Paracrine Wnt Mediators in Decidual-Placental Communication

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Declaration

The experimental work described in this thesis is original and carried out by myself in the School of Women's and Infant's Health, The University of Western Australia under the supervision of Professor Jeffrey Keelan (School of Women's and Infant's Health) and Professor Arun Dharmarajan (School of Anatomy, Physiology and Human Biology, The University of Western Australia and School of Biomedical Sciences, Curtin University), with the financial assistance of a University Postgraduate Award.

This thesis contains co-authored published work and work prepared for publication. The bibliographical details of the work and where it appears in the thesis are outlined under the heading "Publications arising from this work, page xiv" below. All the experimental works, data analysis and the writing of first drafts were performed by me. Corrections and editing were carried out by my supervisors (Professor Keelan and Professor Dharmarajan). Dr. Frank Arfuso made additional edits to the published review.

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Date: Date:

Abstract

The integration of a complex network of signalling molecules promotes implantation of blastocyst and the development of placenta. These processes are crucial for a successful pregnancy and optimal fetal growth and development. The signalling network involves both cell-to-cell and cell-to-extracellular matrix communication. The family of secreted glycoprotein ligands, Wnts, plays a major role in regulating a wide range of biological processes, including embryonic development, cell fate, proliferation, migration, stem cell maintenance, tumour suppression, oncogenesis and tissue homeostasis.

Several studies have previously investigated the expression and significance of Wnt ligands and other signalling components in human endometrium, trophoblast and in placenta. Wnt signalling has also been implicated in various reproductive pathologies. Collectively these studies suggest a tightly regulated control of the expression of Wnt components is essential for a successful pregnancy and maintaining a healthy reproductive system.

Effective reciprocal interactions between the implantation-competent blastocyst and the receptive uterus are required for a successful implantation. Uterus and blastocyst both must undergo synchronous development and differentiation for implantation. However, our understanding of paracrine Wnt signalling interactions between trophoblast and decidua remain largely incomplete; the expression patterns of secreted ligands and antagonists during this time is of particular significance and interest.

The initial aim of this thesis was to characterize the expression of all the known Wnt components in decidualised endometrial cells and in trophoblast cells. Expression of 84 Wnt-associated genes was determined in cells in culture using PCR array technology. Expression of a wide range of Wnt-related genes was observed in both decidual and trophoblast cells using PCR array, with remarkably similar expression profiles.

Our next aim was to determine the effects of tissue cross-talk between trophoblast and decidua on expression and functioning of Wnt signalling proteins. We employed a co-culture model followed by PCR array analysis, with the goal of exploring the effects of secreted factors from decidual and trophoblast cells on cellular function, with a particular interest in the Wnt pathway and its secreted antagonists. Co-culture induced

altered expression of several Wnt-related proteins, with the Wnt inhibitors sFPR4 and DKK1 being among the most differentially expressed genes. Media concentrations of sFRP4 and DKK1 proteins were increased with co-culture, coincident with a decrease in canonical Wnt signalling activity. Collectively, these findings suggest that the close proximity of trophoblast with decidua leads to enhanced production and action of Wnt antagonists and reduced Wnt signalling in trophoblast.

An additional, presumably down-stream, consequence of co-culture was the observed increase in prostaglandin (PG) E_2 production in both the decidual and trophoblast compartment. We measured mRNA of COX1/PTGS1 and COX2/PTGS2 and identified a significant increase in COX1 expression in trophoblast with co-culture, whereas COX2 expression was unaltered. The COX1 expression changes were also seen at the protein level by immunoblotting, adding weight to the possibility that this increase was responsible for the observed increase in PGE_2 levels. The addition of exogenous DKK1 and sFRP4 to trophoblast cultures increased both the production of PGE_2 and the expression of COX1 protein, consistent with the interpretation that the effects of co-culture on trophoblast PG production were, at least in part, mediated through the paracrine actions of these Wnt antagonists.

Our findings of up-regulation of PGE_2 production in decidual-trophoblast co-culture are consistent with the literature showing a role for PGs in decidualisation and trophoblast invasion. PGs produced in the trophoblast most likely act in a synergy with secreted factors from decidua, including decidua-derived PGs, in an autocrine or paracrine manner to modulate trophoblast invasion and promote formation of a healthy, fully functional placenta. Various studies show a positive relationship between Wnt signalling and PG production; this raises the possibility of a reciprocal relationship between Wnt antagonists and PGE_2 in decidual-trophoblast co-cultures, with elevated Wnt antagonist levels leading to increased PGE_2 production which in turn helps to stimulate Wnt antagonist production.

In conclusion, data from this thesis suggest that cell-to-cell interactions between decidua and trophoblast stimulate Wnt antagonist secretion leading to increased placental PG production. This may be important for implantation and placental function.

