.* 1999
Ephrin B	Human	Up/Down	Batlle *et al.* 2002
Bone morphogenetic protein	Human	Up	Kim *et al.* 2002
Claudin-1	Human	Up	Miwa *et al.* 2002
Survivin	Human	Up	Zhang *et al.* 2001
Vascular endothelial growth factor	Human	Up	Zhang *et al.* 2001
Fibroblast growth factor 18	Human	Up	Shimokawa *et al.* 2003
Hath1	Human	Down	Leow *et al.* 2004
Endothelin	Human	Up	Kim *et al.* 2004
c-myc binding protein	Human	Up	Jung *et al.* 2005
Id2	Human	Up	Rockman *et al.* 2001
Tiam1	Human	?	Malliri *et al.* 2006
Nitric Oxide synthase 2	Human	Up	Du *et al.* 2006
Dickkopf	Human	Up	Niida *et al.* 2004
Fibroblast growth factor 9	Human	Up	Hendrix *et al.* 2006
Fibroblast growth factor 20	Human	?	Chamorro *et al.* 2004
Sox9	Human	Up	Blache *et al.* 2004
Gremlin	Human	Up	Klapholz-Brown *et al.* 2007
SALL4	Human	?	Bohm *et al.* 2006
RANK ligand	Human	Down	Spencer *et al.* 2006
CCN1/Cyr61	Human	Up	Si *et al.* 2006

Gene	Organism	Up/Down	Ref
Pituitary tumor transforming gene	Human	?	Zhou *et al.* 2004
Delta like 1	Human	?	Galceran *et al.* 2004
FoxN1	Human	Up	Balciunaite *et al.* 2002
Matrix metalloproteinase 26	Human	?	Marchenko *et al.* 2002
Nanog	Human	Down	Pereira *et al.* 2006
Frizzled-7	Human	Up	Willert *et al.* 2002
Follistatin	Human	Up	Willert *et al.* 2002
Fibronectin	Human	Up	De Langhe *et al.* 2005
Islet 1	Human	Up	Lin *et al.* 2007
Matrix metalloproteinase-2	Human	?	Wu *et al.* 2007
Connexin 43	Mouse	Up	Van der Heyden *et al.* 1999
Stra6	Mouse	Up	Szeto *et al.* 2001
Wrch-1	Mouse	Up	Tao *et al.* 2001
Twist	Mouse	Up	Howe *et al.* 2003
Stromeolysin	Mouse	Up	Prieve *et al.* 2003
Brachyury	Mouse	Up	Arnold *et al.* 2000
Proglucagon	Mouse	Up	Ni *et al.* 2003
Osteocaclin	Mouse	?	Kahler *et al.* 2003
Cdx-1	Mouse	Up	Pilon *et al.* 2007
Cyclooxygenase-2	Mouse	Up	Howe *et al.* 1999
Neurogenin 1	Mouse	Up	Hirabayashi *et al.* 2004
Insulin growth factor 2	Mouse	Up	Longo *et al.* 2002
Vascular endothelial growth factor-C	Mouse	Up	Longo *et al.* 2002
Interleukin-6	Mouse	?	Longo *et al.* 2002
Cdx4	Mouse	Up	Pilon *et al.* 2006
Secreted frizzled receptor	Mouse	Up	Lesher *et al.* 1998
Epidermal growth factor receptor	Mouse	Up	Tan *et al.* 2005
E-cadherin	Mouse	Down	Jamora *et al.* 2003

c. Wnt pathway in early development.

i. During preimplantation embryo development.

Expression of components of the Wnt pathway in the blastocyst has been analyzed by polymerase chain reaction (PCR) and microarray (Hamanati *et al*. 2004; Kemp *et al*. 2005; Lloyd *et al.* 2003; Mohamed *et al*. 2004; Wang *et al.* 2004). With a genome-wide analysis of periimplantation embryos, Wang QT *et al*. detected transcripts encoding ligands (Wnt-1, Wnt-3, Wnt3-a, and Wnt-7b), receptors (Fzd-2 and Fzd-4), intracellular signal transducers and modifiers (Dvl, APC, Axin), and nuclear effectors such as homolog of Drosophila armadillo, Tcf, and groucho. Amongst these genes, expression levels of Dvl-1, Dvl-2, Groucho, Fzd-4 and Wnt-3a between morula and blastocyst stage was strongly increased. By real-time PCR analysis, Kemp C *et al*. have analyzed the expression level of 19 Wnt genes and their 11 potential antagonists in the mouse blatsocysts. They have detected mRNA encoding for Wnt-1, Wnt-3a, Wnt-4, Wnt-6, Wnt-7b, Wnt-9a and Wnt-10b in the blastocyst, as well as a higher expression level of the secreted antagonist sfrp1 and Dkk1. Further characterization of the expression pattern of these genes was performed by immunohistochemistry. According to their results, Wnt-3a, Wnt-7b and Wnt-10b are expressed throughout the blastocyst. Wnt-6 and Wnt-9a are predominantly detected in the trophectoderm, whereas Wnt-1 and sfrp1 are principally expressed in the inner cell mass. Wnt-1, Wnt-3a and Wnt-7b are known to signal through the canonical pathway, and are expressed in the ICM. Regulated expression of members of the Wnt pathway in the preimplantation blastocyst suggests that the Wnt pathway is activated.

To shed light on factors that regulates Wnt genes expression in the blastocyst. Mohamed O *et al*. analyzed the expression profile of several Wnt genes by semi-quantitative PCR. Wnt-7a, -7b, -10b and -13 showed the same regulation in blastocyst developed *in vitro* or *in vivo*. In contrast, Wnt-5a and Wnt-11 were expressed at lower level in the *in vitro* blastocyst. Embryos collected from ovarectomized mice on D4 showed the same expression pattern

for Wnt-1, Wnt-5a, Wnt-5b and Wnt-6, but failed to upregulate Wnt-11 expression. Administration of estradiol or catecholestrogne to ovarectomized females restored expression of Wnt-11 in the blastocyst. In spite of this, treatment of cultured blastocysts with estradiol or its metabolite could not rescue Wnt-11 expression. These results suggest that estradiol regulates gene expression in the blastocyst via uterine factors. Estradiol initiates expression of LIF in uterine glandular cells, and LIF can replace nidatory oestrogen (Kimber *et al*. 2005). However, LIF could not rescue Wnt-11 expression in cultured blastocyst. Taken together these results suggest that estradiol acts on uterine cells to trigger the expression of diffusible molecules that in turn act on the blastocyst to upregulate Wnt-11 expression. Secreted Wnts may modulate uterine and/or blastocyst differentiation (Mohamed *et al.* 2005). All together, these results strengthen the concepts that embryo implantation relies on reciprocal embryo-uterine cross-talk.

Expression of canonical and non-canonical Wnts in the pre-implantation blastocyst suggests a function for the Wnt pathway in cell fate determination. Kemler R *et al*. activated β-catenin signalling by mating a zona pellucida3-cre (Zp3-cre) transgenic mouse line with a mouse line containing an exon3-floxed β-catenin allele, this transgenic mouse line constitutively express stabilized β-catenin. Under constitutive expression of stabilized β-catenin, ectoderm of mutant embryos changed its fate and exhibited a premature epithelial to mesenchymal transition. However, constitutive expression of the stabilized form of β-catenin in the blastocyst did not alter pre-implantation development or embryo implantation. Pre-implantation mutant embryos did not show nuclear localization of β-catenin, which suggest that Wnt pathway could play a role in preimplantation development by acting through a mechanism independent of the phosphorylation of β-catenin by the GSK3β kinase (Kemler *et al*. 2004).

To better define intracellular Wnt signalling in pre-implantation embryos, the expression pattern and regulation of Dvl has been analyzed in mouse blastocyst. Dvl-1, -2, and -3 are expressed in the blastocyst and show

association with cell membrane (Na *et al*. 2007). Besides, at the blastocyst stage, the amount of dephosphorylated β-catenin increases, whereas the expression of Kaiso, a transcriptional suppressor of Wnt responsive genes, decreases significantly. On D5, Dvl-3 shows a higher expression level in the ICM (Na *et al*. 2007). Consequently, Dvl-3 may be involved in stabilizing cytoplasmic β-catenin that is required in the epiblast for the establishment of anterior-posterior axis and the induction of the mesoderm. Meanwhile, Dvl-2, which has been shown to be responsible for Wnt induced cell motility, is specifically enriched at the cell membrane of the trophectodrem (Na *et al*. 2007). In this way, uterine and embryonic Wnt activity may be responsible for Dvl-2 membrane localization, and may facilitate adhesion and invasion of trophectoderm cells into the uterus (Endo *et al*. 2005).

Overexpression of Dvl-GFP fusion protein in a 4-cell stage balstomere generated high levels of cytoplasmic β-catenin in the blastocyst that showed a more rounded shape and formed less coherent clones (Na *et al*. 2007). However, judging by the absence of lacZ signal in BAT-gal Wnt reporter embryos (Kemler *et al*. 2004; Kemp *et al*. 2005), overexpression of Dvl-GFP does not seem to activate the canonical pathway. Cell adhesion defect observed in these blastocysts may be encountered by the activation of RhoA by Dvls, leading to reorganization of the cytoskeleton (Schlessinger *et al*. 2007). Also, overexpression of Dvl may lead to a perturbation in the balance between cytoplasmic and membrane bound pool of β-catenin that may attenuate cell-cell adhesion at the blastocyst stage (Fanto *et al*. 2004).

ii. During embryo implantation.

Genetic studies in mice have established the crucial role of Wnt signalling at the time of embryo implantation (Nusse *et al.* 2005). In adult mouse and human endometrium, Wnt-7a expression is restricted to the luminal epithelium (Miller *et al*. 1998). Mutant mice lacking Wnt-7a are infertile. These mice show an abnormal uterine patterning and absence of gland formation in the uterus (Parr *et al*. 1998). In homozygous null mice, uterine

epithelial cells become stratified and are not responsive to signals emanating from uterine mesenchyme. At the molecular level, there is no expression of Hoxa-10 and Hoxa-11 in the stroma (Miller *et al*. 1998). Expression of these two homeobox transcription factor is up-regulated in human endometrium during decidualization (Lim *et al.* 1999; Taylor *et al.* 1999; Taylor *et al*. 2000; Hsieh-Li *et al*. 1995). Besides, targeted deletion of Hoxa-10 and Hoxa-11 leads to implantation failure and poor decidualization (Taylor *et al*. 1999; Lim *et al*. 1999). It is believed that Wnt-7a plays a decisive function in epithelial-stromal interaction, and during blastocyst attachment to the luminal epithelium (Carta *et al*. 2004). These observations underline the importance of homeobox-Wnt signalling network in implantation and pregnancy establishment. Further evidence reinforces the importance of the homeobox-Wnt pathway in the uterus during the receptive phase. Aberrant uterine expression of Wnt-4 and sFRP4 is detected in Hoxa-$10^{-/-}$ (Daikoku *et al*. 2004). At the time of embryo implantation, sFRP4 show a temporal and cell-specific expression in the uterus. On D1, sFRP4 expression is restricted to the connective tissue in the myometrial bed, while on D4 expression is detected in a select population of stromal cells throughout the endometrium and in stromal cells lying just underneath the myometrium (Fujita *et al*. 2002). After implantation, expression of SFRP4 is restricted to undifferentiated stromal cells that form a barrier between the decidua and circular smooth muscle cells (Fujita *et al*. 2002). Expression of sFRP4 is not under the control of ovarian progesterone or oestrogen (Fujita *et al.* 2002). This means that other regulatory factors, such as LIF, modulate uterine gene function. Hence, in the absence of LIF, sFRP4 expression is decreased on D4 while Msx-1 expression is up-regulated (Daikoku *et al*. 2004). All together, these results reinforce the idea that the Wnt signalling pathway is involved in epithelial-mesenchymal interactions in the endometrium.

One of the recognized characteristics of the Wnt pathway is its involvement in cell proliferation and survival as well as cell fate. Thus, the expression of Wnt-4 in proliferating stromal cells strongly suggests a role for the Wnt pathway in cell proliferation and differentiation during embryo implantation and decidualization (Hou *et al*. 2004; Daikoku *et al*. 2004). To

support this hypothesis, it has been demonstrated that *in vivo* delivery of adenovirally-expressed sFRP2 in ovariectomized mice considerably prevented oestrogen-dependant uterine growth (Hou *et al*. 2004). Moreover, ovariectomized mice treated for a month with estradiol and lithium displayed atypical endometrial hyperplasia. Compared to control mice, the uterus of mice treated with estradiol and lithium presented a higher number of mitotic and BrdU-labelled cells in the luminal and glandular epithelium as well as in stromal and myometrial cells. Treated uteri also expressed lower levels of ER-α, β-catenin and GSK-3β (Gunin *et al*. 2004). Taken together, these results underline the essential role of the Wnt pathway in cell proliferation and differentiation in the uterus in preparation for embryo implantation.

β-catenin plays a central role in canonical Wnt signalling (Gordon *et al.* 2006). By immunohistochemistry using an antibody specific to active β-catenin, it has been demonstrated that under oestrogen stimulation, active β-catenin accumulates solely in the nucleus of luminal and glandular epithelial cells. After oestrogen activation, nuclear localization of β-catenin was detected in wild type and αERKO females (Hou *et al.* 2004). These results confirmed that activation of the Wnt canonical pathway depends on oestrogen stimulation.

d. In humans.

Due to obvious ethical restrictions, direct analysis of embryo-uterine interaction in humans is limited. For these reasons, analysis of the expression pattern of genes at the time of embryo implantation in human is performed by comparing the different phases of menstrual cycle. The proliferative phase, which is under the control of oestrogen correspond to the non-receptive phase, while the midsecretory phase that is under the influence of progesterone is compared to the receptive phase (Jabbour *et al*. 2006).

Using human endometrial biopsies corresponding to the secretory phase compared with the late proliferative phase of the menstrual cyles,

several groups have analyzed the global gene expression pattern during the window of implantation in humans (Horcajadas *et al*. 2007; Popovici *et al*. 2006; Mirkin *et al*. 2005; Giudice *et al*. 2004; Dominguez *et al.* 2003; Carson *et al*. 2002; Kao *et al*. 2002). Microarray analysis detected constitutive expression of Wnt-2, Wnt-4, Wnt-5a, Wnt-7a, and Wnt-7b in the endometrium throughout cycle. A significant upregulation of Wnt3 was detected in proliferative endometrium (Tulac *et al* 2006). Besides, Fzd-6, LRP-6, and the downstream effectors Dvl-1, Gsk-3β and β-catenin did not show any significant change in their expression level between proliferative and secretory phases (Tulac *et al.* 2006). These results suggest that these components of the Wnt pathway are not under the control of steroid hormones in the endometrium. Meanwhile, there is a dramatic change in expression of secreted antagonists of the Wnt pathway, FrpHE (human secreted Frizzled-related protein 4) and Dkk-1, during the cycle (Kao *et al*. 2005). Tulac *et al*. have detected a 22.2 fold down-regulation of FrpHE and 234.3 fold up-regulation of Dkk-1 in the secretory phase. Guidice *et al*. obtained the same data in the expression level of Dkk1 and FrpHE during the window of implantation. These results were further confirmed by RT-PCR. *In vitro*, the induction of stromal cell decidualization by progesterone after E_2 priming, leads to an increase in the expression of Dkk1, and a decrease in the expression of FrpHE. To corroborate these results, it has been demonstrated that FrpHE is strongly up-regulated in endometrial and breast cancer, which are both oestrogen-dependent (Abu-Jawdeh *et al*. 1999). Inhibitors of the Wnt signalling are known to play a crucial role in the regulation of this pathway. However, the mechanism of action of these two inhibitors on endometrial function remains to be elucidated. Nevertheless, it is believed that the secreted antagonists of the Wnt pathway expressed in the receptive endometrium play a role in restricted Wnt signalling at the implantation site.

IX. Rationale.

Although the blastocyst expresses multiple Wnt genes that are known to activate the Wnt canonical pathway, it has previously been demonstrated that this pathway is not activated in the blastocyst (Mohamed O 2004). Therefore, why does the blastocyst express multiple Wnt genes if the canonical pathway is not activated? The most likely explanation was that Wnts might activate the canonical pathway in the uterus.

We hypothesize that Wnt secreted by the blastocyst or by the receptive uterus during implantation activate via paracrine and/or autocrine action the Wnt/β-catenin pathway in the uterus at the time of embryo implantation, and that activation of this pathway is required for the uterus to become receptive to the attachment of the implantation-competent blastocyst.

In this study, I will demonstrate the crucial role of the Wnt/β-catenin in the initial materno-foetal cross-talk that will lead to proper embryo implantation. I will also elucidate the role and the regulation of the Wnt/β-catenin pathway at the time of embryo implantation in the uterus.

CHAPTER II : MANUSCRIPT I

Uterine Wnt/ β-catenin signalling is required for implantation.

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PREFACE

One of the crucial events during mammalian embryogenesis is the process of implantation. In the mouse, attachment of the free-floating blastocyst to the uterine epithelium occurs on D5 of gestation. For proper implantation to occur, the uterus must become receptive to the attachment of the implantation-competent blastocyst. The process of implantation is highly regulated and requires a tightly regulated dialogue between the embryo and the uterus. However, few signalling pathways have been shown to be specifically activated in the uterus in response to signals secreted by the blastocyst. In this paper, we demonstrate that the Wnt signalling pathway plays a crucial role in mediating embryo-uterine cross talk.

ABSTRACT

Successful implantation relies on precisely orchestrated and reciprocal signalling between the implanting blastocyst and the receptive uterus. We have examined the role of the Wnt/β-catenin signalling pathway during the process of implantation and demonstrate that this pathway is activated during two distinct stages. Wnt/β-catenin signalling is first transiently activated in circular smooth muscle forming a banding pattern of activity within the uterus on early day 4. Subsequently, activation is restricted to the luminal epithelium at the prospective site of implantation. Activation at both sites requires the presence of the blastocyst. Furthemore, inhibition of Wnt/β-catenin signalling interferes with the process of implantation. Our results demonstrate that the Wnt/β-catenin signalling pathway plays a central role in co-ordinating uterine-embryo interactions required for implantation.

INTRODUCTION

A crucial event during mammalian embryonic development is the process of implantation, during which the free-living blastocyst attaches to the uterine endometrium. Successful implantation depends on precisely orchestrated and reciprocal signalling between the implanting blastocyst and the receptive uterus (Paria *et al*.2002). For instance, blastocysts can only implant once they have been activated by uterine factor(s) that are regulated by ovarian steroid hormones (Ma *et al.* 2003). Conversely, expression of several uterine genes are regulated by signal(s) emanating from activated blastocysts (Das *et al.* 1994; Das *et al*. 1997). Multiple signalling pathways have been shown to participate in the implantation process and several cytokines and growth factors have been shown to be expressed in the uterus at the time of implantation and play important roles in this process (Dey *et al*. 2005). However, there is increasing evidence that members of other families of growth factors implicated in embryogenesis may also participate in the implantation process. It has been demonstrated that members of the hedgehog, bone morphogenetic (BMP) and Wnt proteins are expressed in the uterus at the time of implantation (Paria *et al.* 2006). However, the precise role of these three different growth factor signalling pathways in the implantation process has not been determined. Furthermore, the cell types within the uterus which respond to these growth factors are not known. In this study, we set out to determine the role of the canonical Wnt/β-catenin signalling pathway in the implantation process. We demonstrate that this pathway is activated during two distinct stages prior to implantation. Signalling is transiently detected in circular smooth muscle forming a banding pattern of activity. Subsequently, activation is restricted to the luminal epithelium at the prospective site of implantation. Activation at both sites requires the presence of the blastocyst. Furthemore, we show that inhibition of Wnt/β-catenin signalling interferes with the process of implantation.