Acknowledgements

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Abbreviations

APC	Adenomatous polyposis coli
BCA	Bicinchoninic assay
CK-1	Casein kinase1
COX	Cyclooxygenase
CRD	Cysteine rich domain
CTB	Cytotrophoblast
d	Day
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
DEPC	Diethylpyrocarbonate
dGTP	Deoxyguanosine triphosphate
DKK1	Dickkopf
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
D-PBS	Dulbecco's phosphate buffered saline
dTTP	Deoxythymidine triphosphate
DVL	Dishevelled
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay

ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
FZD	Frizzled receptors
GSK3β	Glycogen synthase kinase 3
h	hour
HBSS	Hank's buffered salt solution
HOXA10	Homeobox A10
LEF	Lymphoid enhancer-binding factor
LRP	Lipoprotein receptor-related protein
min	Minute
MSX	Msh homeobox
PBS	Phosphate buffered saline
PBS-T	Phosphate Buffered Saline with Tween 20
PCP	planar cell polarity
PCR	Polymerase chain reaction
PET	Polyethylene terephthalate
PGE_2	Prostaglandin E2
PGs	Prostaglandins
PTGES	prostaglandin E synthase
PTGS	prostaglandin-endoperoxide synthase
PVDF	Polyvinylidene fluoride
Q-PCR	Quantitative polymerase chain reaction
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RPM	Rotation per minute

RT-PCR	Reverse transcription polymerase chain reaction
SDHA	Succinate Dehydrogenase subunit A
SDS	Sodium dodecyl sulphate
sFRPs	Secreted FZD-related protein
TBS-T	Tris-Buffered Saline with Tween 20
TCF	T-cell factor
TMB	3,3′,5,5′-Tetramethylbenzidine
WIF1	Wnt inhibitory factor

Publications arising from this work

This thesis contains published work and work prepared for publication, both are co-authored. The bibliographical details of the work and where it appears in the thesis are outlined below.

Chapter 1

1. **Nayeem, S.B.,** Arfuso, F., Dharmarajan, A., and Keelan, J.A. (2014) Role of Wnt signalling in early pregnancy. *Reproduction, Fertility and Development*. 2014 Sep 5. doi: 10.1071/RD14079. [Epub ahead of print].

Chapter 3, 4 and 5

2. **Nayeem, S.B.,** Arfuso, F., Dharmarajan, A., and Keelan, J.A. (2014) Paracrine communication modulates production of Wnt antagonists and COX1-mediated prostaglandins in a decidual-trophoblast co-culture model. Mol Cell Endocrinol. 2015 Apr 15;405:52-62. doi: 10.1016/j.mce.2015.02.003. Epub 2015 Feb 10

Conference presentation

1. **Nayeem SB,** Dharmarajan A, Keelan J; Wnt signalling cross-talk between trophoblast and decidual cells in co-culture. 2014 *Society for Gynaecological Investigations (SRI) 61st Annual scientific meeting*, Florence, Italy (poster presentation).

2. **Nayeem SB,** Dharmarajan A, Keelan J; Role of secreted Wnt signalling inhibitors in cross-talk between trophoblast and decidual cells in co-culture. *Combined ASBMB, ASPS and ANZSCDB meeting*, 2013, Perth, Australia (oral presentation).

3. **Nayeem SB,** Dharmarajan A, Keelan J; Role of secreted Wnt signalling inhibitors in cross-talk between trophoblast and decidual cells in co-culture. *Australian Society for Medical Research Medical Research Week*, WA Scientific Symposium 2013, Perth, Australia (oral presentation).

4. **Nayeem SB,** Dharmarajan A, Keelan J; Wnt antagonists are downregulated in trophoblast decidul co-culture. *Australian Health and Medical Research Congress 2012*. Adelaide, Australia (poster presentation).

5. **Nayeem SB,** Dharmarajan A, Keelan J; Wnt signaling in trophoblast decidul co-culture. *Annual Endocrine and Reproductive Biology Society of WA meeting*, 2012. Perth, Australia (oral presentation).

Chapter 1: Background and literature review.

1.1 Introduction

Implantation of the blastocyst and early development of the placenta is crucial for successful fetal growth, development and delivery (Cartwright *et al.* 2010; Cha *et al.* 2012; Fritz *et al.* 2014). The processes of implantation and trophoblast invasion are key limiting factors for the optimal establishment of the pregnancy. These processes are promoted by the integration of a complex network of signalling molecules that mediate cell-to-cell and cell-to-extracellular matrix communications (Cha *et al.* 2012; Zhang *et al.* 2013a). The limited time span when blastocyst competency is coordinated with the receptive state of the uterus is called the window of implantation (Ramathal *et al.* 2010; Zhang *et al.* 2013a). Implantation fails or is compromised if this coordination is out of phase. Implantation beyond the window can result in spontaneous miscarriage or defective implantation that can generate adverse effects throughout the pregnancy (Cartwright *et al.* 2010).

Reciprocal interactions between the blastocyst and the uterus coordinate the implantation and placentation processes (Cartwright *et al.* 2010; Dimitriadis *et al.* 2010; Cha *et al.* 2012). Recent progress from global genomic microarray screening and other powerful multiplex array techniques, together with transgenic mouse models, has provided us clues and insights into crucial players of blastocyst-uterine dialog (Dimitriadis *et al.* 2010; Ramathal *et al.* 2010; Cha *et al.* 2012; Zhang *et al.* 2013a; Fritz *et al.* 2014). This thesis focusses on wingless-type MMTV integration site family proteins or Wnts, secreted pleiotropic cysteine-rich glycoproteins which act as autocrine and paracrine signalling ligands via binding to cell surface receptors (Frizzled receptors: FZD) and initiating activation of transcription factors.

The Wnt proteins are defined by amino acid sequence, not by any functional properties (Logan and Nusse 2004; Niehrs 2012). Wnts are 350-400 amino acids in length and about 40 kDa in size **(Figure 1.1, 1.2).** Following the signal sequence they carry a number of conserved cysteine residues, one of which is subjected to palmitoylation (Tanaka *et al.* 2002; Clevers and Nusse 2012). Many of the cysteines are hypothesized to form disulphide bonds crucial for the correct folding of the protein. Wnt ligands do

not share any obvious domain structure with other proteins, except the N-terminal signal sequence, which targets them to a secretory pathway (Port and Basler 2010). Recently, the crystal structure of the *Xenopus* Wnt8 ligand bound to its FZD- receptor was determined; it revealed two specific Wnt domains interacting with the receptor (Janda *et al.* 2012). One of these domains contained the palmitoleic acid moiety inserted into a pocket of the receptor's cysteine rich domain (CRD). This configuration confirmed the importance of palmitoylation in Wnt signalling (Janda *et al.* 2012) (**Figure 1.2**).

Since their original discovery in 1982 (Nusse and Varmus 1982), 20 mammalian Wnt proteins have been identified that are divided into 12 subfamilies (Clevers 2006; Cadigan and Peifer 2009; Niehrs 2012). Wnts interact with target cells by binding with a heterodimeric receptor complex, which consists of a FZD- receptor and lipoprotein-receptor-related protein (LRP) 5/6. The FZD proteins are 7 pass transmembrane receptors with an extracellular 120 amino acids long frizzled (FZD) domain (**Figure 1.2**). The extracellular domain is called the N-terminal CRD. The FZD receptor protein family has 10 members, all of which are part of the G-protein coupled receptor superfamily (Wang *et al.* 2006; Clevers and Nusse 2012). In addition, Wnts have also been reported to signal through members of the receptor-like tyrosine kinase (Ryk) and receptor tyrosine kinase-like orphan receptor (Ror) families of single span transmembrane receptors with an intracellular tyrosine kinase domain (Rao and Kühl 2010).

Three pathways are known to be activated by the binding of Wnts to their receptors. The canonical Wnt/β-catenin cascade, the non-canonical planar cell polarity (PCP) pathway, and the non-canonical Wnt/Ca^{2+} pathway (Clevers 2006; Cadigan and Peifer 2009) (**Figure 1.1**). There is no clear cut consensus on which ligand-receptor interactions activate which pathway. Various factors, including cell type, context, receptor expression profile and ligand concentrations have been proposed to influence which cascade is activated (van Amerongen *et al.* 2008). Wnt signalling plays central roles in embryogenesis, organogenesis and tissue homeostasis in adult organisms. Abnormal Wnt signalling is often associated with severe human diseases, including cancer, osteoporosis and other degenerative diseases (Logan and Nusse 2004; Clevers 2006; Cadigan and Peifer 2009; Niehrs 2012).

Accumulating evidence suggests that, among a range of identified signalling pathways, the Wnt signalling pathway plays a particularly important role in implantation and placentation (Chen *et al.* 2009; Sonderegger and Pollheimer 2010). The intricate interplay between the blastocyst and endometrium during implantation shares similarities with the cell-cell communications displayed during organogenesis. The invasive nature of implantation is often compared with cancer, so it is not surprising that Wnts are involved in regulating a variety of processes and pathways common to both.

1.2 Wnt signalling pathways

1.2.1 Canonical signalling

The canonical pathway is the best understood signalling pathway among the pathways induced by Wnt ligands. The central signalling molecule of this pathway is β-catenin, a member of the armadillo family of proteins (Huber *et al.* 1997) (**Figure 1.2**). In the absence of Wnts (off-state), β-catenin is mostly bound with the E-cadherins and α-catenins in the adherens junctions to maintain epithelial integrity (**Figure 1.1**). Excess cytoplasmic β-catenin is complexed with the so called destruction complex composed of the core scaffold protein axin, adenomatous polyposis coli (APC), casein kinase1 (CK1) and glycogen synthase kinase 3 (GSK3). β-catenin is phosphorylated in this complex by CK1 and GSK3, resulting in its degradation through an ubiquitin/proteasome pathway (Aberle *et al.* 1997; Ikeda *et al.* 1998; Liu *et al.* 2002; Stamos and Weis 2013). This results in maintenance of low levels of cytoplasmic β-catenin and therefore minimal transcriptional activation. In the presence of Wnt ligands (on-state), binding of Wnts to the CRD domain promotes FZD-LRP heterodimerisation, which activates dishevelled (Dvl), a key cytoplasmic phosphoprotein (Clevers 2006; Gordon and Nusse 2006). Wnt signalling also promotes LRP5/6 phosphorylation at the cytoplasmic tail by GSK3 and CK1 (Zeng *et al.* 2005). Phosphorylated LRP5/6 recruits axin to the plasma membrane, thereby inactivating the 'destruction complex'. The net result is the formation of a large ribosome-size Wnt-FZD-LRP-DVL 'signalosome' (Bilic *et al.* 2007), with accumulation of β-catenin in the cytoplasm followed by translocation to the nucleus **(Figure 1.1)**.

β-catenin is a coregulator, binding to other transcription factors. The T-cell factor (TCF) family of transcription factors anchor β-catenin in the nucleus; they are responsible for much of the transcriptional response to β-catenin. Besides TCFs, β-catenin can also be recruited by other transcription factors to regulate gene expressions, including paired-like homeodomain 1 (PitX1), forkhead box protein O-1 (FOXO-1) and sex determining region Y-box 17 (SOX17) (Cadigan and Peifer 2009). TCFs are thought to act as bimodal regulators of Wnt signalling. In the absence of Wnts, TCFs bind with the transducing-like enhancer of split (TLE)-groucho-grg corepressor family of proteins to repress target gene expression (Cavallo *et al.* 1998). The β-catenin interaction with the TCF N-terminal antagonizes the repressor complex and recruits co-activators to convert it from a repressor to an activator (Willert and Jones 2006). Once formed, this activator complex modulates expression of a plethora of genes (Willert *et al.* 2002; Klapholz-Brown *et al.* 2007; Wetendorf and DeMayo 2012) **(Figure 1.1)**. These genes, in turn, play a central role in important cellular processes such as embryogenesis and tissue homeostasis.