EXPERIMENTAL PROCEDURES.

Mating and Experimental Manipulation of Transgenic Animals.

The generation and characterization of the TCF/Lef-LacZ transgenic mice have been described in Mohamed *et al.* 2004. Transgenic females were either mated with fertile males or vasectomized males and the day of plug was considered to be day 1. The numbers of transgenic females tested for each experiment is shown in Table 3. TCF/Lef-lacZ transgenic females used to determine whether the embryo was required to activate the Wnt pathway in the uterus were ovariectomized from one side only, or the junction between the oviduct and the uterus was tightly ligated with 6-0 silk thread (Ethicon, Somerville, NJ). Animals were left to recover for 2–3 weeks and then mated with fertile males. The uteri were recovered at the indicated time points and processed for β-galactosidase activity. TCF/Lef-lacZ females used for embryo transfer experiments were mated with vasectomized males, and then 5–10 blastocysts were transferred into each uterine horn on the morning of day 4. Uteri were recovered at the times indicated in the *Results and Discussion* and processed for β-galactosidase activity. TCF/Lef-lacZ females used for artificial decidualization were mated with vasectomized males, and 10 μl of sesame oil was injected into each horn in the morning of day 4. Uteri were recovered from these animals and processed for β-galactosidase activity. TCF/Lef-LacZ transgenic females used for the estrogen dependency study were ovariectomized on the morning of day 4 of pregnancy between 7:00 a.m. and 9:00 a.m. Immediately after surgery, mice received a s.c. injection of only progesterone (2 mg per mouse) or a s.c. injection of progesterone and β-estradiol (50 ng per mouse). Uteri were recovered and processed for β-galactosidase activity.

Injection of Wnts and Secreted Frizzled-Related Proteins (sFRPs).

Parental L cells, Wnt5a cells, and Wnt7a cells were generated as follows. The cDNA fragments of Wnt5a and Wnt7a were provided by Jan Kitajewski (University of North Carolina, Chapel Hill) (Araki *et al*. 2003). These cDNA fragments were subcloned into the retrovirus vector, pHAN(puro). To prepare ecotropic retrovirus, Phoenix-ECO packaging cells

were transfected with retrovirus vectors by using Lipofectamine (Invitrogen). Viral supernatants were harvested 30 h after transfection and used to infect L cells (American Type Culture Collection CRL-2648) in the presence of 8 μg/ml polybrene (Sigma). Infected cells were selected 24 h after infection with 5 μg/ml puromycin (Sigma). Selected Wnt-expressing cells were grown in 100-mm dishes, washed twice with PBS, and lysed in 0.5 ml radioimmunoprecipitation assay buffer (50 mM Tris·HCl, pH 7.5/150 mM NaCl/0.5% Nonidet P-40/0.1% deoxycholate) containing protease inhibitor mixture (Roche). Cell extracts were collected and spun in a microcentrifuge at 15,000 × *g* for 5 min. Total proteins (5 μg) were separated by 10% SDS/PAGE and transferred to Immobilon-P (Millipore). The membranes were probed with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies at 1:5,000 (Bio-Rad) and developed by using ECL Plus (Amersham Pharmacia Biosciences). Membranes were exposed to BioMax film (Kodak). The primary antibody used in this work was anti-HA at a 1:5,000 ratio (HA7 clone, Sigma).

Parental L cells, Wnt5a cells, and Wnt7a cells were cultured to 80–90% confluency and the conditioned medium was harvested and concentrated 2- to 3-fold by using a centricon column. Five μl of parental and Wnt-conditioned medium was injected in each uterine horn in the morning of day 4. Uteri were isolated on day 5 and stained for β-galactosidase activity. To coat Cytodex-3 beads (Amersham Pharmacia Biotech) with parental or Wnt-expressing L cells, cells were cultured in the presence of beads according to the manufacturer's specifications. Fully coated beads were collected and washed in PBS, and three to five cell-coated beads were injected in one uterine horn as done for the conditioned medium.

Purified human sFRP1 and mouse sFRP2 were purchased from R & D Systems. Isolated blastocysts along with 500 ng of BSA, sFRP1, or sFRP2 were transferred into pseudopregnant females on day 4, and the uteri were isolated on day 6. Stably transfected sFRP2-expressing ES cells were generated and characterized in Austin Smith's laboratory (Institute for Stem Cell Research, University of Edinburgh, Edinburgh) (Shimizu *et al.* 1997).

sFRP2-conditioned medium was collected in the same manner as Wnt-conditioned medium. Embryoid bodies were generated by culturing the ES cells in the absence of leukemia inhibitory factor according to standard procedures. Five to eight embryoid bodies of sizes similar to a blastocyst were transferred in each uterine horn along with 5 μl of conditioned medium.

Detection of β-Galactosidase Activity.

Uteri were dissected in PBS, pH 7.3, rinsed in 100 mM sodium phosphate, pH 7.3, and then fixed in 0.2% glutaraldehyde/2 mM MgCl2/5 mM EGTA/100 mM sodium phosphate, pH 7.3, for 10 min at room temperature. Uteri were then washed three times in wash buffer (0.02% Nonidet P-40/0.01%deoxycholate/2 mM MgCl2/100 mM sodium phosphate, pH 7.3) for 15 min each at room temperature. To reveal β-galactosidase activity, uteri were incubated in 1 mg/ml X-gal/5 mM K3Fe(CN)6/5 mM K4Fe(CN)6/0.02% Nonidet P-40/0.01% deoxycholate/2 mM MgCl2/100 mM sodium phosphate, pH 7.3, overnight at 37°C. The uteri were then rinsed with wash buffer and PBS and postfixed overnight in 4% paraformaldehyde in PBS at 4°C.

Preparation of Progesterone and Estrogen.

Progesterone (4-pregnen-3, 20-dione) was purchased from Steraloids (Newport, RI) (catalog no. Q2600-000) and was dissolved in sesame oil at a concentration of 20 mg/ml. Each mouse received a s.c. dose of 100 μl (2 mg per mouse). Estrogen [1,3,5(10)-estratriene-3, 17 β-diol] (β-estradiol) was purchased from Sigma (catalog no. E-8875) and was dissolved in sesame oil at a concentration of 500 ng/ml. Each mouse received a s.c. dose of 100 μl (50 ng per mouse).

RESULTS AND DISCUSSION.

To define the sites of active Wnt/β-catenin signaling in the uterus, we used a TCF/Lef-LacZ transgenic mouse reporter line that faithfully monitors activation of this pathway (Mohamed *et al*. 2004). In the uteri of nonpregnant transgenic females, Wnt/β-catenin signaling activity was found to be constitutively active in the oviducts at all stages of the estrous cycle, but no signaling was detectable in the uterus of nonpregnant females (Fig. 1*A*). This observation allowed us to determine whether this pathway was activated during the implantation period. Analysis of uteri isolated during the periimplantation period demonstrated a dynamic pattern of signaling activity. Activity was first detected at ≈1500 hours on day 4 in groups of cells that formed a series of bands visible in whole-mount β-galactosidase uterine preparations. The bands of Wnt/β-catenin signaling activity first appeared in the uterine region closest to the ovary (Fig. 1B), and, by late day 4, five to seven bands of activity were evenly distributed along each uterine horn (Fig. 1C). The number of bands present in each uterus was found not to correlate with the number of blastocysts because a greater number of bands were observed compared with the number of embryos present in each uterus. At this stage, activity was restricted to the circular smooth muscle cells of the myometrium on the antimesometrial side of the uterus, although weak activity was sometimes detected on the mesometrial side (Fig. 1 D and *E*). By the morning of day 5, activity was no longer detected in this region. Recently, the Wnt antagonist sFRP4 was shown to be expressed in this region at this time, which may account for silencing of this pathway (Daikoku *et al.* 2004).

At 1200 hours on day 5, Wnt/β-catenin signaling became active at specific sites within the uterus (Fig. 2*A*). At this stage, signaling activity was only detected on the antimesometrial side of the luminal epithelium directly apposed to the implanting blastocyst (Fig. 2 B and *C*). By 1600 hours, activity had intensified and spread to the mesometrial side (Fig. 2 D and *E*). Importantly, analysis of serial sections confirmed that endometrial Wnt/β-catenin activity was observed only where a blastocyst was present (Figure 6 A–*C*,). Wnt/β-catenin signaling was maintained in the uterine epithelium

throughout day 5 but was no longer detected by day 6 (data not shown). Thus, Wnt/β-catenin signaling was transiently activated in the regions of the uterine endometrium directly apposed to the blastocyst at the time of implantation.

On the morning of day 5, weak Wnt/β-catenin signaling was also detected in the endometrial glands (Fig. 2*B* and Fig. 6, speckled staining). However, unlike Wnt/β-catenin signaling activity in the luminal epithelium, which was restricted to the site where the embryo was present, activity in the endometrial glands was detected throughout the uterus. Furthermore, in uteri that contained embryos in only one of the uterine horns, Wnt/β-catenin signaling activity was also present in the endometrial glands of the uterine horn that did not contain embryos (Fig. 2G). However, in the uteri of mice mated with vasectomized males, which contained no embryos, no Wnt/β-catenin signaling activity was detected in the endometrial glands (Fig. 2F). These results demonstrate that activation of Wnt/β-catenin signaling activity in the endometrial glands is not directly activated by the blastocysts but is rather a global response of the uterus because of the presence of embryos.

Because activation of uterine Wnt/β-catenin signaling is only observed where blastocysts were present, we wanted to determine whether activation of this pathway depended on the presence of the embryo. We first mated transgenic females with vasectomized males and examined the uteri of these pseudopregnant females. No activity was detected on either day 4 or day 5 (Fig. 2*F* and data not shown). Next, we prevented the entry of embryos into one uterine horn by either removing one ovary while keeping the other intact or by tightly ligating the junction between the oviduct and the uterus on one side, and, 2 weeks after surgery, we mated those females with fertile males. Active Wnt/β-catenin signaling was detected only on the side in which the ovary was kept intact or the uterotubal junction were kept intact (Table 3 and Fig. 2G). These results clearly demonstrate that activation of this pathway in the uterus requires the presence of the embryo. To determine whether activation of this pathway in the uterus was a result of a decidualization response, we induced artificial decidualization in pseudopregnant transgenic females by injecting sesame oil into each uterine horn on the morning of day

4. No Wnt/β-catenin signaling activity was detected on the evening of day 4 or throughout day 5, although deciduas were visible by day 6 (Table 3 and data not shown). Thus, activation of the Wnt/β-catenin signaling pathway in the uterus is likely triggered by signals emanating from the embryo before implantation.

In mice, implantation depends on an ovarian estrogen surge that occurs during the morning of day 4 (McCormack *et al*. 1974 ; Yoshinaga *et al*. 1966). To evaluate whether activation of Wnt/β-catenin signaling was regulated by estrogen, we removed both ovaries from pregnant transgenic females before the onset of the estrogen surge and administered animals with either progesterone alone or a mix of progesterone and estrogen. After this treatment, activation of this pathway was not detected in the uteri of females that received progesterone alone, whereas normal activation was detected in females that received progesterone and estrogen (Table 3 and Fig. 2 *H* and *I*). Thus, estrogen injection, which is required to reactivate dormant blastocysts in utero and the resumption of implantation (McCormack *et al*. 1974 ; Yoshinaga *et al*. 1966), is also required for blastocysts to trigger Wnt/β-catenin signaling in the uterine endometrium.

We next determined whether, independently of the presence of the blastocyst, Wnt proteins could activate this pathway in the uterus. Two members of the Wnt family of proteins were selected for this study, Wnt5a and Wnt7a. Genes coding for both Wnt molecules are expressed in the blastocyst at the time of implantation (Mohamed *et al.* 2004). Wnt5a is known not to signal through the canonical Wnt/β-catenin pathway, whereas Wnt 7a does signal through this pathway (Sheldahl *et al.* 1999 ; Kuhl *et al.* 2000 ; Caricasole *et al.* 2003). Freshly collected conditioned medium from parental L cells or L cells expressing Wnt5a or Wnt7a was injected in the uteri of pseudopregnant transgenic females on the morning of day 4. On day 5, the uteri were isolated and stained for β-galactosidase activity. No Wnt/β-catenin signaling activity was detected in uteri injected with conditioned medium obtained from the parental cells or from the cells expressing Wnt5a (Fig. 3*A* and data not shown). These results demonstrate that the parental cells do not

secrete any molecules capable of activating the Wnt/β-catenin signaling pathway in the uterus. Furthermore, Wnt5a, which has been shown to activate the noncanonical Wnt pathway (Torres *et al*. 1996 ; Ishitani *et al*. 2003; Topol *et al*. 2003) and antagonizes the canonical Wnt/β-catenin activity in Xenopus embryos and mammalian cells (Torres *et al*. 1996 ; Ishitani *et al*. 2003; Topol *et al*. 2003), was incapable of activating the Wnt/β-catenin signaling pathway in the uterus (Fig. 3*A*). In contrast, robust activation of the Wnt/β-catenin signaling pathway was observed when Wnt7a-conditioned medium was injected in one or both uterine horns (Fig. 3 B and *C*). Furthermore, activation of Wnt/β-catenin signaling was detected throughout the uterine horns, demonstrating that the uterus is capable of activating this pathway along its entire length and not just in spatially restricted domains. These results demonstrate that Wnt proteins known to activate the canonical Wnt pathway can selectively activate Wnt/β-catenin signaling in the uterus.

We have previously demonstrated that the blastocyst expresses multiple Wnt genes (Mohamed *et al*. 2004), suggesting that Wnt proteins secreted by the blastocyst may be responsible for activating Wnt/β-catenin signaling in the uterus. We therefore tested whether a small group of Wnt-expressing cells of the size of a blastocyst were capable of activating Wnt/β-catenin signaling in the uterus. To achieve this, Cytodex-3 beads of sizes similar to the blastocyst (Fig. 3*D*) were coated with either Wnt5a- or Wnt7a-expressing cells (Fig. 3E), and three to five cell-coated beads were injected in each uterine horn of pseudopregnant transgenic females on the morning of day 4. On day 5, the uteri were isolated and stained for β-galactosidase activity. No Wnt/β-catenin signaling activity was detected in uteri injected with beads coated with Wnt5a-expressing cells (Fig. 3F). In contrast, in the uteri injected with beads coated with Wnt7a-expressing cells, Wnt/β-catenin signaling activity was detected in discrete bands along the uterine horn (Fig. 3G), which mimics the banding pattern observed in naturally mated females (Fig. 2A). These results demonstrate that a small group of cells expressing Wnt7a is sufficient to activate Wnt/β-catenin signaling activity in discrete bands along the uterus, supporting the possibility that Wnts secreted by the blastocyst activate Wnt/β-catenin signaling in the endometrium.

Having demonstrated that Wnts were sufficient to activate signaling in the uterus, we next determined whether activation of the Wnt/β-catenin signaling pathway before implantation was required for successful implantation. To achieve this, we took advantage of the ability of sFRP proteins to physically interact with Wnt proteins and thereby prevent the activation of the Wnt/β-catenin signaling pathway (Jones *et al.* 2002 ; Kawano *et al*. 2003). Eight to 10 blastocysts were transferred along with BSA, purified sFRP1 protein, or purified sFRP2 protein in each uterine horn of wild-type pseudopregnant females. Embryonic development was allowed to proceed until day 6, when recipient females were killed and the ability of the transferred blastocysts to implant was assessed (Table 1). Transfer of blastocysts with BSA or sFRP1 had no significant effect on embryo implantation (Table 1 and Fig. 4 *A* and *B*). However, transfer of blastocysts along with purified sFRP2 resulted in a significant decrease in the frequency of implantation (35%) (Table 1 and Fig. 4C). Furthermore, although embryos did implant and formed deciduas, sites of implantation were always located close to the junction of the two uterine horns and no implantation occurred in the more proximal region of the uterus as observed with blastocysts incubated with either BSA or sFRP1. Because the embryos and sFRP2 were transferred in the proximal region of the uterine horn and that implantation occurred only in the distal region raises the possibility that these embryos may have implanted in regions where sFRP2 protein concentrations were low.

We next tested whether sFRP2 could inhibit normal implantation of blastocysts in naturally mated wild-type females. Conditioned media from wild-type ES cells or ES cells overexpressing sFRP2 were collected. In addition, to favor continued expression of sFRP2 in the uterus, embryoid bodies were generated from both ES cell lines. Conditioned medium and embryoid bodies were injected together into one uterine horn of naturally mated wild-type females on the morning of day 4. The second uterine horn was left intact as a control. Injection of four to five wild-type embryoid bodies along with conditioned medium from wild-type ES cells had no effect on implantation, because a similar number of implanted embryos were observed

as compared with control (Table 2). In contrast, injection of four to five embryoid bodies overexpressing sFRP2 along with sFRP2-conditioned medium resulted in a 57% decrease in the implantation rate (Table 2). Although embryos were found in each decidua, the embryos that had implanted on the treated side consistently showed a developmental delay of ≈1 day as compared with embryos implanted on the control untreated side (Fig. 4*E*). Interestingly, injection of sFRP2 on the evening of day 4, when activation of this pathway had already occurred, had no effect on blastocyst implantation and embryonic development (data not shown). This result suggests that the growth retardation observed in embryos treated with sFRP2 on the morning of day 4 is likely due to delayed implantation of these embryos rather than a growth retardation effect caused by sFRP2.

We have demonstrated that the Wnt/β-catenin signaling pathway is active in two distinct regions of the uterus during the periimplantation period. Wnt/β-catenin signaling is first transiently activated in circular smooth muscle forming a banding pattern of activity within the uterus on early day 4. Subsequently, activation is restricted to the luminal epithelium at the prospective site of implantation. We have shown that the embryo is required for activation of Wnt/β-catenin signaling in the luminal epithelium, demonstrating that this pathway is active in embryo–uterus communication. Finally, we have shown that inhibiting Wnt signaling at the time of implantation severely reduces the rate of embryo implantation, demonstrating an essential role of the Wnt pathway in the process of implantation.

Our results are consistent with the model shown in Fig. 5. On the morning of day 4, shortly after the estrogen surge, the blastocyst emits a signal that results in the activation of the Wnt/β-catenin signaling pathway in discrete groups of cells within the circular smooth muscle that form bands along the uterus. Because of the substantial distance between the location of the embryo and the domain of activation of the pathway, it is unlikely that this activation is mediated by Wnts secreted by the blastocyst. A more likely explanation would be that the blastocyst emits a signal(s) that directly or indirectly activates the Wnt/β-catenin signaling pathway in circular smooth muscle cells.