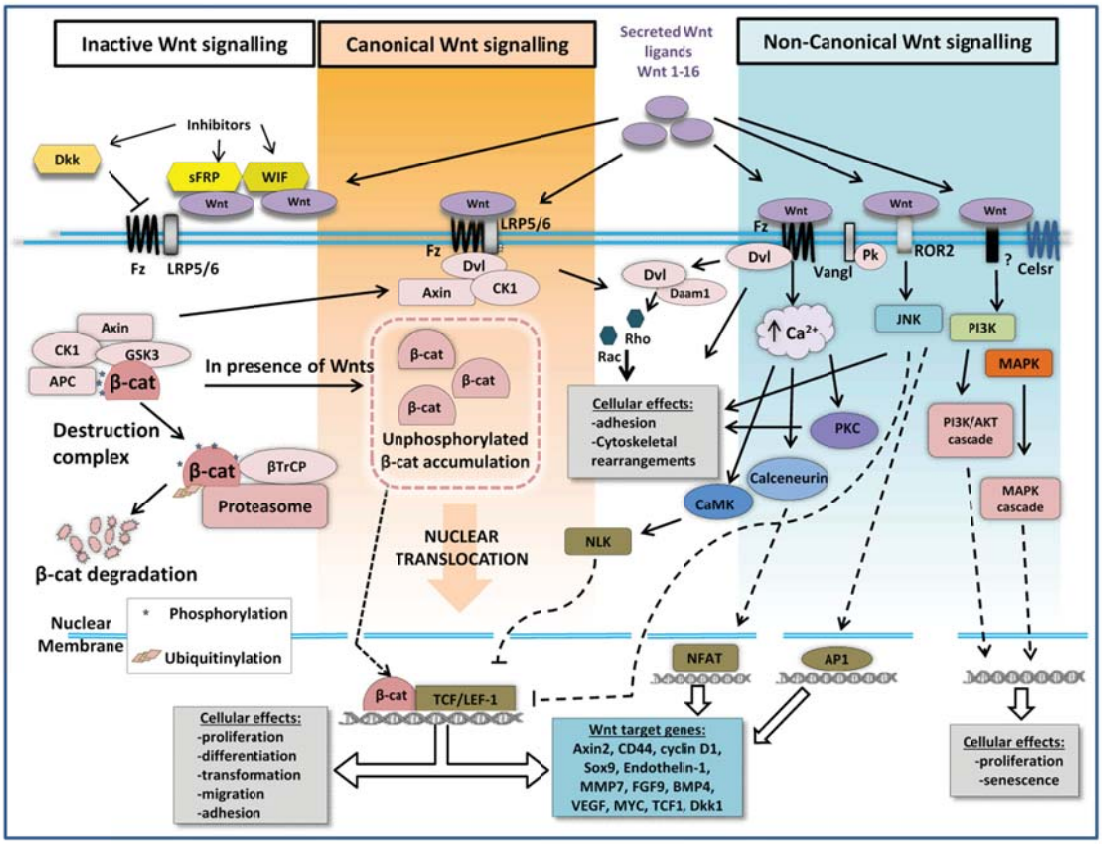


Figure 1.1: Canonical and non-canonical Wnt signalling pathway: a simplified representation. In the absence of Wnt ligands (far left), β-catenin (β-cat) is bound with the destruction complex and later degraded. In the canonical pathway, Wnts bind with the FZD receptor and co-receptor lipoprotein receptor-related protein (LRP) 5/6; a 'signallosome' is formed, resulting in accumulation of β-catenin, which translocates to the nucleus and modulates gene expression. Non-canonical Wnt signalling is initiated following binding of Wnt ligands with FZD or receptor tyrosine kinase-like orphan receptor 2 (ROR2). The mechanisms are not well understood, and may be largely determined by cellular context. The secreted antagonist secreted FZD-related protein (sFRP) and Wnt inhibitory factor (WIF) can block Wnt signalling by binding with extracellular Wnts and sequestering ligand. Dickkopf (DKK) can block Wnt signalling by competitively binding with LRP 5/6. The dashed arrows indicate translocation across membranes. *Abbreviations:* AP1, activator protein 1; APC, adenomatous polyposis coli; AKT, protein kinase B; BMP4, bone morphogenic protein 4; βTrCP, β-transducin repeat-containing protein; CaMK, calmodulin dependent kinase; CK1, casein kinase1; CD44, cluster of differentiation 44; Daam1, dishevelled-associated activator of morphogenesis1; DKK, dickkopf; GSK3, glycogen synthase kinase 3; LEF-1, lymphoid enhancer-binding factor 1; LRP, lipoprotein-receptor-related protein; DVL, dishevelled; FGF9, fibroblast growth factor 9; JNK, Janus kinase; LEF, MMP7, matrix metalloproteinase 7; NFAT, nuclear factor of activated T-cells; NLK, nemo-like kinase; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; Pk, prickle; PKC, protein kinase C; ROR2, receptor tyrosine kinase-like orphan receptor 2; TCF, t-cell factor; VEGF, vascular endothelial growth factor.

1.2.2 Non-canonical signalling

Non-canonical Wnt signalling may be loosely defined as the Wnt signalling pathway not involving the nuclear accumulation of β-catenin. Non-canonical signalling is usually associated with major cell movements, directed migration and anteroposterior extension of the body axis (Keller 2002; Mlodzik 2002). The non-canonical pathway can be broadly classified into 2 branches based upon phenotypic response: the planar cell polarity (PCP) pathway and the Wnt/Ca^{2+} pathway (**Figure 1.1**). Signalling starts following the binding of Wnt ligands with FZD receptors or other receptors such as receptor tyrosine kinase-like orphan receptor 2 (ROR2). In the PCP pathway, binding of Wnt triggers the formation of a complex comprised of DVL and DVL-associated activator of morphogenesis 1 (Daam1). This complex, in turn, activates Rho and subsequently Rho associated kinase (Rock) and myosin. Stimulation of GTPase and activation of Rac also occurs, but is independent of Daam1; Rac subsequently induces Janus kinase (JNK) activation (Marlow *et al.* 2002). However, the downstream events following JNK activation are poorly understood and the coordinating mechanism linking Rac and Rho is also unclear (Sugimura and Li 2010).

The second branch of non-canonical signalling involves the initial activation of phospholipase C (PLC) through heterotrimeric G-proteins, which in turn increases cytoplasmic Ca^{2+} levels via the release and activity of inositol 1,4,5-trisphosphate (IP3). The increased Ca^{2+} levels activate several Ca^{2+} responsive kinases. Three of them are well known to be activated by Wnt/Ca^{2+} signalling: calmodulin dependent kinase II (CaMKII), protein kinase C (PKC) and calcineurin. Wnt/Ca^{2+} signalling is associated with cell adhesion, cytoskeletal rearrangements, and other developmental processes (Kohn and Moon 2005) **(Figure 1.1)**.

Non-canonical Wnt ligands, such as, Wnt3A and Wnt16b, may also activate mitogen-activated protein kinase (MAPK) and phosphatidylinositol-4,5-bisphosphate 3-kinase/protein kinase B (PI3K/AKT) signalling in a β-catenin independent manner (Kao *et al.* 2002; Yun *et al.* 2005; Kim *et al.* 2007).

1.2.3 Wnt signalling antagonists

Genetic and biochemical evidence has demonstrated that an important aspect of regulation of Wnt signalling is its modulation through several extracellular antagonists. These antagonists are broadly divided in two classes (Kawano and Kypta 2003). The first class of inhibitors, termed the sFRP class, includes secreted frizzled related proteins (sFRPs), Wnt inhibitory factor-1 (WIF1) and Cerberus. These proteins have structural similarity with the extracellular domains of the FZD receptors (Rattner *et al.* 1997). The second class includes the members of the dickkopf (DKK) family. Proteins from the first class bind directly to the Wnt ligands, whereas the second class of antagonists bind to the LRP5/LRP6 component of the Wnt receptor complex, causing a rapid removal of the LRP receptors via Kremen-mediated endocytosis (Glinka *et al.* 1998; Bafico *et al.* 1999). In theory, the first class have the capacity to antagonize both Wnt canonical and non-canonical Wnt pathways, whereas the dickkopf class of proteins specifically inhibit only the canonical pathway (Semënov *et al.* 2001; Kawano and Kypta 2003) (**Figure 1.2**).

1.2.4 Wnt target genes

Wnt signals ultimately activate transcriptional programs and there is no intrinsic restriction in the type of biological events that can be controlled by these programs. Thus, Wnt signals may modulate all major cellular activities (Clevers 2006; Clevers and Nusse 2012). Some selected genes that are activated by Wnt signalling are matrix metalloproteinase-7 (MMP-7), urokinase-type plasminogen activator receptor (UPAR), CD44, c-Myc, c-Jun, fos-related antigen-1 (FRA-1), CCND1 (Cyclin-D1), peroxisome proliferative activated eeceptor-delta (PPAR-δ), transcription factor-1 (TCF1), fibronectin , slug, gastrin, and PTGS-2/cyclooxygenase-2 (Cox-2) amongst others (Lim 2013).