These results demonstrate that the uterus exhibits regionalization along it proximodistal axis before implantation and, more intriguingly, that activation of the Wnt/β-catenin pathway occurs at regularly spaced intervals along the uterus. The function of Wnt/β-catenin signaling in these cells is currently not known. Because circular smooth muscle cells have been suggested to play a role in proper spacing of the embryos (Crane *et al.* 1991) and because activity occurs at regularly spaced intervals, it is possible that Wnt/β-catenin signaling in these cells may be involved in regulating embryo spacing.

On late day 4 to early day 5, the blastocyst activates the Wnt/β-catenin signaling pathway in the luminal epithelium of the uterus. Activation first occurs on the antimesometrial side of the uterus, where the embryo will implant, and is later detected throughout the luminal epithelium. By late day 5, Wnt/β-catenin signaling is no longer active in the uterus. Whether Wnt proteins secreted by the blastocyst or other blastocyst-secreted factors are responsible for this activation is not known. However, several lines of evidence suggest that blastocyst-secreted Wnt proteins may be involved. We have previously shown that the blastocyst expresses multiple Wnt genes before implantation (Mohamed *et al*. 2004). Furthermore, beads coated with Wnt-expressing cells are sufficient to activate Wnt/β-catenin signaling in a fashion similar to the blastocyst, and injection of sFRP2 inhibits activation of this pathway in the uterus. Taken together, these findings strongly suggest that Wnt secreted by the blastocyst activates the Wnt/β-catenin signaling pathway in the luminal epithelium of the uterus. Interestingly, none of the individual Wnt gene mutations have resulted in preimplantation lethality because of implantation failure. Because the blastocyst has been shown to express multiple Wnt genes at the time of embryo implantation (Mohamed *et al*. 2004), it is possible that several of these Wnt genes may have redundant functions and are able to activate the Wnt/β-catenin signaling pathway in the luminal epithelium of the uterus. Thus, the inactivation of multiple Wnt genes expressed by the blastocyst would be required to affect implantation. Although our results support the idea that the blastocyst-secreted Wnt proteins directly activate Wnt/β-catenin signaling, we cannot exclude the possibility that the blastocyst secretes a factor that activates the expression of Wnt genes in the

luminal epithelium, which, in an autocrine fashion, activates signaling.

Inhibiting Wnt/β-catenin signaling in the uterus by the addition of sFRP2 significantly decreases implantation, demonstrating the importance of this signaling pathway during this process. That Wnt/β-catenin signaling is only active on days 4 and 5 and is not active during the decidualization process suggests that it plays a role early during implantation. Activation of the Wnt/β-catenin signaling pathway in the luminal epithelium most probably results in modulating the expression of target genes, which allows successful implantation to take place. Indeed, the expression of several genes, such as HB-EGF and COX-2, have been shown to be activated in the endometrium by the presence of the blastocyst (Dey *et al*. 2004). The signaling pathway as well as the mechanism of activation of these genes are still unknown. The Wnt/β-catenin signaling is an excellent candidate pathway to be involved in the activation of some of these genes. Supporting this view, it has been demonstrated that Wnt/β-catenin is involved in the activation of the COX-2 promoter (Araki *et al*. 2003).

Although embryo–uterus cross-talk is known to play an essential role in facilitating implantation, few signaling pathways specifically activated in the uterus in response to signals secreted by the blastocyst have previously been identified. Our results demonstrate that the blastocyst activates Wnt-dependent β-catenin signaling in the uterine endometrium and that activation of this pathway is required for implantation, strongly suggesting that this paracrine signaling mechanism plays a central role in coordinating uterus–embryo interactions required for implantation.

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Figure 1: The Wnt/β-catenin signalling pathway is activated in the circular smooth muscle cells of the myometrium.

(A) Wholemount β-galactosidase staining of a uterus from a non-pregnant transgenic female. (B) Wholemount β-galactosidase staining of a uterus from a TCF/Lef-lacZ transgenic female at mid-day 4. (C) Wholemount β-galactosidase staining of a uterus from a TCF/Lef-lacZ transgenic female on the evening of day 4. (D) Sagittal and (E) transverse section of the uterus in (C) showing activity in the circular smooth muscle cells (csm).

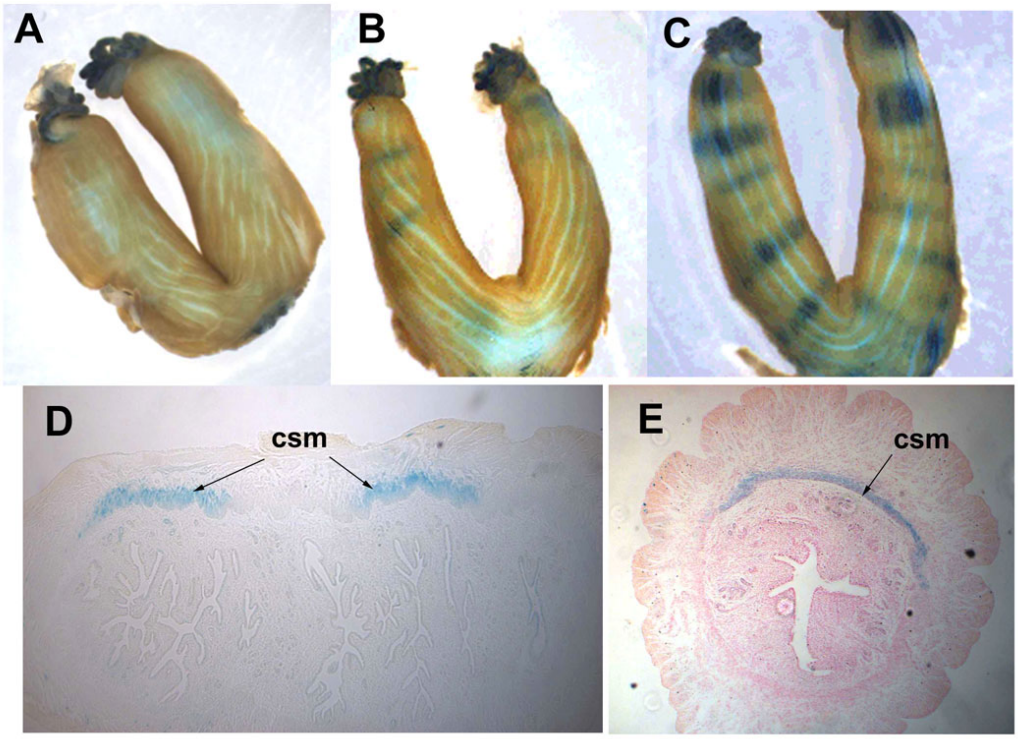


Figure 2: Activation of the Wnt/β-catenin signalling pathway in the lumenal epithelium requires the presence of the embryo and is oestrogen dependent.
(A) Wholemount β-galactosidase staining of a TCF/Lef-lacZ transgenic uterus at 12:00 pm on day 5 showing restricted activity within the uterus. (B). Transverse section from a uterus at 12:00 pm on day 5, showing activation of the reporter gene in the luminal epithelium (le) adjacent to the blastocyst (bl) only on the anti mesometrial side (ams) of the uterus and not on the mesometrial side (ms). (C) Higher magnification of (B). (D). Transverse section from a TCF/Lef-lacZ uterus at 16:00 pm on day 5 demonstrating that activity has spread throughout the luminal epithelium by this stage. (E) Higher magnification of (D). (F) Wholemount β-galactosidase staining of a uterus from a TCF/Lef-lacZ transgenic female mated with a vasectomized male. (G) Wholemount β-galactosidase staining of a uterus from a TCF/Lef-lacZ transgenic female where the junction between the oviduct and the uterus was tightly ligated on one side. (H) Uterus from a pregnant TCF/Lef-lacZ transgenic female in which both ovaries were removed on the morning of day 4, prior to the oestrogen surge, and injected with progesterone alone or (I) both progesterone and oestrogen.

Figure 2: Activation of the Wnt/β-catenin signalling pathway in the lumenal epithelium requires the presence of the embryo and is oestrogen dependent.

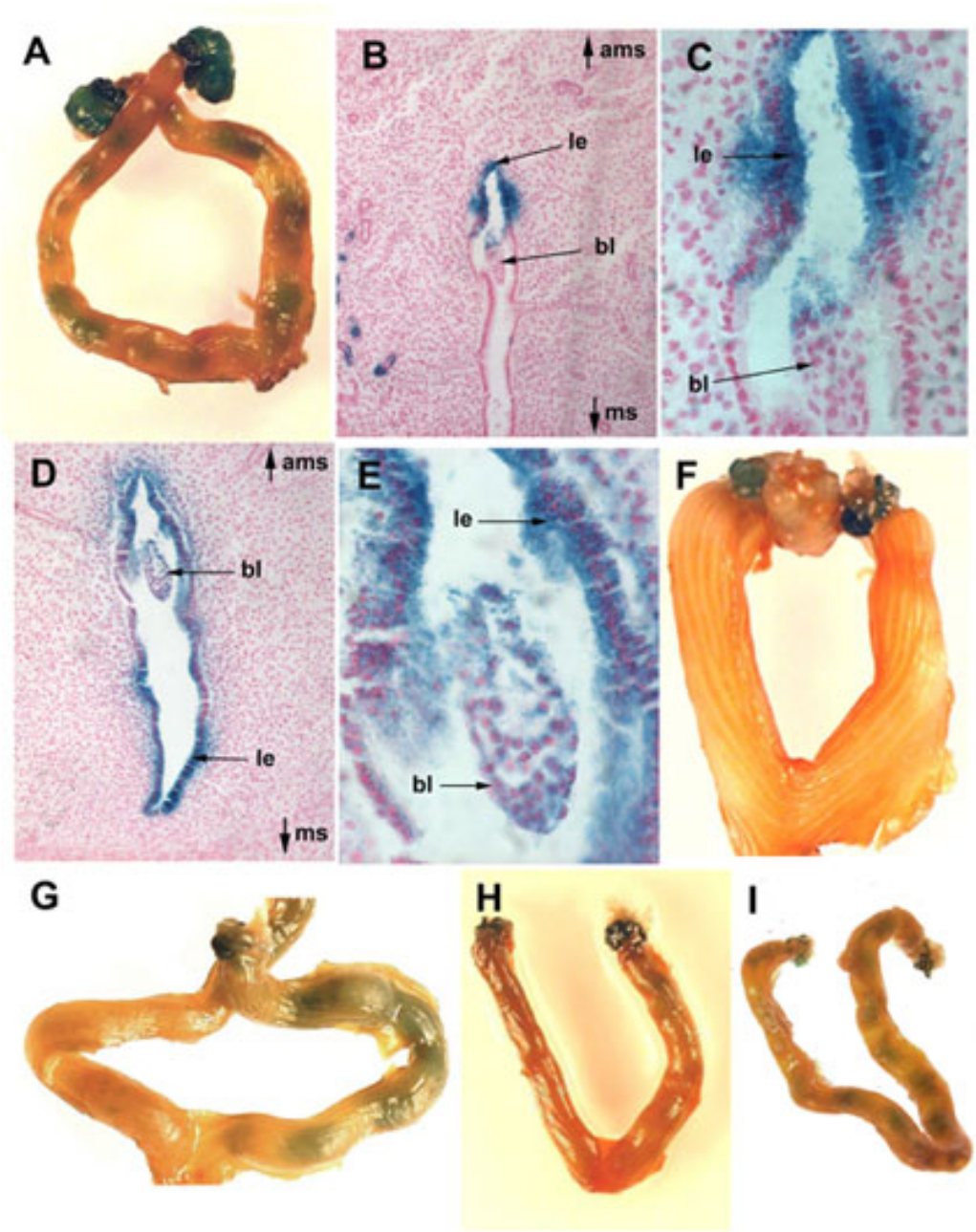


Figure 3. Wnt proteins known to activate the canonical Wnt pathway can selectively activate Wnt/β-catenin signalling in the uterus.

(A) Wholemount β-galactosidase staining of a TCF/Lef-lacZ transgenic uterus injected with Wnt5a conditioned media in both uterine horns. (B) Wholemount β-galactosidase staining of a TCF/Lef-lacZ transgenic uterus injected with Wnt7a conditioned media in both uterine horns. (C) Wholemount β-galactosidase staining of a TCF/Lef-lacZ transgenic uterus injected with Wnt7a conditioned media only in the right uterine horn. (D) A Cytodex-3 bead (bd) is approximately the same size as a wild-type blastocyst (bl). (E) A Cytodex-3 bead coated with Wnt7a expressing cells. (F) Wholemount β-galactosidase staining of a TCF/Lef-lacZ transgenic uterus injected with Wnt5a coated Cytodex-3 beads. (G) Wholemount β-galactosidase staining of a TCF/Lef-lacZ transgenic uterus injected with Wnt7a coated Cytodex-3 beads.

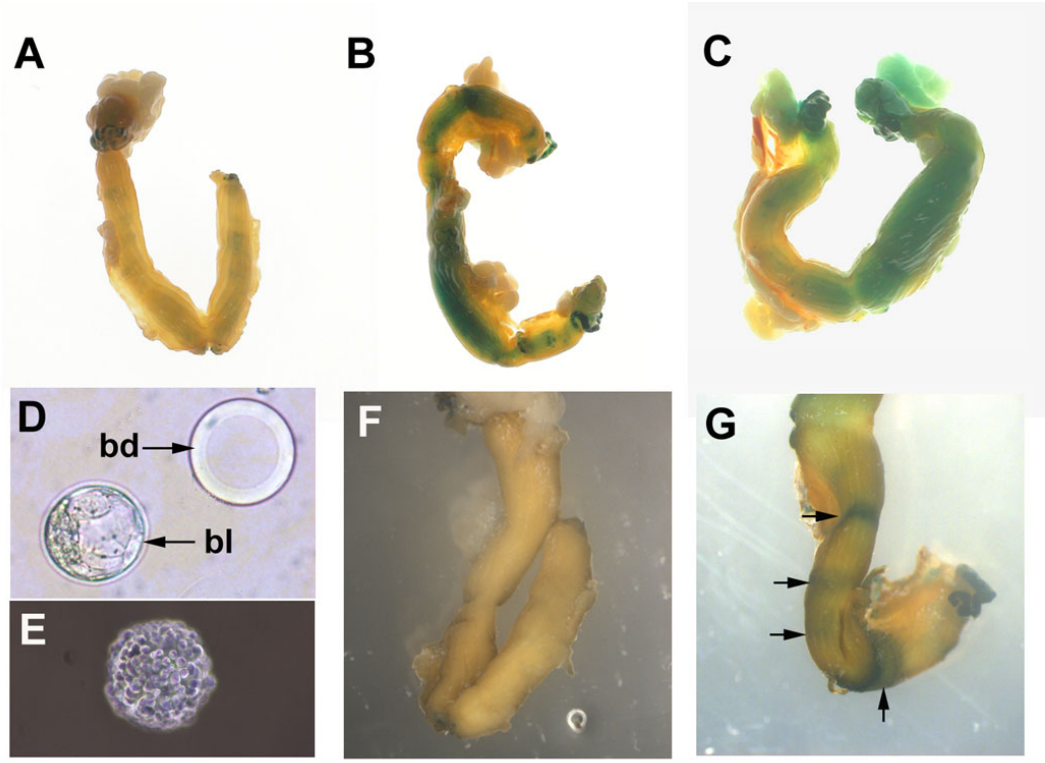
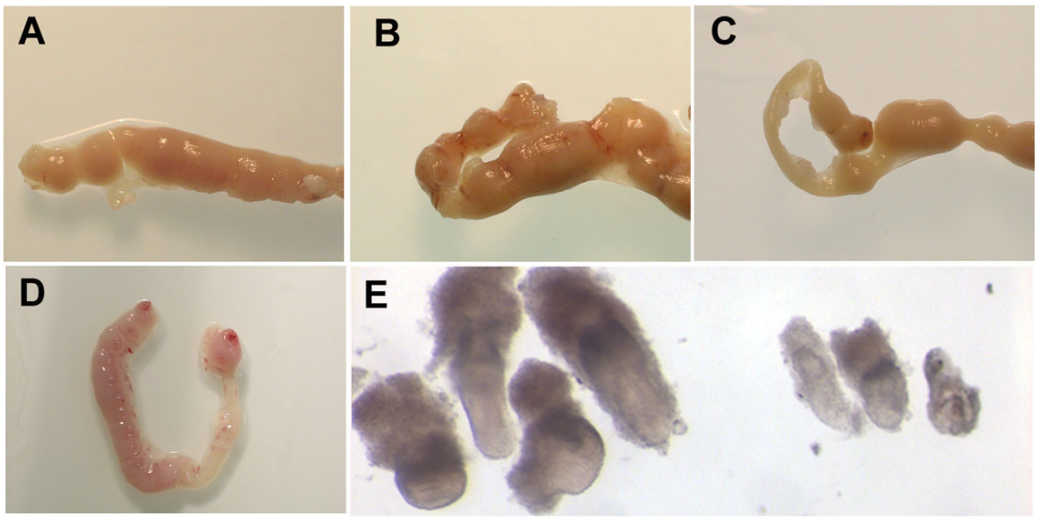


Figure 4: Activation of the Wnt/β-catenin signalling pathway in the uterus is required for proper blastocyst implantation.

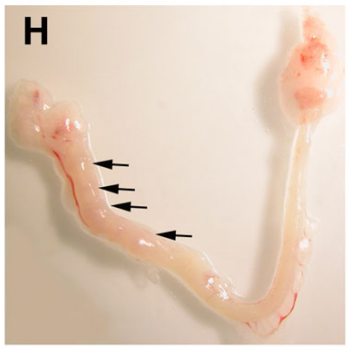
(A) Uterine horn on day 6 in which blastocysts along with bovine serum albumin had been transferred on day 4. (B) Uterine horn on day 6 in which blastocysts along with sFRP1 had been transferred on day 4. (C) Uterine horn on day 6 in which blastocysts along with sFRP2 had been transferred on day 4. (D) Uterus on day 6 in which sFRP2 conditioned media along with embryoid bodies expressing sFRP2 had been injected in the right uterine horn on the morning of day 4. The left horn was not injected and served as a control. (E) Embryos dissected from a uterine horn that had been injected with sFRP2 conditioned media along with embryoid bodies expressing sFRP2 (right) and embryos from the same uterus from the control uterine horn (left). (F) Table showing the results of injection of wild-type conditioned media and embryoid bodies as compared to injection of sFRP2 conditioned media and embryoid bodies. (G). Table showing the results of implantation of wild-type chimeras versus sFRP2 expressing chimeras. (H) Day 5 uterus in which wild-type chimeras had been transferred into the left uterine horn and sFRP2 chimeras into the right horn. Arrows show implantation sites.

Figure 4: Activation of the Wnt/β-catenin signalling pathway in the uterus is required for proper blastocyst implantation.



F

Sample Injected	# of Uterus	# of embryos Control side	# of embryos Experimental side	% implantation
Conditioned Media Parental cell + WT EB	4	27	24	89%
sFRP2 Conditioned Media + sFRP2 EB	4	28	12	43%



G

Aggragated with	# of Embryos Transfered	# of Embryos Implanted	% of Embryos Implanted	% of Control
Wild Type ES cells	44	20	45%	100%
sFRP2 Expressing ES cells	99	17	17%	37%

Figure 5. Model for blastocyst-induced Wnt/β-catenin signalling in the uterus. On the morning of day 4, the blastocyst emits a signal which results either directly or indirectly in the activation of the Wnt/β-catenin signalling pathway in discrete group of cells within the circular smooth muscle which form bands along the uterus. On late day 4 to early day 5, the blastocyst activates the Wnt/β-catenin signalling pathway in the luminal epithelium of the uterus. Activation of the Wnt/β-catenin signalling pathway results in the modulation of target genes in the uterus which facilitate implantation.