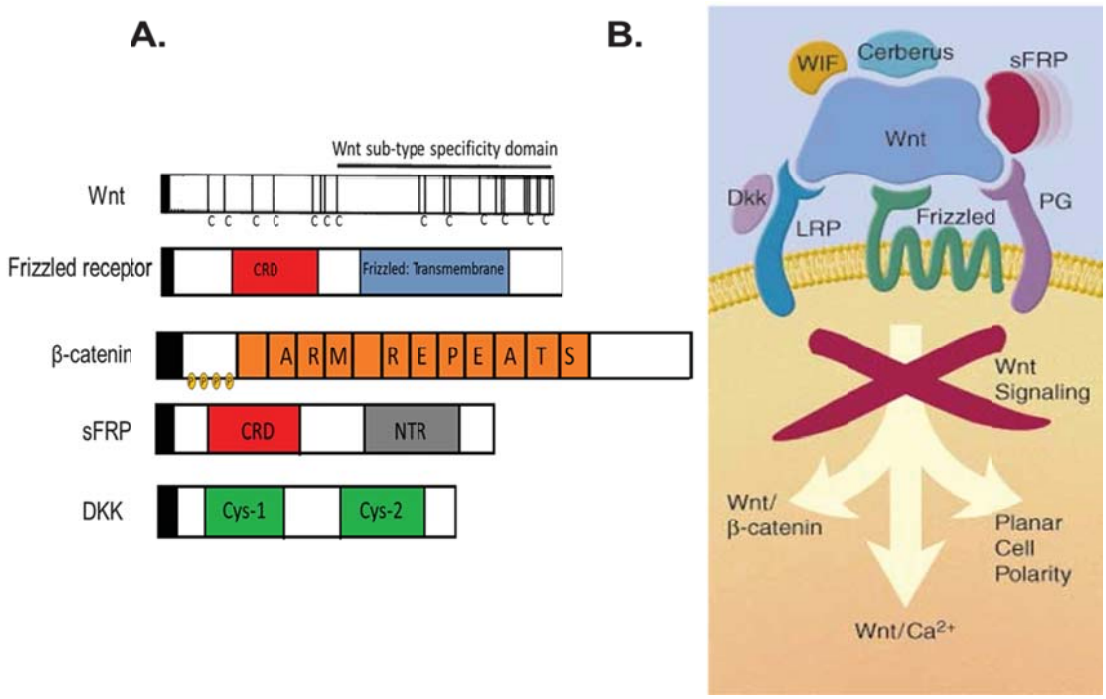


Figure 1.2: Some of the major proteins of the Wnt signalling pathway and the mode of action of Wnt antagonists. (A) Some of the major proteins of the Wnt signalling pathway. The black domain at the left hand size (N-terminal) of each protein is the signal sequence. Wnt ligands are~ 350 amino acids long with ~ 22 conserved cysteine residues. Frizzled (FZD) receptors are~ 700 amino acids long with a Cysteine rich domain (CRD) and transmembrane domains. β-catenin is~ 1000 amino acids long and contains 12 armadillo (ARM) repeats. sFRPs (soluble frizzled-related proteins) are~ 300 amino acids in length and contain a CRD and a netrin-like domain (NTR). DKK (Dickkopf) protein has 2 cysteine-rich domains, Cys-1 and 2. (B) Binding partners and modes of action of different Wnt antagonists. Adapted from from the Wnt signaling diagram on the Wnt homepage and R&D biosystems website (Lim 2013).

1.3 Early stages of pregnancy

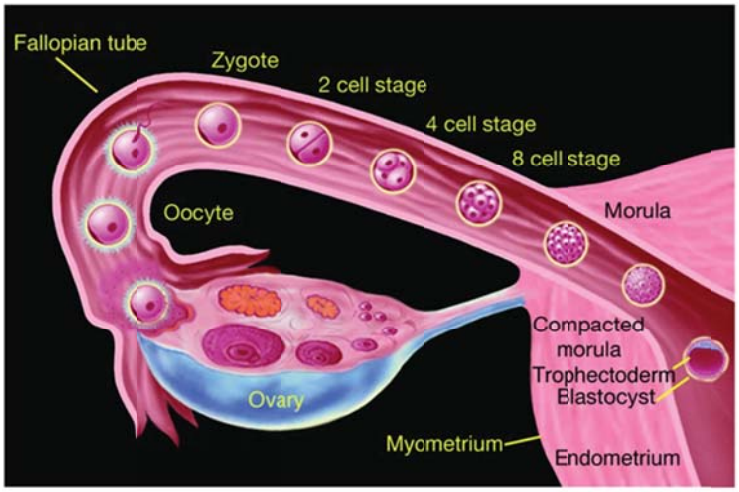
1.3.1 Blastocyst formation and Implantation

In humans, the start of a new life begins with the union between a sperm and an egg (ovum) culminating in fertilization (Johnson 2007). The one-cell fertilized egg undergoes several mitotic cell divisions (**Figure 1.3**). At the 32 to 64-cell stage the conceptus begins to transform itself into a differentiated tissue called the blastocyst. At this stage, the conceptus reaches uterus. Blastocyst contains two types of cell, surrounded by zona pellucida (ZP) (Johnson and Ziomek 1981; Johnson 2007) : an outer rim of trophoblast cells surrounding a blastocoelic cavity, and an inner group of

pluriblast inner cell mass (ICM). The trophoblast cells are the first extraembryonic tissues, as they do not contribute to the embryo or fetus itself. The blastocyst eventually hatches from the ZP in uterus and is now ready for implantation. A two-way interaction between the blastocyst and maternal uterine, especially endometrium, initiates the process of implantation (Ramon Pinon 2002; Louis Vontver 2006; Johnson 2007) (**Figure 1.3**).

Implantation is the process involving the attachment, penetration, and embedding of the blastocyst in the endometrial lining during the early stages of prenatal development (**Figure 1.3**). Decidualisation, conversely, is the process that transforms the endometrial lining into a secretory lining in preparation of accepting the embryo. Decidualisation is a hallmark of the endometrium of pregnant uterus (Kasahara *et al.* 2001; Louis Vontver 2006). Human implantation is invasive, which means the conceptus breaks through the surface epithelium to invade the underlying stroma. In the invasive phase, the surface epithelium underlying the conceptus becomes eroded. In humans the invasion is so deep that the surface epithelium is restored over the blastocyst and the conceptus is said to implant interstitially. Implantation is a dynamic process. A plethora of regulatory molecules has been identified which play functional roles in this process. These regulatory molecules include both secreted and adhesion/contact factors, such as, hormones, cytokines, neuropeptides, adhesion molecules and proteases (Red-Horse *et al.* 2004; Dimitriadis *et al.* 2010; Cha *et al.* 2012; Zhang *et al.* 2013a; Fritz *et al.* 2014) (**Figure 1.3**).

A.



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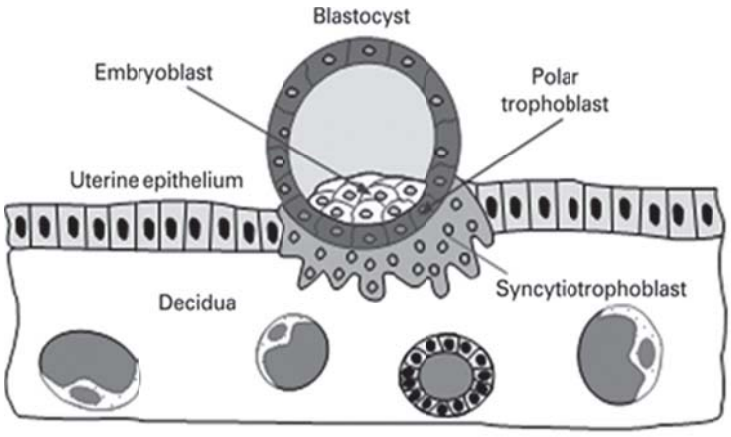

Figure 1.3: From fertilization to implantation. (A) The early stages of human development from fertilization to blastocyst formation. Adapted from (Red-Horse *et al.* 2004). (B) A simplified illustration of implantation. Adapted from (Huppertz 2008).

1.3.2 Placentation

Placenta is the first fetal organ to develop. It is a specialised organ of mammalian pregnancy, supporting the growth and development of the fetus. During early stages of pregnancy it mediates implantation and later establishes the interface for nutrients, metabolites, hormones and gas exchange between the maternal and fetal circulation.

The placenta is developed from two major cell types. The trophoblast cell lineage originates from the trophoectodermal cells of the blastocyst (**Figure 1.3**). The other major component is the stromal and vascular component derived from the allantois. It grows out from the embryo proper to interact with the trophoblast layer (Cross 2005; Knöfler and Pollheimer 2013). The early progenitor of trophoblast cells (cytotrophoblast) differentiate along two different lineages: the villous and the extravillous pathways. The mononucleated cytotrophoblasts (CTBs) undergoes a differentiation process involving intercellular fusion to form the multinucleated primitive syncytium. After forming syncytiotrophoblast layer CTBs emanate to generate primary villi by proliferation and invasion through the primitive syncytium. Throughout pregnancy these primary villous transform into secondary and tertiary villi by invasion, villous branching and vascularization. The trophoblast cells of extravillous pathway emerge from the proliferative cell columns at the basal plate of the anchoring villi. These cells, known as extravillous cytotrophoblast (EVTs) have the ability to remodel the maternal endometrium and its vasculature (Bischof and Irminger-Finger 2005; Aplin 2010; Knöfler and Pollheimer 2013; Menkhorst *et al.* 2014). A subset of EVTs, invasive endovascular cytotrophoblasts plugs the maternal arterioles to prevent premature onset of blood flow into the intervillous space (Pijnenborg *et al.* 2006). Another subset, interstitial cytotrophoblasts migrate into the maternal decidua and likely to participate in cross-talking with diverse cell types, including natural killer cells, macrophages and decidual stromal cells. Possibly these interactions is indispensable for immunological acceptance of the placenta/ fetus.

1.4 Pre-implantation Wnt signalling

1.4.1 Wnt signalling in blastocyst formation

Most of our understanding of the role of Wnt signalling in implantation comes from studies in rodents. After fertilization, the one-cell fertilized zygote transforms itself into morulae through successive mitotic cell divisions to form the blastocyst. Many Wnt ligands, receptors and related regulators are extensively expressed throughout mouse pre-implantation embryo development (Lloyd *et al.* 2003; Mohamed *et al.* 2004; Kemp *et al.* 2005) **(Table 1.1)**. However, various studies have demonstrated that canonical

Wnt signalling is not required for blastocyst formation. A gene knock-out study in mice showed that mutant embryos without *β-catenin* develop into blastocysts (Haegel *et al.* 1995), although this study did not preclude possible compensatory contributions from residual maternal β-catenin. Another study in mice, with a conditional deletion of *β-catenin* in their oocytes, demonstrated that lack of both zygotic and maternal β-catenin does not impair blastocyst development (De Vries *et al.* 2004). Furthermore, silencing of the Wnt/β-catenin pathway using small molecular inhibitors or over-expression of the Wnt inhibitor Dkk1 did not adversely affect the development of pre-implantation embryos (Xie *et al.* 2008). Canonical Wnt signalling components were down-regulated in porcine parthenogenetic embryos compared with normal embryos, and the development of these embryos was largely unaffected by the inhibition of Wnt signalling using a small molecule drug (IWP-2); however, blastocyst hatching and trophectoderm development was blocked. Excessive activation of Wnt induced apoptosis and blastocyst hatching was also impaired (Huang *et al.* 2013).