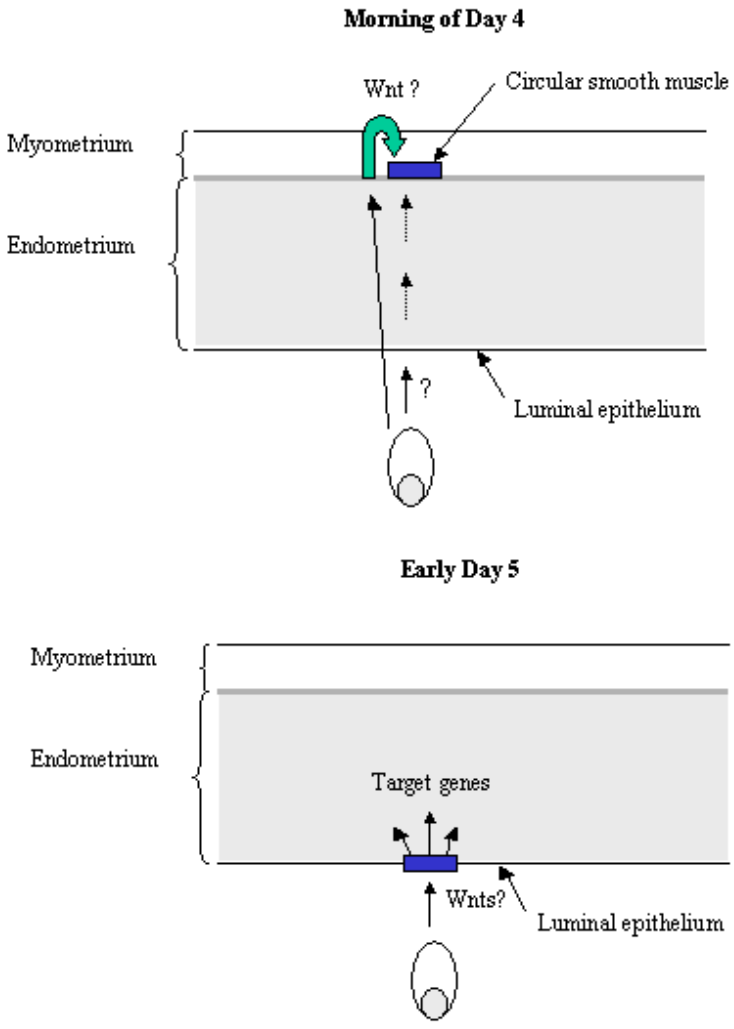
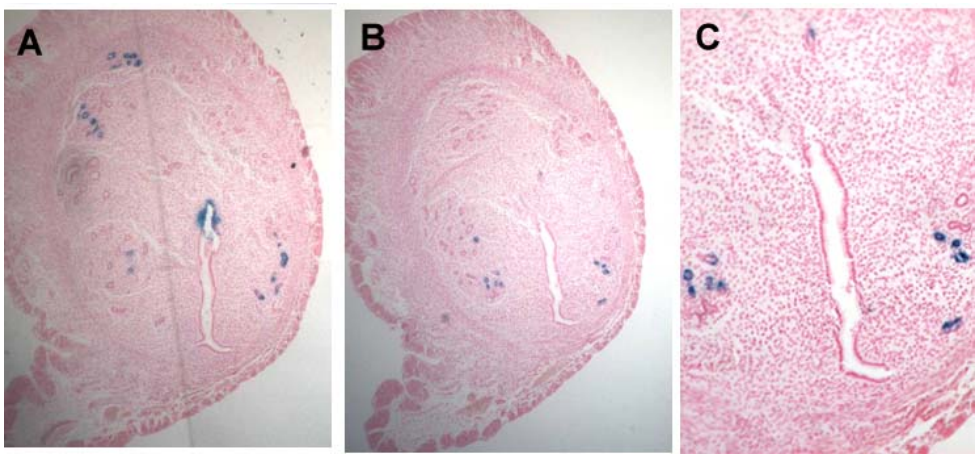


Table S1: Activation of reporter gene in the uteri of Tcf-LacZ during peri-implantation stages:

Treatment	Number of animals used	Activation of the reporter gene in the oviduct region		Specific activation of the reporter gene in the uterus	
		In Day 4	In Day 5	In Day 4	In Day 5
None	32	14	18	9	14
Mated with Vas males	13	5	8	0	0
Mated with Vas males + blastocyst transfer	7	3	4	2	3
Ovariectomy on one side	8	ND	8	ND	5
Oviduct-uterus junction ligation	9	ND	9	ND	6
Artificial Decidualization	17	4	13	0	0
Ovariectomy + Progesterone	7	ND	7	ND	0
Ovariectomy + Progesterone + Oestrogen	8	ND	8	ND	5

Figure S1:. Endometrial Wnt/β-catenin activity is detected only where a blastocyst is present.

(A)Transverse section from a uterus at 12:00 pm on day 5, showing activation in the luminal epithelium adjacent to the blastocyst. (B) Section through the same uterus as (A), 200 microns further. (C) Higher magnification of (B).



CHAPTER III: MANUSCRIPT II

Leukemia inhibitor factor modulates the expression of components of the Wnt signalling pathway in the uterus

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Short title: LIF and Wnt signalling in implantation

Summary sentence: Lif is required for luminal epithelial cells to respond to Wnt signals emminating from the blastocyst.

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Key words: LIF, implantation, Wnt signalling, LRP5, LRP6, luminal epithelium

PREFACE

In the first paper, we have demonstrated that the Wnt signalling pathway plays a crucial role in mediating embryo-uterine cross-talk. Our results have demonstrated that it is through Wnt signalling that the uterus can sense the presence of the embryo and thereby prepare to be receptive for embryo implantation. We have shown that the oestrogen surge was required for activation of the Wnt signalling pathway by the embryo. Since LIF is believe to be one of the most important mediators of oestrogen function in the uterus, in the following study, we set out to determine the relationship between LIF signalling and Wnt signalling pathway.

ABSTRACT

The process of implantation is a highly regulated process involving several signalling that must coordinate uterine receptivity and blastocyst competency for implantation. Both the LIF and Wnt signalling pathways have been shown to be involved in the implantation process. However, it is unclear whether these two pathways function autonomously or if they interact in coordinating successful implantation. To address this issue, we have determined whether the Wnt signalling pathway was activated at the time of implantation in LIF homozygous mutant females. Our results demonstrate that in the absence of LIF, luminal epithelium cells fail to respond to Wnt proteins. Injection of LIF in these mutant females is sufficient to restore activation of the Wnt pathway at the time of implantation. We further demonstrate that there is a dynamic pattern of expression of the components of the Wnt signalling pathway and that LIF may be involved in the regulation of a subset of these components. Taken together, our results demonstrate the LIF and WNT signalling pathways interact and that the LIF signalling is required for luminal epithelial cells to respond to Wnts.

INTRODUCTION

Attachment of the implantation competent embryo to the luminal epithelium of the receptive uterus is a crucial event during mammalian embryogenesis. The process of implantation relies on tightly regulated cross-talk between the implanting blastocyst and the receptive uterus (Dey 2004). This embryo-maternal dialogue has been shown to involve the complex interplay between steroid hormones, cytokines, growth factors, adhesion molecules, lipid mediators, and homeobox transcription factors (Dey *et al.* 2003; Edwards *et al*. 2003; Carson *et al*. 2000). Although several factors involved in cell signalling have been identified, the interaction between these various pathways, that are required for successful implantation, are not well understood.

A variety of cytokines have been proposed to regulate implantation (Salamonsen *et al.* 2007; Makrigiannakis *et al*. 2006; Chaouat *et al*. 2005), and to date; only leukemia inhibitory factor (LIF) has been shown to be crucial for uterine preparation and implantation (Cheng et al. 2002). LIF expression is induced to high levels in the endometrial glands of the uterus shortly after the oestrogen surge on day 4 and then declines by day 5 (Shen *et al*. 1992; Bhatt *et al.* 1991). Gene targeting experiments have demonstrated that LIF homozygous mutant females are sterile due to a failure of blastocyst implantation (Stewart *et al*. 1992; Benson *et al.* 1996; Wang *et al*. 1998). In the mouse, LIF expression has been postulated to be regulated by oestrogen and may be one of its crucial target genes required for implantation since it can replace nidatory oestrogen in ovariectomized mice (Chen *et al*. 2000). Although LIF signalling is known to be crucial for implantation, the exact role of LIF during this process remains elusive.

Recently, the Wnt signalling pathway has been demonstrated to play a role in the implantation process (Mohamed *et al*. 2004; Mohamed *et al*. 2005; Kemp *et al*. 2005; Daikoku *et al*. 2004). Activation of the Wnt signalling pathway was shown to be activated in the luminal epithelium of the uterus at the site of implantation (Mohamed *et al*. 2005). Activation of the Wnt pathway

in the uterus was dependent on the presence of the embryo and more importantly, activation of the pathway was shown to be required for implantation (Mohamed *et al*. 2005). Wnt genes encode a large family of cysteine-rich secreted glycoproteins that function as signalling molecules and play key roles in a wide variety of cellular and developmental processes, (Logan *et al*. 2004). To date, 19 Wnt genes have been identified in humans and mice. Wnt proteins act by binding to members of the Frizzled protein family (Logan *et al*. 2004). These transmembrane receptors contain an N-terminal, cysteine-rich extracellular domain, a putative seven-transmembrane domain, and a variable-length cytoplasmic C-terminus (Logan *et al*. 2004). Ten Frizzled proteins have been identified in humans and mice. Within the cell, multiple pathways have been identified through which Wnt signals may be transduced. In the canonical pathway, binding of Wnt ligands to their Frizzled receptors and the low-density lipoprotein receptor-related protein (LRP) co-receptors, LRP-5 and LRP-6, results in the activation of Disheveled protein (Dvl), which in turn inhibits GSK-3β activity, thereby leading to the stabilization and accumulation of the hypophosphorylated form of β-catenin (Fig 4) (Yost *et al*. 1996; Willert *et al*. 1998; Pinson *et al*. 2000). β-catenin then translocates to the nucleus, where it interacts with members of the TCF/Lef family of transcription factors and activates downstream target genes (Barker *et al*. 2000; Sharpe *et al*. 2001). Several extracellular secreted inhibitors of the Wnt signalling pathway have also been identified (Nierhs *et al.* 2006; Kawano *et al*. 2003). These secreted antagonists can be grouped in two categories according to the mechanism by which they inhibit the Wnt signalling pathway. The first group composed of the soluble Frizzled-Related Proteins (SFRPs), which resemble the ligand-binding CRD domain of the Frizzled family of Wnt receptors, bind to Wnt proteins to prevent their interactions with their receptors (Hoang *et al*. 1996). The second group composed of the secreted Dickkopf (Dkk) proteins, inhibit Wnt signalling by direct binding to LRP5/6 preventing activation of the intracellular pathway leading to the stabilization of β-catenin (Glinka *et al*. 1998).

Two members of the Wnt signalling pathway, Wnt4 and sFRP4, have been shown to be aberrantly expressed in the uterus of LIF homozygous mice (Daikoku *et al*. 2005). This suggests that the activity of these two signalling pathways may interact in coordinating the implantation process. We have examined the possible interaction of the LIF and Wnt signalling pathways and demonstrated that the luminal epithelial cells in LIF mutants are incapable of activating the Wnt signalling pathway in the presence of the embryo or of high concentration of Wnt7a protein. Using RT-PCR assays, we characterized the expression of the Frizzled receptors, LRP co-receptors, SFRPs, DKKs and Wnt genes, in both wild type and LIF mutant luminal epithelial cells on days 3 to 5. We show that there is a dynamic pattern of expression of the components of the Wnt signalling pathway at the time of implantation. Furthermore, we demonstrate that LIF signalling is required for the expression of a subset of these Wnt components in luminal epithelial cells at the time of implantation. Our results demonstrate that the LIF and Wnt signalling pathway form a network involved in coordinating the process of implantation

MATERIALS AND METHODS

Mating and Experimental Manipulation of Transgenic Animals.

All animal care and experimental procedures were approved by the Animal Care Committee of the Royal Victoria Hospital and were in accordance with the regulations established by the Canadian Council on Animal Care. The generation and characterization of the TCF/Lef-LacZ transgenic mice have been described in (Mohamed *et al*. 2004). Transgenic females were mated with fertile males and the morning of vaginal plug was considered to be day 1. The uteri were recovered at the indicated time points and processed for β-galactosidase activity. All mice were housed in filtered-topped isolator cages under a 12-hr light-dark cycle (7:00 AM to 7:00 PM), and allowed free access to food and water. The LIF knockout females were obtained from Dr. A. Smith. PCR analyzis of tail genomic DNA was used for genotyping mutant mice, using primers specific for the neomycin (Neo) resistance cassette, and the wild-type LIF sequence. Neo primers sequences were neo forward, GGC TAT GAC TGG GCA CAA CAG ACA ATC; Neo reverse, AGC TCT TCA GCA ATA TCA CGG GTA GC. LIF primers sequences were LIF forward CGC CTA ACA TGA CAG ACT TCC CAT ; LIF reverse AGG CCC CTC ATG ACG TCT ATA GTA. The PCR conditions were as follows: denaturation at 94°C for 60 sec, annealing at 58°C for 60 sec, elongation at 72°C for 60 sec for 30 cycles for tail DNA and for 35 cycles for embryos. The LIF rescue was performed by intraperitoneal injections of recombinant LIF on the morning of D4 and the uteri were isolated at 6:00PM for RNA extraction and real-time PCR analysis.

Detection of β-Galactosidase Activity.

Uteri were dissected in PBS, pH 7.3, rinsed in 100 mM sodium phosphate, pH 7.3, and then fixed in 4% paraformaldehyde/PBS, pH 7.3, for 10 min at room temperature. Uteri were then washed three times in wash buffer (0.02% Nonidet P-40 /0.01%deoxycholate /2 mM $MgCl_2$/ 100 mM sodium phosphate, pH 7.3) for 15 min each at room temperature. To reveal β-galactosidase activity, uteri were incubated in 1 mg/ml X-gal/5 mM

$K_3Fe(CN)_6$/5 mM $K_4Fe(CN)_6$/0.02% Nonidet P-40/0.01% deoxycholate/2 mM $MgCl_2$/100 mM sodium phosphate, pH 7.3, overnight at 37°C. The uteri were then rinsed with wash buffer and PBS and postfixed overnight in 4% paraformaldehyde in PBS at 4°C. Stained uteri were then dehydrated through a graded series of ethanol, incubated twice in xylenes and embedded in paraffin. Paraffin-embedded uteri were then subjected to serial transverse histological sectioning (0.7 nm) and stained with nuclear fast red solution (Sigma catalog no. N-3020).

In situ hybridization

Embryo isolation and in situ hybridization using digoxigenin-labeled RNA probes was performed as described (Henrique *et al*. 1995) on uterine cryosections (10um). Hybridization was carried out with probes for FZ-2 (Gregorieff *et al*. 2005); FZ-6 (Gregorieff *et al*. 2005); LRP-5 (Wang *et al*. 2005); LRP-6 (Wang *et al*. 2005); WNT5A (Gregorieff *et al*. 2005); WNT7A (Gregorieff *et al.* 2005); WNT11 (Kemp *et al*. 2005); DKK3; and SFRP-2 (Stump *et al*. 2003).

Semi-quantitative Real-time PCR

All experiments were performed 5 times, with 5 different mice, and the expression level was normalized with GAPDH. The calculations take in account the amplification efficiency of each reaction that was calculated using the LinRegPCR analysis program (Ramakers *et al*. 2003). The expression of the components of the Wnt pathway was analyzed in the LE cells of the uterine at 3 preimplantation stages: D3, D4 and D5. The primers used and their specific conditions are shown in table 1. Amplification was carried out on cDNA from uterine LE cells using Quiagen SYBRgreen kit (cat# 204141). The PCR cycling was carried out on a real-time PCR Light Cycler from Roche.

Uterine epithelial cells isolation

Endometrial epithelial cells were enzymaticaly isolated from the mouse uterus according to the method described by McCormack & Glasser, 1974.

Uteri were removed and placed into a Petri dish containing sterile PBS (without Ca^{2+} and Mg^{2+}). After washing with PBS and trimming off the remaining fatty and connective tissues, the uteri were sliced longitudinally. The sliced uteri were incubated in PBS supplemented with 7.5 mg ml^{-1} trypsin, for 60 min at 4°C, and then at room temperature for another 60 min. After the enzyme digestion, the test-tube containing PBS and the tissues was shaken gently for 30 sec. Uterine tissue was carefully removed and centrifuged at 1000 *g* for 5 min. The cell pellet was resuspended in Trizol for RNA extraction.

RESULTS

The Wnt signalling pathway is not activated in the uterus of LIF mutant females.

Since it had previously been shown that components of the Wnt signalling pathway were aberrantly expressed in the endometrium of homozygous LIF uteri (Daikoku *et al*. 2004), we tested whether the canonical Wnt pathway was activated in the luminal epithelium of LIF mutant mice as had been shown in wild type mice (Mohamed *et al*. 2005). To achieve this, we mated mice containing the Wnt reporter transgene (Mohamed *et al*. 2004) with LIF homozygous females. Homozygous females carrying the Wnt reporter gene were mated with wild type males and the uteri were isolated on day 5. Staining of the uteri for β-galactosidase activity, to assay whether the Wnt pathway had been activated, demonstrated that the canonical Wnt pathway failed to be activated in LIF homozygous females (Figure 1A and B). To ensure that the absence of Wnt signalling in homozygous female was solely due to the absence of LIF and not a developmental defect in this mutant, we injected LIF protein on the morning of day 4, when LIF is normally expressed (Chen *et al*. 2000), and assayed for activation of the Wnt pathway on day 5. Injection of LIF was sufficient to restore activation of the Wnt signalling pathway in the luminal epithelium of LIF mutant uteri (Figure 1C and D). This demonstrates that LIF is required for activation of the Wnt pathway in luminal epithelial cells.

To address this in more detail, we tested whether the luminal epithelial cell in LIF mutant uteri were capable of responding to high concentrations of Wnt protein. We mated wild type and $LIF^{-/-}$ females with vasectomized males, and on day 4, we injected Wnt7a conditioned media in each uterine horn. The uteri were dissected on day 5 and stained for β-galactosidase activity. Injection of Wnt7a in wild type uteri resulted in strong activation of the Wnt pathway (Figure 1E). In contrast, the Wnt pathway failed to be activated by injection of Wnt7a in LIF mutant uteri (Figure 1F), demonstrating that the luminal

epithelium in LIF mutants is incapable of activating the Wnt pathway in response to Wnt7a.

Components of the Wnt signalling pathway show a dynamic expression pattern in LE cells of the uterus at the time of implantation.

One possible explanation for the inability of LE cells in LIF mutants to respond to Wnt7a, may be due to an absence of downstream Wnt signalling components in these cells. Since the expression pattern of these components in the LE at the time of implantation were not known, we first determined by real-time PCR the pattern of expression of the Frizzled receptors, LRP co-receptors, the sFRP and DKK secreted antagonists, as well as several Wnt genes during the preimplantation period.

We first examined the expression of the Frizzled receptors and LRP co-receptors during the peri-implantation period. Three Frizzled receptors were found to be expressed in the LE during this period, FZ-2, FZ-4 and FZ-6. Moderate expression of FZ-2 was detected on days 3 and 4, which decreased to low levels by day 5 (Figure 2A). High levels of expression of FZ-6 were found on day 3 and the levels of expression decreased reaching low levels by day 5 (Figure 2A). FZ-4 on the other hand showed high levels of expression only on day 4 whereas no or low expression was detected on days 3 and 5 (Figure 2A). Since activation of the canonical Wnt pathway requires the presence of an LRP co-receptor (Pinson *et al*. 2000), we also examined the expression of both LRP5 and LRP6. LRP5 was expressed on days 3 and 4 and absent on day 5, whereas LRP6 was only expressed on day 3 and no or low expression was detected days 4 and 5 (Figure 2A). To confirm the dynamic pattern of expression of these receptors and co-receptors, in situ hybridizations were performed on sections of day 4 and day 5 uteri. On day 4, FZ-2 was strongly expressed both in the LE and stromal cells adjacent to the LE cells. By day 5, FZ-2 was absent from the LE cells but was highly expressed in proliferating stromal cells surrounding the implanted blastocyst. In contrast, FZ-6 showed a strong expression in the LE and stromal cells on day 4, but on day 5 FZ-6 was clearly undetectable either in the LE cells or the stroma. Both LRP-5 and LRP-6 were expressed at low levels in the LE on day 4, but were

barely detectable on day 5. These results demonstrate the highly dynamic spatio-temporal expression profile of FZ-2, FZ-6, LRP-5 and LRP-6 during the preimplantation period.

Antagonists of growth factors play key roles in monitoring intracellular signalling networks in many developing systems. sFRPs and DKKs are secreted antagonists that negatively regulate the Wnt pathway by physically interacting with Wnt proteins and LRP co-receptor, respectively (Kawano *et al.* 2003). To gain insights into the involvement of Wnt antagonists during embryo implantation, the expression pattern of sFRP and DKK transcripts were analyzed at different preimplantation stages. Of the three DKK genes, only DKK3 was found to be expressed in the LE cells. Moderate expression was observed on day 3, which increased on day 4 and returned to lower levels by day 5 (Figure 4A). In situ hybridizations demonstrated that DKK3 was specifically expressed in the LE on day 4 and expression could no longer be detected by day 5 (Figure 4B and C). Expression analysis was also performed on five sFRP genes of which three showed a dynamic pattern of expression during the peri-implantation period. High levels of expression were detected for both sFRP 2 and sFRP4 on day 3. The level of expression of both genes drastically decreased on day 4 followed by an increase in expression on day 5. sFRP5 expression was only detected on day 5. These results demonstrate that there is a drastic decrease in the expression of sFRP2 and sFRP4 on day 4 of gestation, which coincides with the period where activation of the Wnt pathway is observed.

Uterine Wnt4 expression had previously been shown to be aberrant in the uterus of LIF homozygous females. Since it was not known which Wnt genes were specifically expressed in LE cells at the time of implantation, we examined the expression of Wnt genes that had previously been proposed to be expressed in the uterus. Both Wnt5a and Wnt11 were shown to be abundantly expressed in LE cells at the time of implantation. Wnt5a expression was highest on day 3 and the levels slightly declined by day 5 (Figure 3A). Wnt11 expression levels were high on day 3 and increased on day 4 followed by a decrease on day 5 (Figure 4A). Weak expression of Wnt4,

Wnt5b, Wnt6, Wnt7b and Wnt11 were also detected in LE cells. Wnt3 on the other hand was not expressed (Figure 4A). In situ hybridizations demonstrated that Wnt 5a and Wnt11 expression was specific to the LE cells (Figure 4B to E).

Expression of components of the Wnt signalling pathway in LIF mutant LE cells.

Having identified the Wnt signalling components expressed in the LE of wild type mice during the peri-implantation period, we next examined the expression of these genes in the LE of LIF mutant females. In LIF mutant LE cells, FZ2 was expressed at high levels on day 3 and expression decreased to low levels on day 4 and low levels of expression was maintained on day 5 (Figure 5a). As observed in wild type LE, no FZ4 expression was detected on day 3 in LIF mutant LE cells (Figure 5A). However, unlike wild type, where high levels of FZ4 expression were detected on day 4 (Figure 2A), no expression of FZ4 was detected in LIF mutant LE cells at this stage. In fact, FZ4 expression was not detected in LE of LIF mutants at any of the peri-implantation stages examined. FZ6 expression was detected at moderate levels on day 3 and significantly decreased by days 4 and 5. The LRP5 coreceptor was expressed on day 3 but no expression was found on days 4 and 5, whereas LRP6 expression was not detected in the LE cell during this period (Figure 5A). A similar pattern of expression to these receptors was also observed for Wnt5a and Wnt11. High levels of expression for both Wnt5a and Wnt11 were found on day 3 but expression was absent or significantly decreased by days 4 and 5 (Figure 5B). Finally, no expression for DKK3, sFRP1 and 2 were detected in the luminal epithelium of LIF mutant mice (Figure 5C)

To determine if the expression of any of these genes could be rescued by activating the LIF signalling pathway, purified LIF protein was injected in homozygous mutant females on the morning of day 4, when LIF is normally expressed. Injection of LIF protein did not have any significant effect on the expression of FZ2, FZ4 or FZ6. However, an increase in expression of both Lrp5 and 6 was observed (Figure 5A) upon injection of LIF. Similarly, a drastic increase in expression of both Wnt5a and Wnt11 was also observed

upon injection of LIF. No increase in sFRP1 and sFRP2 expression was observed but expression of DKK3 was significantly increased upon injection of LIF. These results demonstrate that injection of LIF increased the expression of LRP5 and 6, Wnt5a and Wnt11 as well as DKK3 suggesting that the LIF signalling pathway may be regulating the expression of these genes.

DISCUSSION

Embryo implantation is a highly regulated process involving multiple signalling pathways (Dey *et al*. 2003; Edwards *et al.* 2003; Carson *et al*. 2000). One of the remaining challenges is to elucidate how these signalling pathways interact to coordinate the implantation process. Here we have investigated the possible interaction of the LIF and Wnt signalling pathways and shown that LIF signalling is required for luminal epithelial cells to respond to Wnt proteins and activate the canonical pathway. We demonstrated that in LIF mutant females, the canonical Wnt signalling pathway is not activated at the time of implantation. Furthermore, injection of high concentration of Wnt7a protein in the uterus of LIF mutant females was not able to activate the pathway; however, injection of LIF protein on the morning of day 3 was sufficient to restore the ability of luminal epithelial cells to activate the Wnt pathway in response to Wnt7a. This demonstrates LIF can regulate the ability of the luminal epithelial cells to respond to Wnts.

One possible mechanism by which LIF could regulate Wnt signalling is by modulation one of the Wnt downstream effectors. Since the expression of most Wnt downstream effectors had not been characterized, we determined which of the effectors were expressed during the peri-implantation period. Our results demonstrate that there is a dynamic pattern of expression for many of the Wnt effectors. The most dramatic modulation of Wnt effectors occurs on day 4, just prior to implantation of the embryo. At this stage, there is an increase in expression of both FZ2 and FZ4 receptors as well as for the co-receptor LRP5. This increase in receptors and co-receptor coincides with the time at which active Wnt signalling is detected in the uterus (Mohamed *et al*. 2005). Interestingly, during this period of day 4, there is a drastic decrease in two of the Wnt inhibitors, sFRP2 and sFRP4. These results demonstrate that on day 4, there is an increase in Wnt receptor expression, which is accompanied by a decrease in Wnt inhibitor expression.

Interestingly, by late day 5 when the Wnt pathway is no longer activated. There is a decrease to very low levels of expression of FZ2, FZ4

and LRP5 and an increase in expression of the Wnt inhibitor sFRP2. This modulation in the level of expression of these genes during the implantation period may be responsible for restricting the activation of the Wnt pathway to days 4 and 5.

Analysis of the expression of the Wnt effectors during the peri-implantation period in LIF mutant epithelial cells has demonstrated that FZ2, FZ4 and LRP5 are not expressed at the time of implantation. Interstingly, injection of LIF protein, to rescue implantation, had no effect on the expression of FZ2 or FZ4. Thus the increase observed on day 4 in wild type mice is not due to LIF expression. Furthermore, since the Wnt pathway is activated in LIF mutants injected with LIF protein suggests that FZ2 and FZ4 may not be required or can be substituted for by other FZ receptors.

Activation of the canonical Wnt pathway requires both FZ receptor as well as one of the co-receptors LRP5 or LRP6 (Logan *et al*. 2004). We have shown that in LIF mutants, neither of the co-receptors are expressed on days 4 or 5. The absence of coreceptors could account for the inability of the luminal epithelial cells on LIF mutants to respond to Wnt proteins. Further supporting this possibility is that injection of LIF protein, which rescues activation of the Wnt pathway, leads to an increase in expression of both LRP5 and LRP6.

In conclusion, we have demonstrated that LIF is required for luminal epithelial cells to respond to Wnt proteins present in the lumen and activate the Wnt pathway. This inability to activate the Wnt pathway in LIF mutants may be due to an absence in Wnt co-receptors. Our results also show that there is a dynamic pattern of expression of Wnt downstream effectors at the time of implantation showing an increase in Wnt receptors and co-receptor and a decrease in Wnt inhibitors.

Figure 1: The Wnt signaling pathway is not activated in LIF mutant uteri.

A and B): Day 5 LIF mutant uteri stained for β-galactosidase activity.

C and D): LIF mutant uteri injected with LIF on the morning of day 4 and stained for β-galactosidase activity on day 5.

E and F): Wild-type (E) and LIF mutant (F) uteri injected with Wnt7a conditioned media on day 4 and stained for β-galactosidase activity on day 5.

le: luminal epithelium; ge: glandular epithelium.

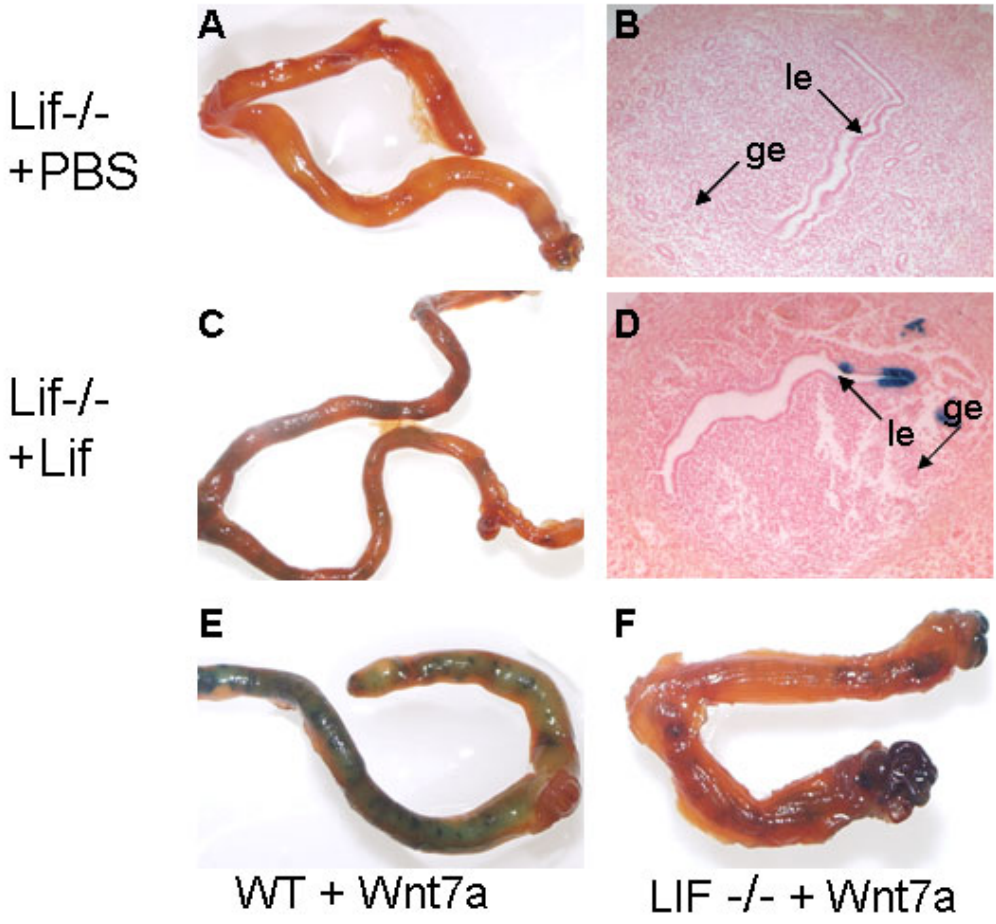


Figure 2: Expression of Frizzled receptors and co-receptors in wild-type uteri. A): real-time PCR analysis of FZ and LRP expression from day 3 to day 5. Different superscripts above each bar indicate statistically significant differences at p≤0.05. B, C, D, and E): *In situ* hybridization of FZ2, FZ6, LRP5 and LRP6 on day 4 uteri. F, G, H, and I): *In situ* hybridization of FZ2, FZ6, LRP5 and LRP6 on day 5 uteri. le: luminal epitelium.

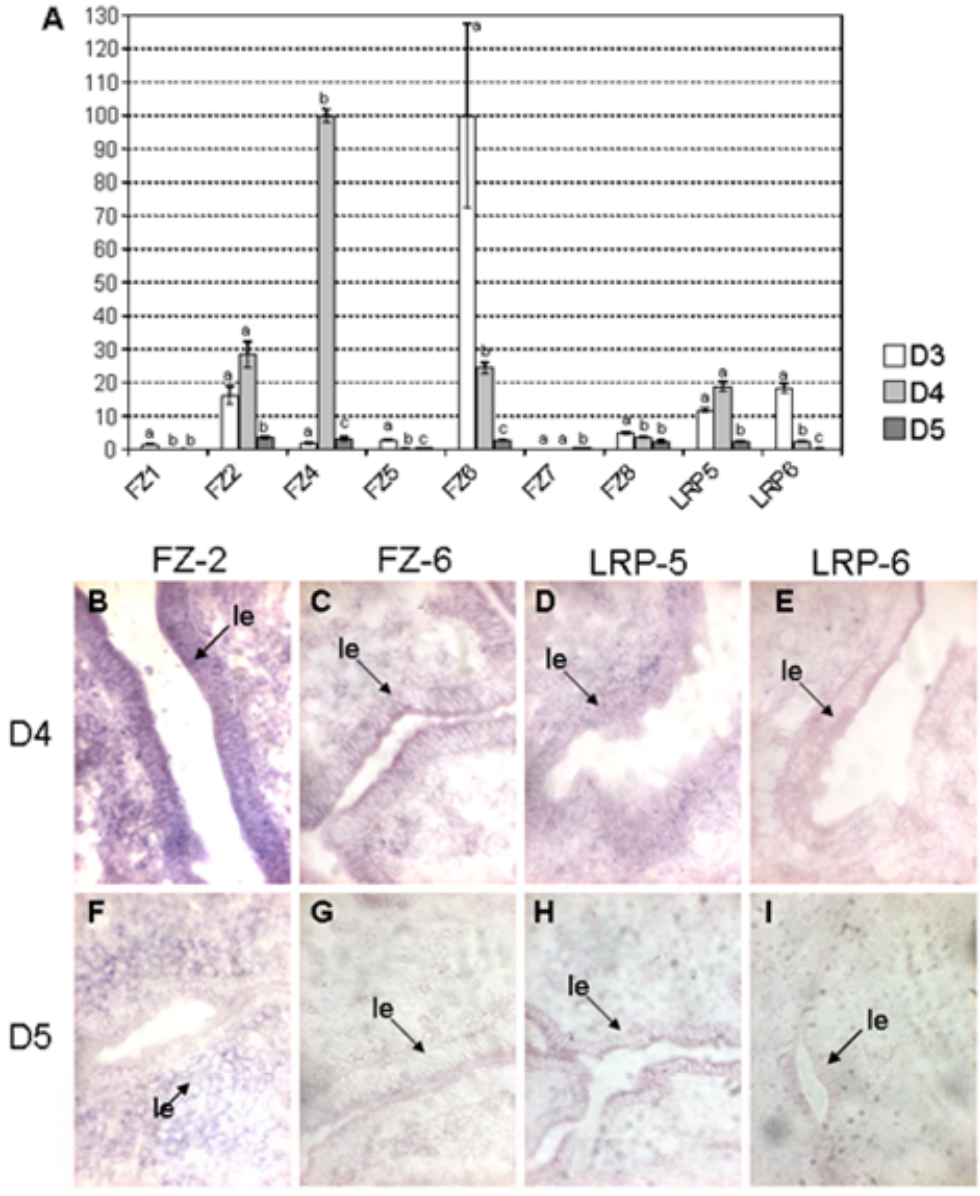


Figure 3: Expression of Wnt in the wild-type uteri.

A): real-time PCR analysis of indicated Wnts expression from day 3 to day 5. Different superscripts above each bar indicate statistically significant differences at $p \leq 0.05$. Wnt5a *in situ* hybridization on day 4 (B), and on day 5 (D). Wnt11 *in situ* hybridization on day 4 (C), and on day 5 (E). le: luminal epithelium; ge: glandular epithelium.

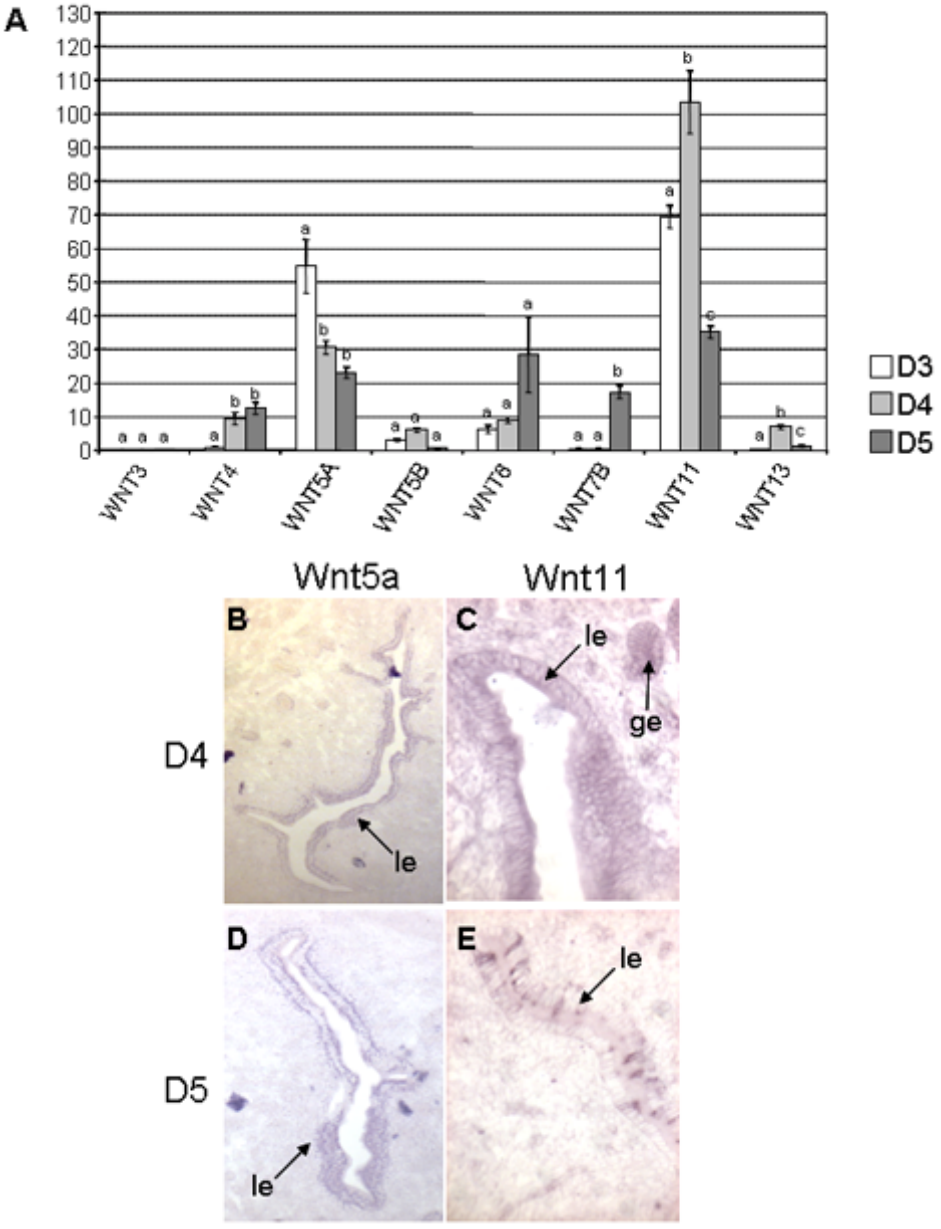


Figure 4: Expression of DKK and sFRP in the wild-type uteri.

A): real-time PCR analysis of DKK and sFRP expression from day 3 to day 5. Different superscripts above each bar indicate statistically significant differences at $p \leq 0.05$. *In situ* hybridization of DKK on day 4 (B), and on day 5 (C). le: luminal epithelium.

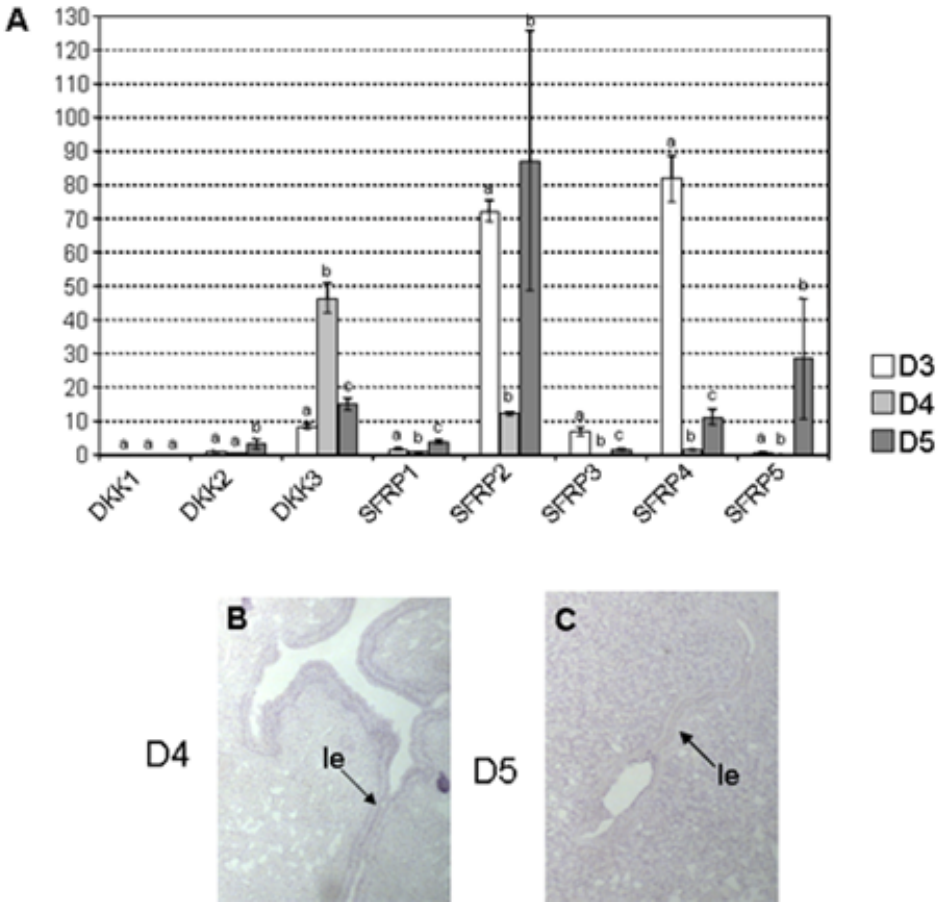
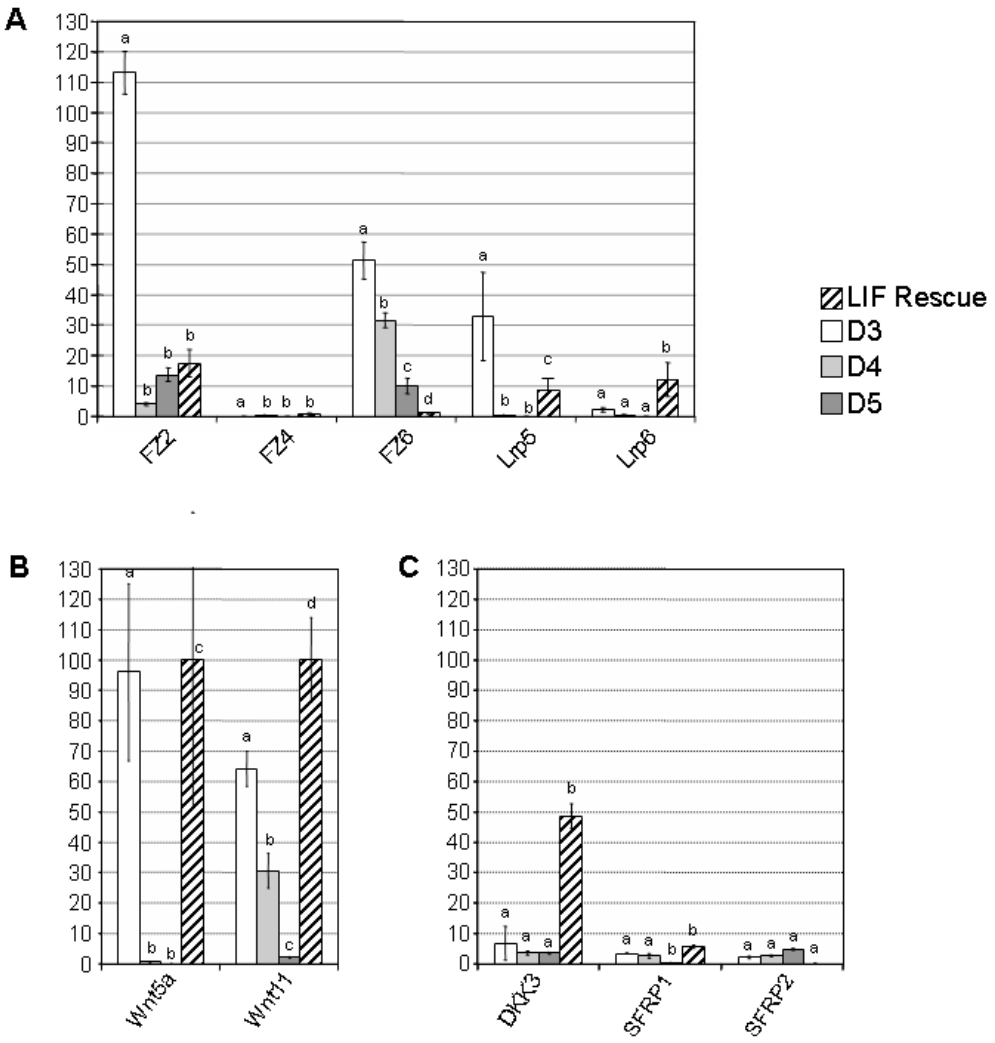


Figure 5: Expression of components of the Wnt signaling patway in LIF mutant uteri.

Real-time PCR analysis of indicated FZ and LRP expression from day 3 to day 5 (A), Wnt5a and Wnt11 (B), and DKK3, sFRP1, and sFRP2 (C). LIF rescue: LIF protein was injected on themorning of day 4. Different superscripts above each bar indicate statistically significant differences at $p \leq 0.05$.



CHAPTER IV: MANUSCRIPT III

Identification of Wnt downstream target genes in the luminal epithelium of the uterus.

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Short title: Wnt signalling in implantation

Summary sentence: Wnt/β-catenin signalling pathway activates the expression of cell differentiation genes in the luminal epithelium of the uterus.

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PREFACE

Although we have shown that the Wnt/β-catenin signalling pathway is required for implantation, we currently do not know the function of this pathway in the luminal epithelium of the uterus during implantation. In order to understand the function of the Wnt/β-catenin signalling pathway in the luminal epithelium at the time of implantation, in this study, we will identify target genes of this pathway in the uterus.

ABSTRACT

Implantation, which is exclusive to mammals, enables the embryo to invade the uterine endometrium and to gain access to the maternal circulation. Several signalling pathways have been shown to be involved in the intricate dialogue between the blastocyst and the uterus. Wnt signalling has been shown to be active in luminal epithelial cells at the future site of implantation and required for successful implantation. As an initial step in elucidating the role of the Wnt pathway in the implantation process, we have identified target genes whose expression is up-regulated in the luminal epithelium in response to Wnts. We identified FGF-1 and IGF-2 as downstream target genes of the Wnt/β-catenin pathway in the luminal epithelial cells of the uterus at the time of embryo implantation.

INTRODUCTION

One of the crucial events during mammalian embryogenesis is the process of implantation. The attachment of the implantation competent embryo to the luminal epithelium of the receptive uterus relies on a tightly regulated embryo-maternal dialogue. This intricate cross talk requires the coordination of endocrine, cellular and molecular events, in a dynamic manner. Despite the discovery of numerous molecules and signalling pathways involved in this process, the precise sequence and hierarchy of events leading to successful embryo implantation remains to be elucidated. We need to understand whether these signalling pathways function independently, in parallel or converge to a common pathway to establish successful reciprocal dialogue between the embryo and the uterus. We have previously demonstrated that activation of the Wnt/β-catenin pathway in the luminal epithelium of the uterus at the site of implantation was required for implantation (Mohamed *et al*. 2005). At the time of embryo attachment, the blastocyst has been shown to express multiple genes (Kemp *et al*. 2005; Mohamed *et al*. 2004). Interestingly, activation of the Wnt/β-catenin pathway in the uterus was shown to be dependent on the presence of the embryo suggesting that Wnt secreted by the embryo may be involved in activation of the Wnt pathway in the luminal epithelial cells of the uterus (Mohamed *et al*. 2005).

Wnt genes encode a large family of cysteine-rich secreted glycoproteins that function as signalling molecules and play key roles in a wide variety of cellular and developmental processes (Logan *et al*. 2004). At least three intracellular signalling pathways have been identified through which Wnt signals may be transduced (Yost *et al*. 1996; Willert *et al*. 1998). The best characterized is the canonical pathway where binding of Wnt ligands to their Frizzled receptors and the low-density lipoprotein receptor-related protein (LRP) co-receptors, LRP-5 and LRP-6, results in the stabilization and accumulation of the hypophosphorylated form of β-catenin in the cytoplasm. β-catenin then translocates to the nucleus where it interacts with members of

the TCF/Lef family of transcription factors and activates downstream target genes (Pinson *et al*. 2000; Barker *et al*. 2000).

As an initial step in elucidating the function of Wnt signalling in the implantation process, we have set out to identify downstream target genes of the Wnt pathway in luminal epithelial cells. We have made use of the Wnt reporter strain of mice, TCF/Lef-LacZ, to identify luminal epithelial cells in which the Wnt pathway had been activated. Using a microarray approach, we have compared the gene expression profile of cell in which the Wnt pathway had been activated for four hours versus cells where the Wnt pathway had not been activated. Using this approach, we have identified IGF-2 and FGF-1 as Wnt/β-catenin target genes.

MATERIAL AND METHODS

Mating and Experimental Manipulation of Transgenic Animals.

All animal care and experimental procedures were approved by the Animal Care Committee of the Royal Victoria Hospital and were in accordance with the regulations established by the Canadian Council on Animal Care. The generation and characterization of the TCF/Lef-LacZ transgenic mice have been described in Mohamed *et al.*, 2004. Transgenic females were mated with fertile males and the morning of vaginal plug was considered to be day 1. All mice were housed in filtered-topped isolator cages under a 12-hr light-dark cycle (7:00 AM to 7:00 PM), and allowed free access to food and water.

Injection of Wnts

Parental L cells and Wnt7a cells were generated as follows. The cDNA fragments of Wnt5a and Wnt7a were provided by Dr. Jan Kitajewski (Harris *et al.* 2003). These cDNA fragments were subcloned into the retrovirus vector, pHAN(puro). To prepare ecotropic retrovirus, Phoenix-eco packaging cells were transfected with retrovirus vectors by using lipofectoAMINE (Invitrogen). Viral supernatants were harvested 30 hrs posttransfection and used to infect L cells (ATCC CRL-2648) in the presence of polybrene (Sigma, 8 micro g/ml). Infected cells were selected 24 hrs postinfection with puromycin (Sigama, 5 μg/ml). Selected Wnt-expressing cells were grown in 100-mm dishes, washed twice with PBS and lysed in 0.5 ml radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris HCl, pH 7.5; 150 mM NaCl; 0.5% Nonidet P-40; 0.1% deoxycholate) containing protease inhibitor cocktail (Roche). Cell extracts were collected and spun in a microcentrifuge at 13,000 rpm for 5 min. Total proteins (5 micro g) were separated by 10% SDS-PAGE and transferred to Immobilon-P (Millipore). The membranes were probed with primary antibodies, followed by HRP-conjugated secondary antibodies at 1:5000 (Bio-Rad), and developed using ECL™ Plus (Amersham Biosciences). Membranes were exposed to

BIOMAX film (Kodak). Primary antibodies used in this work: anti-HA (HA7 clone, Sigma, 1:5,000).

Parental L cells and Wnt7a cells were cultured to 80-90% confluency and the conditioned medium was harvested and concentrated two- to three-fold using a centricon column. Five ul of parental and Wnt conditioned media was injected in each uterine horn in the morning of day 5.

Uterine epithelial cell isolation

Endometrial epithelial cells were enzymaticaly isolated from the mouse uterus according to the method described by McCormack & Glasser (1980) with slight modifications (Chan *et al.* 1997). Uteri were removed and placed into a Petri dish containing sterile PBS (without Ca^{2+} and Mg^{2+}). After washing with PBS and trimming off the remaining fatty and connective tissues, the uteri were sliced longitudinally. The sliced uteri were incubated in PBS supplemented with 7.5 mg/mL trypsin, for 60 min at 4°C, and then at room temperature for another 60 min. After the enzyme digestion, the test-tube containing PBS and the tissues were shaken gently for 30 sec. Uterine tissue was carefully removed and centrifuged at 1000 *g* for 5 min. The cell pellet was resuspended in Trizol for tRNA extraction.

Quantitative Real-time PCR

All experiments were performed 3 times, and the expression level was normalized with GAPDH. The calculations take in account the amplification efficiency of each reaction. The primers used and their specific conditions are shown in Table 1. Amplification was carried out on cDNA from uterine LE cells using Quiagen SYBRgreen kit (cat# 204243). The PCR cycling was carried out on a real-time PCR Light Cycler from Roche.

Immunofluorescence Confocal Microscopy

Uteri obtained from *CD-1* matings were fixed in 4% PFA (4% paraformaldehyde) overnight at 4°C. Fixed embryos were incubated for 24 hrs in 30% sucrose solution at 4°C and embedded in Shandon Cryomatrix medium (Thermo Electron Corporation) before freezing in liquid nitrogen, and stored

at -80°C. 7μm sections were cut on cryostat, mounted on Superfrost slides, air-dried, and stored at -80°C. Representative tissue sections were equilibrated at room temperature, rinsed 5 min. in PBS, washed three times 15 min. in 0.1% Triton-X in PBS at room temperature. Tissue sections were then incubated for 1 hr at room temperature in blocking solution (20% heat inactivated sheep serum, 0.1% Triton-X in PBS). Tissue sections were incubated overnight at 4°C with primary antibodies: mouse anti-IGF-2 (1:100) and mouse anti-IGF-1 (1:100) and 1 hr at room temperature with secondary antibody antibody, FITC-conjugated anti-rabbit (1:200). Sections were then mounted in mowiol (Calbiochem) and examined using a Zeiss 510 confocal scanning laser microscopic imaging system.

RESULTS

Data analysis

In order to activate the Wnt/β-catenin pathway in the luminal epithelial cells of the uterus, we injected Wnt7a in the uteri of pseudopregnant TCF/Lef-LacZ transgenic reporter mice on the morning of D5 (D1= day of plug) for four hours. Injection of Wnt7a in the uterus results in the activation of the Wnt/ß-catenin in the luminal epithelial cells (LE). LE cells were isolated, stained for ß-galactosidase activity and sorted by FACS to isolate positive cells (activated) from negative cells (nonactivated). cRNA was generated and used to screen the microarray. The data were analyzed with Genespring. Fold change ratios between groups were subsequently derived, and a difference of 5-fold, was applied to identify up-regulated genes. Nonparametric testing was further applied, using a *P* value of 0.05 to identify statistical significance between the two groups. With this strategy, we identified 393 up-regulated genes. Table 2 shows, in descending order, the fold increase for each up-regulated gene in positive uterine LE cells.

Clustering

Up-regulated genes included those involved in cell differentiation (Numb gene homolog, Fibroblast Growth Factor 1), cell cycle (Insulin Growth Factor 2, Cyclin D1), cell proliferation (Bone morphogenic protein receptor type II, endothelin 1), immune response (Caspase 3, Selectin), enzyme regulators (regulator of G-protein signalling, tissue inhibitor metalloproteinase 1), and cell adhesion (coxsackievirus and adenovirus receptor, cadherin 13). Additional genes that were up-regulated included several members of the Wnt signalling pathway (Wnt2b, LRP6, Rho associated coiled-coil containing protein kinase 2, Lymphoid enhancer binding factor 4), and known Wnt target genes (E-cadherin, Fibroblast growth factor 9, Jagged, Cyclin D1, Fos-like antigen 1, Krupple-like factor 5). Several of the up-regulated genes had previously been shown to be expressed in the uterus or involved in the implantation process. These included: Numb, cadherin 13, Insulin growth factor 2, Fibroblast growth factor 1, Prostacyclin, and Vascular cell adhesion

molecule. Furthermore several known downstream target genes of the canonical Wnt signalling pathway were also identified. These included Hoxc6, Gata6, Fgf1, Igf2, Notch 4, Timp1, Numb, Rgs4, and Keratin 14.

Validation by qPCR.

We validated the expression of nine selected up-regulated genes (FGF-1, Gata6, Hoxc6, IGF-2, K14, Notch4, Numb, RGS4, Timp1) using real-time PCR from activated uterine LE cells and nonactivated cells. We selected these nine up-regulated genes because they are known target genes of the Wnt/β-catenin pathway and/or known to be involved in the implantation process. The level of expression of each gene in each sample was normalized using GAPDH expression, and ratios were determined. The real-time PCR data (Figure 1) demonstrate clear up-regulation of FGF-1, Gata6, Hoxc6, IGF-2, Notch4, Numb, K14, and Timp1. These data are consistent with the observations from the microarray data.

The temporal and spatial expression of IGF-2 and FGF-1 was analyzed by immunofluorescence in the endometrium of pseudopregnant females activated for four hours with Wnt7a on the morning of D5. We compared the expression pattern of IGF-2 and FGF-1 in the endometrium of mice injected with Wnt7a conditioned medium, L cells control medium, and in normal pregnant females on D4 (Figure 2). Wnt7a induced the expression of FGF-1 in the luminal epithelium of pseudopregnant females, whereas no expression was found in the luminal epithelium in females injected with control L cell medium. Moreover, FGF-1 expression was detected in the LE of naturally mated females on late D4. These results indicate that FGF-1 is a downstream target gene of the Wnt/β-catenin pathway. Contrarily, IGF-2 is only present in the LE of pseudopregnant females treated with Wnt7a.

DISCUSSION

One of the crucial events during mammalian embryogenesis is the process of embryo implantation. During this process, the activated blastocyst establishes close contact with the uterine luminal epithelium. The intricate dialogue between the blastocyst and the uterus involves the coordination of endocrine, cellular and molecular events, in a dynamic manner. Although much effort has been made to identify factor responsible for uterine receptivity, principal effectors that determine success or failure of implantation have not been identified yet. Since this process shares characteristics of epithelial to mesenchymal transition, it is expected that development genes participate in the embryo-maternal interplay. Hence, we have recently highlighted the crucial role of the Wnt/β-catenin signalling at the time of embryo implantation (Mohamed *et al*. 2005). We have demonstrated that the Wnt/β-catenin pathway was activated in the luminal epithelium at the prospective site of embryo implantation, on D4. Consequently, the purpose of this investigation was to identify target genes in the LE whose expression is regulated by the Wnt/β-catenin pathway. This study allowed us to identify downstream target genes of the Wnt/β-catenin signalling pathway in the luminal epithelium of the uterus by injection of Wnt7a in the lumen of uteri.

Many studies have been performed with the aim of identifying a "molecular signature" characteristic of a receptive endometrium. The murine studies have used several paradigms to identify receptivity genes. These include comparisons of: implantation vs intersite tissue or luminal epithelium only, prereceptive vs receptive uteri and delayed vs nidatory oestrogen treated uteri. These groups have tried to identify genes whose expression is activated by the blastocyst without much success. This is most likely due to the fact that either all the luminal epithelium or chunks of uterus isolated at various stages were used. The luminal epithelium immediately surrounding the implanting blastocyst differs greatly from the rest of the luminal epithelium in its gene expression. For example, example, cyclooxygenase-2 and basigin are

specifically expressed in the luminal epithelium surrounding the implanting blastocyst (Lim *et al*. 1997). Since only a few cells within the luminal epithelium respond to signals from the blastocysts and from Wnt7a, the increase in gene expression within these cells could not be detected amongst the large amount of material isolated. Our approach allowed us to eliminate this problem since we only isolated cells that had activated the Wnt signalling pathway.

We identified genes that have been previously shown to be expressed in the luminal epithelium of naturally mated females at the time of embryo implantation, such as connexin 31, Cadherin 13, Selectin, Cyclin D1, and Notch4. Since the expression of these genes is up-regulated in Wnt activated cells suggest that their expression at the site of implantation is regulated by Wnts. We did not detect changes in the expression of the genes encoding Cyclooxygenase-2, amphiregulin, epiregulin, and HB-EGF, which have previously been shown to be activated by signals eminating from the embryo. Thus, this suggests that the Wnt signalling pathway is not involved in the regulation of these genes.

The process of implantation involved cell-cell interactions between blastocyst and uterus, cell-type specific proliferation and differentiation of the uterus, and immunological responses of the mother to the semi-allogenic embryo. Consequently, our data showed a broad diversity of genes that are modulated during implantation. The majority of highly ranked genes activated by Wnt7a were involved in cell differentiation process, such as Hoxc6, Gata6, FGF-1, Notch4, IGF-2, and Numb. The upregulation of these genes by Wnt7a in uterine luminal epithelial cells was confirmed by real-time PCR. It is known that the process of embryo implantation triggers a cell differentiation process in the endometrium that allows syncitiotrophoblast invasion and that leads to decidualization and placentation.

Growth factors such as IGF-2 and FGF-1 are believed to mediate and modulate the actions of hormones at their target tissues through autocrine/paracrine mechanisms. IGFs have proliferative, differentiative and

metabolic effects. Specifically, IGF-2 gene expression is associated with endometrial differentiation (Irwin *et al*. 2002). In humans, Endometrial stromal cells produce insulin-like growth factors I and II (IGF-1 and IGF-2) as well as the high-affinity IGF binding proteins (IGFBPs), whereas epithelial cells and, in a lesser amount, also stromal cells contain cell membrane receptors for IGFs (Badinga *et al.* 1999; Rutanem *et al*. 1998). The recognized functions of FGFs in tissue plasticity are potentially important for the cyclic growth, differentiation, and neovascularization that characterize uterine responses to sex steroids.

In conclusion, we have identified target genes of the Wnt/β-catenin signalling pathway in the LE at the time of embryo implantation. The nature of these genes suggests a role for the Wnt/β-catenin signalling pathway in cell differentiation at the time of embryo implantation. Analysis of the function of these genes will allow a better understanding of the implantation process.

Table 1: List of primers.

Gene	Accesion number	Sequences 5' to 3'		Annealing Temp.
Fgf1	NM_010197	mFGF1-F	GAAGCATGCGGAGAAGAACTG	60C
		mFGF1-R	CGAGGACCGCGCTTACA	
Gata6	NM_010258	mGATA6-F	GAAGCGCGTGCCTTCATC	55C
		mGATA6-R	GTAGTGGTTGTGGTGTGACAGTTG	
Hoxc6	NM_010465	mHOXC6-F	ACCAGAAAGCCAGTATCCAG	58C
		mHOXC6-R	CTTTTCCTCTTTTCCGCCCA	
IGF-2	NM_010514	mIGF2-F	GTGGCATCGTGGAAGAGTGC	60C
		mIGF2-R	GGGGTGGGTAAGGAGAAACC	
K14	NM_016958	mk14-F	GGATGTGAAGACAAGGCTGGA	60C
		mK14-R	AAGCCTGAGCAGCATGTAGCA	
Notch4	NM_010929	mNOTCH4_F	CAGAACGTGGATCCCCTCAAGTTGC	58C
		mNOTCH4_R	AGGCAGAGAGAGGGCAAGGACTCAT	
Numb	NM_010949	mNUMB_F	GGGATTTCCTGCTCTTAGCC	55C
		mNUMB_R	GGTCAGCTTCAGAGGGAGTG	
RGS4	NM_009062	mRGS5_F	TCTCCTCCAGAAGCCAGACT	55C
		mRGS5_R	GGAAGCCTGACCAGATGACT	
Timp1	NM_011593	mTIPM1_F	CCACCTTATACCAGCGTTAT	60C
		mTIPM1_R	CTGGGACTTGTGGGCATATC	

Table 2: List of genes up-regulated 10x and more.

Gene symbol	Description
CELL DIFFERENTIATION	
Dhrs9	Dehydrogenase/reductase (SDR family) member 9
Ablim1	Actin-binding LIM protein 1
Numb	Numb gene homolog (Drosophila)
Fgf1	Fibroblast growth factor 1
Adcyap1r1	Adenylate cyclase activating polypeptide 1 receptor1
Snrk	SNF related kinase
Acin1	Apoptotic chromatin condensation inducer 1
Ptpn11	Protein tyrosine phosphatase, non-recptor type 11
Casp3	Caspase 3
Myst3	MYST histone acetyltransferase (monocytic leukemia) 3
Robo4	Roundabout homolog 4 (Drosophila)
Efn5	Ephrim A5
Notch4	Notch gene homolog 4 (Drosophila)
Timp1	Tissue inhibitor of metalloproteinase 1
Flt1	FMS-like tyrosine kinase 1
Ccnd1	Cyclin D1
Gata6	Transcription factor
Wnt2b	Wingless related MMTV integration site 2b
Hoc6	Homeobox C6 transcription factor
Hoxc9	Homeobox C6 transcription factor
CELL CYCLE	
Anln	Anilin, actin binding protein (scrpas homolog, Drosophila)
Hpgd	Hydroxyprostaglandin dehydrogenase 15 (NAD)
Ptprv	Protein tyrosine phosphatase, receptor type V
Ptpn11	Protein tyrosine phosphatase, non-receptor type 11
Bmpr2	Bone morphogenetic factor 2
Igf2	Insulin growth factor 2
CELL PROLIFERATION	
Evi1	Ecotropic viral integration site 1
Edn1	Endothelin1
Col8a1	Procollagen, type VIII, alpha 1
IMMUNE RESPONSE	
Chst4	Carbohydrate (chondroitin 6/keratin) sulfotransferase 4
Gbp5	Guanylate nucleotide binding protein 5
Selp	Selection, platelet
Kng1	Kininogen 1
Aoc3	Amine oxydase, copper containing 3
Ptgis	Prostaglandin I1 synthase
ENZYME REGULATORY ACTIVITY	
Rasal2	Ras protein activator like 2
Arhgef15	Rho guanine nucleotide exchange factor (GEF) 15
Rgs5	Regulator of G-protein signalling 5
Rapgef5	Rap guanine nucleotide exchange factor (GEF) 5
Rgs4	Regulator of G-protein signalling 4
Kng1	Kininogen 1
Timp1	Tissue inhibitor of metalloproteinase 1
Rasgrp3	RAS guanyl releasing protein 3

Gene	description
CELL ADHESION	
Flot2	Flotilin 2
Jup	Junction plakoglobin
Fblim1	Filamin binding LIM protein 1
Cdh13	Cadherin 13
Cxdar	Coxsackievirus and adenovirus receptor
Msln	Mesithelin
Amigo2	Adhesion molecule with Ig like domain 2
Selp	Selectin, platelet
Cldn15	Claudin 15
Vcam1	Vascular cell adhesion molecule 1
Pcdhb2	Protocadherin beta 2
Vwf	Von willerbrand factor
Col8a1	Procollagen type VIII
Aoc3	Amine oxydase, copper containing 3

Figure 1: Analysis by real-time PCR of selected up-regulated genes in LacZ positive cells. The selected up-regulated genes are FGF-1, Gata6, Hoxc6, IGF-2, Notch4, Timp1, Numb, Rgs4, and K14.

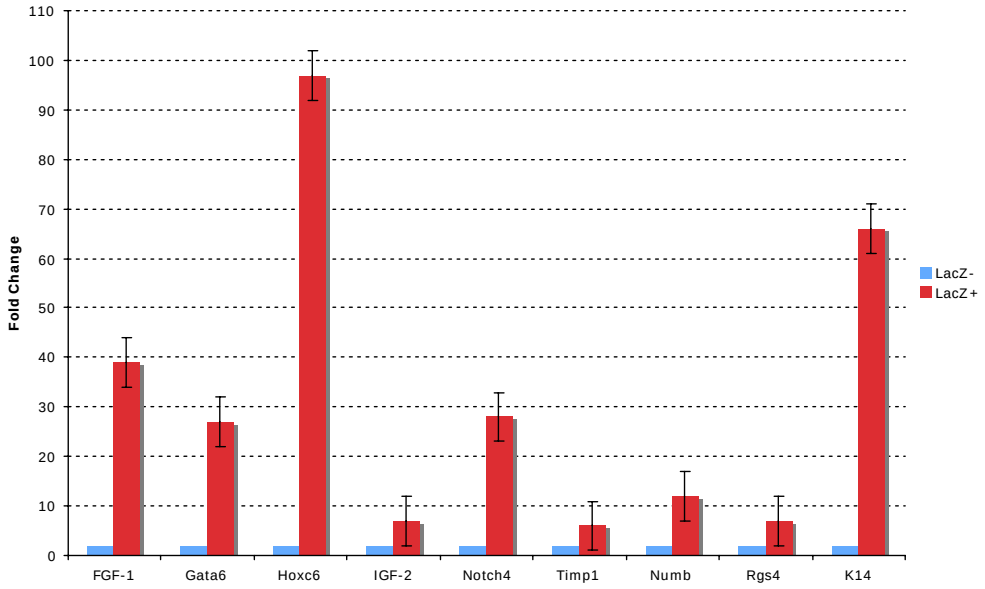
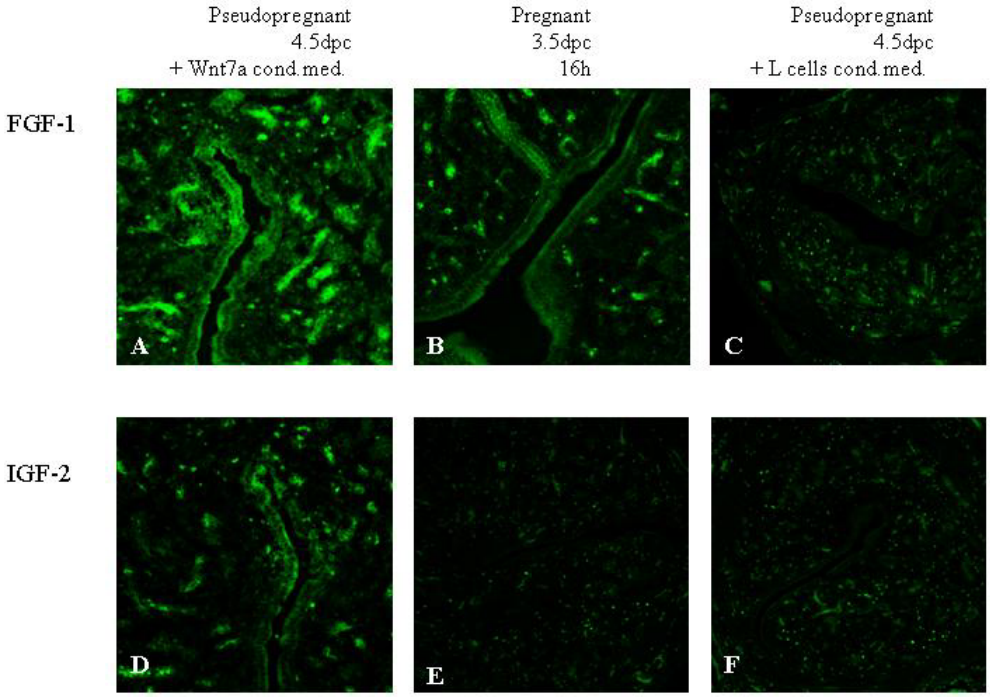


Figure 2: Expression of FGF-1 and IGF-2 in the uteri of pseudopregnant females. Expression of FGF1 (A) (B) (C) and IGF-2 (D) (E) (F) in the uteri of pseudopregnant females injected with Wnt7a conditioned medium on D4.5 (A) (D); not injected (B) (E); and injected with L cells control conditioned medium (C) (E).



DISCUSSION

Early pregnancy loss has become a major social and economic concern. Although the human population is growing rapidly, 15% of couples worldwide are childless because of infertility. Many underlying causes of human infertility have been overcome by in vitro fertilization and embryo transfer techniques; implantation rates, however, remain disappointingly low. Implantation failures are generally related to inadequate endometrial receptivity in two-thirds of cases and abnormalities of the embryo in one third (Simon *et al*. 1998). There is, therefore, a continuing needs to unravel the complexities of preimplantation embryonic development and implantation to address two contrasting global issues: improving fertility and developing novel contraceptives. This has led to an increasing interest in exploring and defining the molecular road map during the critical time of implantation. In this thesis, we identified the Wnt/β-catenin pathway as a major effector of uterine receptivity for embryo implantation. This is the first comprehensive analysis of the expression of components of the Wnt pathway, the regulation of Wnt signalling and the identification of specific Wnt/β-catenin target genes during embryo implantation in the mouse.

Cell proliferation, cell differentiation and specific epithelial-mesenchymal interaction in the uterus are regulated by the action of oestrogen and progesterone. These hormones act through their cognate receptors to alter the rates of specific gene transcription (Tsai et al. 1994; Hall et al. 2001; Li *et al*. 2003). The wnt signalling pathway plays roles in mediating epithelial to mesenchymal transition and cellular organization during embryonic and postembryonic development that involve cell proliferation and differentiation, cell fate specification and cell-to-cell communication (Cadigan *et al.* 1997; Wodraz *et al*. 1998; Smalley *et al*. 1999). One of our hypotheses was that *Wnt* genes expressed in the receptive uterus during implantation regulate establishment and maintenance of pregnancy via autocrine and/or paracrine actions through FZD receptors. In this study, we demonstrated the spatiotemporal expression pattern of components of the Wnt pathway in the endometrium at the time of embryo implantation in the mouse. We demonstrated that the activation of the Wnt/β-catenin pathway, and the

regulation of Wnt (Wnt-5a, Wnt-4, and Wnt-11), Frizzled (Fzd-2, Fzd-4, and Fzd-6), LRP co-receptors, and secreted antagonist of the Wnt pathway (sFRP-2 and Dkk-3) are under the control of ovarian oestrogen and LIF.

The majority of the functional information concerning the role of the Wnt family in adult mouse uterus comes from the study of Wnt4, which is required for the establishment of materno-foetal communication, for stromal decidualization, and for the progression of implantation in human and mouse (Lee *et al*. 2007; Li *et al*. 2007). Wnt4 is a downstream target gene of BMP-2 signalling in stromal decidualization. During decidualization, BMP-2 coordinates stromal cell differentiation (Paria *et al*. 2001; Lee *et al*. 2007; Li *et al*. 2007). In this study, we demonstrate that there is an increase in the expression level of Wnt4 from D4 to D5, which correspond to the beginning of decidualization. Thus, Wnt4 is a critical regulator of stromal decidualization linked with BMP-2.

Wnt-5a and Wnt-11 are known to signal through the non-canonical pathway. It is currently unknown whether the non-canonical Wnt pathway is important for embryo implantation in mice. This signalling pathway has been shown to be involved in the regulation of cell polarization and movement, including orientation of cilia (Ross *et al.* 2005) and sensory hair cells (Jones *et al*. 2007). Indeed, I have demonstrated the spatio-temporal expression of Wnt-5a and Wnt-11 in the uterus. Expression of Wnt-5a and Wnt-11 is restricted to the luminal epithelium on the antimesometrial side of the uterus on D4 and D5. Thus, Wnt-5a and Wnt-11 may play a role in orienting the implantation chamber and in specifying the implantation site in the uterus at the time of embryo implantation.

In order to mediate their signals, Wnt protein must interact with their cognate receptor Fzd and their co-receptor LRP. We have demonstrated that Fzd-2, Fzd-4, and Fzd-6 are expressed in the uterus at the time of embryo implantation. Previous studies have shown that Fzd-2 is present in the mouse uterus (Hou *et al.* 2004), Fzd-4 is involved in retinal angiogenesis (Robitaille *et al.* 2002), and Fzd-6 is expressed in the endometrial epithelia during early

pregnancy in sheep (Hayashi *et al*. 2007). While the specific receptor-ligand binding elationship have yet to be established in the uterus, Fzd-2, Fzd-4 and Fzd-6 were expressed in the luminal epithelium of the uterus at the time of embryo implantation. Thus, we hypothesize that Wnt genes expressed in the adult mouse uterus mediate intracellular signal transduction via these three receptors, and play critical roles for implantation, including stromal decidualization.

Two secreted antagonists of the Wnt pathway, Dkk-3 and sFRP-2 are also spatio-temporally regulated in the uterus at the time of embryo implantation. Dkk-3 is upregulated on D4. Then the level of expression of Dkk-3 decreases on D5. An explanation for this is that Dkk-3 may regulate the Wnt signaling pathway in a negative feedback loop.

We have demonstrated that the Wnt/β-catenin pathway was activated in the uterus during the peri-implantationo period. At this time, the blastocyst and the receptive uterus express a multitude of Wnt genes (Mohamed *et al*. 2004). It is not clear whether Wnt secreted by the blastocyst or by the uterus are esponsible for the activation of the Wnt/β-catenin in the uterus. Wnt constitute a familu of cystein-rich glycoproteins that are highly insoluble (Hendriks *et al*. 2002). Indeed, they tend to stick to the membrane of the cells from which they are secrted and have a short range of action. Thus, it is unlikely that Wnt secreted by the blastocyst activate the Wnt/β-catenin in the uterus, unless the blastocyst comes into very close contact with the luminal epithelium. However, at the time of embryo apposition, when the blastocyst establishes the first contact with the uterine luminal epithelium, the Wnt/β-catenin is already activated. It is most likely that Wnt secreted by the luminal epithelium acts in a paracrine manner to activate the Wnt/β-catenin in the uterus. Althought the activation of the Wnt/β-catenin signalling is probably not triggered by Wnt secreted by the blastocyst, it requires the presence of the blastocyst to occur.

In mice, Wnts have been shown to play important roles in female reproductive tract development after birth. The levels and the sites of Wnt expression in adult mouse uteri fluctuate during estrous, suggesting a continued role in the adult (Miller *et al.* 1998). Available studies demonstrate that components of the Wnt pathway show a dynamic expression pattern in the endometrium of ovine and humans during oestrous cycle, suggesting a role for steroid hormones in the regulation of the Wnt pathway (Tulac *et al.* 2003; Hayashi *et al.* 2007). Therefore, the canonical Wnt signalling pathway could act as a conserved regulator of endometrial function across mammals, and of embryo-uterine cross-talk. Moreover, the wnt pathway could be the mediator of steroid hormone action in the female reproductive tract.

Although we have demonstrated that the inhibition of the Wnt/β-catenin signalling in the uterus by injection of sFRP2 drastically reduced the embryo implantation rates, none of the individual Wnt gene mutations have resulted in infertility due to implantation failure (Table 4). Loss of function studies of components of the Wnt pathway is complicated by the complexity of the Wnt network, involving a number of homologs of Wnt, Frizzled and secreted antagonists. Moreover, promiscuity of Wnt-frizzled interactions may generate functional redundancy (Gordon and Nusse 2006). Likewise upstream signalling converges to the same intracellular effectors, compromising in this way analysis of downstream Wnt pathways components. Consequently, the specific role of Wnt, Frizzled and secreted antagonists during embryo implantation can hardly be studied with mouse knockout models. The use of morpholino oligonucleotide targeting various components of the Wnt pathway, such as LRP5/6 and Dishevelled, is an alternative to classical knockout mouse models. Morpholino have been used successfully to knockdown the expression of Cox-2, PC6 and calbindins in the uterus at the time of embryo implantation (Tyson-Capper AJ 2006; Nie G 2005; Luu KC 2004). Using morpholino oligonuclotides could help unravel the specific roles of components of the Wnt pathway in the uterus at the time of embryo implantation.

There is a need to identify reliable markers of uterine receptivity and to develop the means to extend uterine receptivity or to treat nonreceptivity to improve the pregnancy rate in *in vitro* fertilization and embryo transfer techniques. Overcoming these challenges will lessen the need to transfer multiple embryos to increase the pregnancy rate and the resulting complication of multiple pregnancies. In this study, we identified the Wnt/β-catenin pathway as a marker of endometrial receptivity in the mouse endometrium. The ability of the uterus to respond to the presence of the embryo by activating the Wnt/β-catenin patway could be used as a marker of endometrial receptivity.

Genome-wide analysis of the human endometrium has detected the modulation of numerous genes during the window of implantation including those for cholesterol trafficking and transport, prostaglandin biosynthesis and action, proteoglycan synthesis, IGF binding protein, signal transduction, extracellular matrix components, neurotransmitter synthesis and receptors, immune modulators, detoxification genes and genes involved in water and ion transport. In this study, we identified the downstream target genes of the Wnt/β-catenin pathway in the uterus at the time of embryo implantation. Genes up-regulated by this pathway are involved in cell proliferation, cell differentiation, cell-to-cell communication, and cell adhesion. We have demonstrated that this pathway is involved in the expression of genes required for successful implantation to occur, such as IGF-2 and FGF-1. This study allowed us to clarify the role of the Wnt/β-catenin pathway, and to shed light on the hierarchy of the signalling network that directs embryo implantation. We hypothesized that Wnt genes expressed by the blastocyst at the time of embryo implantation activate the Wnt/β-catenin pathway in the uterus to indicate to the endometrium that the blastocyst is ready to implant. Activation of the Wnt/β-catenin pathway in the uterus requires the presence of oestrogen surge and LIF secretion. The Wnt/β-catenin pathway acts downstream the oestrogen surge and is involved in the activation of the expression of target genes that are crucial for embryo implantation. This study establishes a novel level of hierarchy in the process leading to successful embryo implantation.

The data presented here offer the opportunity to develop an endometrial database of genes expressed during the window of implantation. The current study validates using microarray technology to investigate global changes in gene expression in human endometrium and can be extrapolated to defining the genetic profiles of human receptivity. This study sets the stage to develop a screen for candidate genes in patients with infertility and for targeted drug discovery for enhancing (or inhibiting) implantation for infertility treatment (or contraception).

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Annex 1: Ethic protocol

ACTION	✓	DATE
CCs		
DB	✓	Nov 26/08
APPROVED		

www.mcgill.ca/researchoffice/compliance/animal/forms/

For Office Use Only:
Protocol #: 5261
Approval end date: Sept 30, 2009
Facility Committee: RVH
Renewal#: 1st (2nd)

McGill University Animal Care Committee
RENEWAL of Animal Use Protocol

For: Research ☒ Teaching ☐ project

Principal Investigator: Daniel Dufort **Protocol #** 5261

Protocol Title: Deciphering the signaling pathways involved in embryo-uterine communication during implantation **Category:** C

Unit, Dept. & Address: Obstetrics and Gynecology, RVH, Women's pavilion Room F3-24

Email: daniel.dufort@mcgill.ca **Phone:** 934-1934 X34743 **Fax:** 843-1662

Funding source: CIHR 89734

Start of Funding: April 2007 **End of Funding:** March 2012

Emergency contact #1 + work AND home phone #s: Daniel Dufort: Work# 934-1934 X34743 Home# 514 376-9436

Emergency contact #2 + work AND home phone #s: Yuefei Lou: Work# 834-1934 X36968 Home# 450 441-6645

FR=SOK

1. Personnel and Qualifications

List the names of the Principal Investigator and of all individuals who will be in contact with animals in this study and their employment classification (investigator, technician, research assistant, undergraduate/ graduate student, fellow). Indicate if the Principal Investigator is not handling animals. If an undergraduate student is involved, the role of the student and the supervision received must be described. Training is mandatory for all personnel listed here. Refer to *www.animalcare.mcgill.ca* for details. Each person listed in this section must sign. *(Space will expand as needed)*

Name	Classification	Animal Related Training Information		Occupational Health Program *	Signature *"Has read the original full protocol"*
		UACC on-line Theory course	Workshops + others		
Daniel Dufort	P.I.	Yes (McGill)	Yes (McGill)	yes	
Youfei Lou	Research Ass	(McGill)	(McGill)	yes	Youfei Lou
Craig Park	Grad Student	(McGill)	(McGill)	yes	
Sophia Akl	Grad Student	(McGill)	(McGill)	yes	Sophia AKL

*** Indicate for each person, if participating in the local Occupational Health Program, see http://www.mcgill.ca/researchoffice/compliance/animal/occupational/ for details.**

Approved by: FACC approved à Rev. Modif. (Sec. 3) clp

2. Approval Signatures

Principal Investigator/ Course Director		**Date:** Oct 2, 2008
Chair, Facility Animal Care Committee A. C. O.	Jean C. Patterson	**Date:** 12 Nov 2008
Chairperson, Ethics Subcommittee *(D level or Teaching Protocols Only)*		**Date:** Nov, 98 08
Approved Animal Use Period	**Start:** Oct. 1, 2008	**End:** Sept 30, 2009

26 NOV. 2008

☐ **Renewal requires submission of full Animal Use Protocol form**

Form version July 2007

3. Summary (in language that will be understood by members of the general public)
AIMS AND BENEFITS: Describe, in a short paragraph, the overall aim of the study and its potential benefit to human/animal health or to the advancement of scientific knowledge *(was section 5a in main protocol).*

A crucial event during mammalian embryonic development is implantation, during which the free-living blastocyst attaches to the uterine endometrium. Successful implantation depends on precisely orchestrated and reciprocal signaling between the implanting blastocyst and the receptive uterus. For example, blastocysts can implant only after they have been activated by uterine factor(s) that are regulated by ovarian steroid hormones. Conversely, expression of several uterine genes are regulated by signal(s) emanating from active blastocyst. Thus far, however, no specific signaling pathway has been identified that could mediate this critical embryo-maternal interaction.We have demonstrated that the Wnt signaling pathway may be involved in this embryo-uterus communication. We have shown that the blastocyst expresses many Wnt genes and that the Wnt pathway is activated in the uterus before implantation. We are currently trying to determine if this pathway is required for implantation. Also, we are trying to identify genes whose expression is regulated by this pathway to better understand how the embryo and uterus communicate and prepare for implantation.A better understanding of this embryo-uterine communication will greatly improve the successful rate of embryo implantation both in IVF patients as well as in couples experiencing repeated implantation failures.

Aim 1. Is Lif* required for Wnt signaling in the luminal epithelium.
Aim 2. How does Lif regulate Wnt signaling in the luminal epithelium.
Aim 3. What are the downstream target genes of the Wnt/b-catenin signaling pathway.

* LIF = Leukemia Inhibitory Factor

4. Has there been any animal care issues?	**YES** ☐	**NO** ☒	**if yes, supply details:**

5. If creating genetically modified animals or new combinations of genetic modifications, complete and attach a *Phenotype Disclosure form.*
If mice expressing new phenotype have been produced, submit a *Phenotype Disclosure form.*
Blank forms at *http://www.mcgill.ca/researchoffice/compliance/animal/forms/*

6. Procedures

a) For B and C level of invasiveness,

The procedures are the same as the original protocol: **YES** ☒ **NO** ☐

IF NO, complete the following:
Detail new procedures that are different from section 10a of the original protocol, including amendments *(include a copy of the entire revised procedure section 10a of the original protocol with the changes and/or new procedures in CAPS):*

b) For D level of invasiveness,

Include here ALL procedures described in the original protocol. New and changed procedures in CAPS *(was section 10a in main protocol); Please only attach SOPs related to new and changed procedures to this renewal form.*

7. Endpoints

a) For B and C level of invasiveness,

The procedures are the same as the original protocol: YES☒ NO☐

IF NO, supply new endpoints that are different from the original protocol:

Experimental endpoints:

Clinical endpoints:

b) For D level of invasiveness,

Include here ALL endpoints, including the ones described in the original protocol as well as new and changed endpoints in CAPS:

Experimental endpoints:
In all experimental groups, females will be mated with either stud or vasectomized males. On day 3 after plug, mice plug with stud males will be sacrified as described and embryos isolated. Females that have been mated with vasectomized males will be anaesthisized and 4-5 embryos will be transfer in each uterine horn. On day 5 (two days after surgery), these females will be sacrificed and the uterus isolated. Male and female Breeders will be kept for 8 months.

Clinical endpoints:
All our experiments will involve first mating the mice and on day 3 after mating, either embryos or wnt proteins will be introduced within the uterus. These mice will then be sacraficed on Day 5. If any distress is observed in these mice, such as excessive weight loss (greater than 20%), or lack of grooming, the animal will be sacrificed.

Animals will be monitored on a regular basis at least three times weekly. If any distress is observed, such as excessive weight loss (greater than 20%), or lack of grooming, the animal will be sacrificed.

8. Hazards (check here if none are used: ☐)

a) Are the hazards different from original protocol? (infectious, radioactive, toxic, carcinogen, tumours)

YES☐ NO☒ if yes, supply details (material, risks, precautions):

b) Have the cell lines been tested for human and animal pathogens? YES:☐ NO:☐ None used:☒

9. Description of Animals to be used in the coming year (only):

Quality Control Assurance: To prevent introduction of infectious diseases into animal facilities, a health status report or veterinary inspection certificate may be required prior to receiving animals from all non-commercial sources or from commercial sources whose animal health status is unknown or questionable. Quarantine and further testing may be required for these animals. *If more than 6 columns are needed, please attach another page*

	Sp/strain 1	Sp/strain 2	Sp/strain 3	Sp/strain 4	Sp/strain 5	Sp/strain 6
Species	Mouse	Mouse	Mouse	Mouse	Mouse	Mouse
Supplier/Source	Charles River	Charles River	in house	in house	in house	in house
Strain	CD1	CD1	FTF	LIF	Nodal-LacZ	Floxed-Nodal
Sex	Female	Male	F & M	F & M	F & M	F & M
Age/Wt	5-7 weeks	2-9 months	2-9 months	2-9 months	2-9 months	2-9 months
# To be purchased	400	60	0	0	0	0
# Produced by in-house breeding	0	0	100	100	60	100
# Other (e.g.field studies)						
TOTAL# /YEAR	**400**	**60**	**100**	**100**	**60**	**100**

10. Explanation of Animal Numbers:

BASED ON THE EXPERIMENTAL OBJECTIVES OF THE PROJECT, describe the number of animals required for one year. Include information on experimental and control groups, # per group, and failure rates.

For breeding, specify how many adults are used, number of offspring produced, and how many offspring are used in experimental procedures. The arithmetic explaining how the total of animals for each column in the table above is calculated should be made clear.

Most of our mice requirements will be for CD1 females. We expect to perform 20 experiments per year and each expeiment will require the purchase of 20 females. These will be used to 1) isolate embryos (for aim 1) or 2) used as recepients for embryo transfer. Thus 20 X 20 = 400 mice per year.

We will need 15 stud males as well as 15 vasectomized males at all times. These males will be used to generate embryos (stud Males) and to generate pseudo pregnant females for embryo transfer (vasectomized males). These males will be kept for 6 months, Therefore we will require 60 males per year.

In order to study the role of FTF in maintaining pregnancy, we will need to generate approximately 100 mice per year. We will have 5 breeding pairs to generate heterozygous FTF females.

Homozygous LIF females are sterile and we will be examining the role of LIF signaling during implantation as well as its relationship to Wnt signaling. We will need 10 breeding pairs to generate at least 100 homozygous LIF females. We will need approximately 80 females to identify the downstream components of the LIF signaling pathway. Furthermore, we will need heterozygous females to mate with other strains of mice (TCF/Lef-LacZ, Nodal). 80 + 20 = 100

Nodal has been shown to be expressed in the uterus at the time of implantation. We will need to fully characterize the the expression pattern during the pre-implantation and post-implantation stages. To do this we will require 40 Nodal heterozygous mice. Furthermore, as for Lif mice, we will need 20 Nodal heterozygous mice to cross to various other strains of mice. 40 + 20 = 60

We have shown that Nodal is expressed at the time of implantation. We will now perform a uterine specific deletion of the Nodal gene. To achieve this we will use the homozygous Floxed Nodal mice. We will these mice with a uterine cre specific trangenic stain (see below). To characterize Nodal phenotype, we will need 10

Additional table to add to 'Description of animals' in full Animal Use Protocol form as well as Renewal form						
	Sp/strain	**Sp/strain**	**Sp/strain**	**Sp/strain**	**Sp/strain**	**Sp/strain**
Species	Mouse	Mouse				
Supplier/Source	in house	in house				
Strain	PR-Cre	TCF/LacZ				
Sex	F & M	F & M				
Age/Wt	2-9 months	2-9 months				
# To be purchased	0	0				
# Produced by in-house breeding	30	140				
# Other (e.g.field studies)						
#needed at one time						
# per cage	4	4				
TOTAL# /YEAR	**30**	**140**				

Additional table to add to 'Description of animals' in full Animal Use Protocol form as well as Renewal form						
	Sp/strain	**Sp/strain**	**Sp/strain**	**Sp/strain**	**Sp/strain**	**Sp/strain**
Species						
Supplier/Source						
Strain						
Sex						
Age/Wt						
# To be purchased						
# Produced by in-house breeding						
# Other (e.g.field studies)						
#needed at one time						
# per cage						
TOTAL# /YEAR						

breeding pairs to generate Nodal females. We will need approximately 80 females to characterize the Nodal phenotype. Furthermore, we will need heterozygous females to mate with other strains of mice (TCF/Lef-LacZ, Nodal, LIF). 80 + 20 = 100

To generate the uterine specific deletion of Nodal we will use the PR-Cre transgenic mice. We will maintain 5 breeding pairs to have males available at all times. We will use approximately 10 males per 6 month periods. Thus 5 breeding pairs (10 mice) + 10 males (X2 for 12 months = 30 mice

We have generated a Wnt reporter transgenic line called Tcf/Lef-LacZ. We will use these mice to cross the transgene into the FTF, Nodal, LIF and PTC mice to examine the effect of these mutations on Wnt signaling. We have also had a high demand for exporting these mice. Thus, we will need 10 breeding pairs to maintain the strain as well as export the mice. We will also need approximately 40 animals for crossing the transgene in various knock-out lines described previously. Since the main focus of this grant is to understand the role of the Wnt signaling pathway during implantation, we will nee to generate a large number of heterozygous females for our studies. We will need 100 females to be used as recepient females for embryo transfer per year. 40 + 100 = 140

Submit to your local Facility Animal Care Committee. Please note that after two renewals, a full protocol needs to be submitted.

This approval does not imply that space will be made available. If a major increase of space needs is anticipated, please contact the appropriate animal facility manager.