In contrast to these rather negative studies, others suggest that a functional Wnt signalling system does exist during pre-implantation in the bovine embryo (Denicol *et al.* 2013). These investigators demonstrated that the activation of canonical signalling decreases development and blastocyst cell numbers. The inhibition of Wnt/β-catenin signalling by Dkk1 did not seem to be beneficial (Denicol *et al.* 2013). Therefore, it is likely that the diverse Wnt signalling components expressed in the pre-implantation embryo function predominantly through the non-canonical pathways, involving Ca^{2+}-dependent signalling, and/or MAPK activation. These pathways are known to be necessary for pre-implantation development (Pey *et al.* 1998; Wang *et al.* 2004; Xie *et al.* 2005). In addition, recent observations on the dishevelled (DVL) family of proteins showed their potential roles in regulating cell-cell adhesion in blastocyst development, which reinforced the notion of alternate roles of Wnt signalling components at this stage (Na *et al.* 2007). Collectively, these studies indicate that a functional Wnt signalling system exists in the pre-implantation embryo of mammals; however, canonical Wnt signalling seems to be expendable with respect to blastocyst formation to a large extent. The non-canonical Wnt pathway may contribute to the pre-implantation embryo development by regulating cell-cell adhesion. Studies in humans are needed to provide a comprehensive understanding of the role of Wnt signalling in pre-implantation embryonic development.

1.4.2 Wnt signalling in blastocyst activation

A variety of signalling molecules have been found to be critical in orchestrating uterine receptivity for implantation (Dimitriadis *et al.* 2010; Sinderen *et al.* 2013; Zhang *et al.* 2013a; Fritz *et al.* 2014). However, our information about signalling networks that regulate blastocyst activation is rather limited (Paria *et al.* 1998; Wang *et al.* 2003; Hamatani *et al.* 2004). Early expression studies in mice have provided evidence that some Wnt ligands may play a role during blastocyst activation (Mohamed *et al.* 2004). Subsequently, it was shown that female mice with conditional deletion of *β-catenin* in oocytes produce fewer pups than wild-type female mice when cross-bred with normal mice; however, conditional deletion of both *β-catenin* and *E-cadherin* in oocytes resulted in a normal number of pups (De Vries *et al.* 2004). From these findings it was suggested that loss of maternal β-catenin results in insufficient canonical Wnt signalling. After fertilization, paternally derived β-catenin is incorporated in adherens junctions along with E-cadherens and is therefore not available for Wnt signalling. In contrast, when both *β-catenin* and *E-cadherens* were deleted in oocytes, paternal β-catenin was free to restore Wnt signalling (De Vries *et al.* 2004). In another study, DKK1 and a small molecular inhibitor (PKF115-584) were used to block canonical Wnt signalling in a mouse embryo culture. The study showed that silencing of nuclear β-catenin signalling blocks blastocyst competency to implant, while the simultaneous activation of canonical Wnt signalling combined with blocking of the non-canonical Wnt-RhoA signalling pathway confirmed blastocyst competency to implantation (Xie *et al.* 2008).

The environmental toxicant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) affects embryo development, implantation and fertility in humans. In a co-culture study of trophoblastic spheroids (embryo surrogate) cultured with endometrial cells, TCDD inhibited the attachment of spheroids to the endometrial cells, as well as β-catenin and E-cadherin expression. Induction of Wnt signalling by Wnt3A or lithium chloride treatment rescued the attachment (Tsang *et al.* 2012). These findings suggest that Wnt signalling is at least one pathway determining blastocyst competency for implantation. However, the information on hierarchical signalling networks is rather limited. In an earlier study in mice, it was shown that catecholestrogens, formed in the uterus from endogenous estrogen, participate in blastocyst activation (Paria *et al.* 1998). Later it was demonstrated that catecholestrogens are capable of inducing canonical Wnt activation in

the uterus (Hou *et al.* 2004; Ray *et al.* 2008). It would be interesting to explore the potential interactions of catecholestrogens and the canonical Wnt signalling pathway to help elucidate some potential signalling cross-talk. In summary, canonical Wnt signalling seems to be one of the required signalling pathways to achieve blastocyst competency for implantation; however, an understanding of its biological role and its interactions with other molecular factors is far from complete. A greater insight into the role of Wnt signalling and its molecular basis of blastocyst competency for implantation might help to improve pregnancy rates in human *in vitro* fertilisation (IVF) programs.

1.5 Wnt signalling and uterine receptivity

1.5.1 Wnt signalling in uterine development

The female reproductive tract in mammals is comprised of oviducts (fallopian tubes), uterus, cervix and vagina. Mammalian embryos have two pairs of genital ducts before sexual differentiation; the Wolffian ducts and the Müllerian ducts. Müllerian ducts give rise to the female reproductive tract by differentiating into the oviducts, uterus, cervix and upper portion of the vagina (Yin and Ma 2005). In mice, the Müllerian duct is formed around embryonic day 11.5 and evolves into the oviduct, uterus and cervix. It is formed by the epithelial cells from the coelomic wall. These are Wnt4 expressing cells and, after duct formation, Wnt4 is expressed at high levels by the surrounding mesenchymal cells (Kobayashi *et al.* 2004) **(Table 1.1).** Both male and female *Wnt4* mutant mice lack a Müllerian duct and, as a result, mutant females completely lack a reproductive tract. On the other hand, mutant males appear normal (Stark *et al.* 1994; Vainio *et al.* 1999). These data suggest that Wnt4 is critical in the initial stages of duct development before sexual differentiation occurs.

Wnt7A is another Wnt ligand critically required for the development of reproductive tracts in both sexes. In male mice, Wolffian ducts develop instead of the Müllerian duct because of a secreted Müllerian-inhibiting substance from Sertoli cells of the testes. The primitive Müllerian duct extends to and interacts with the Wolffian duct. *Wnt7A*-null male mice fail to undergo regression of the Müllerian duct as a result of the absence of the receptor for Müllerian-inhibiting substance. These mice have a persistent Müllerian duct (Parr and McMahon 1998; Yin and Ma 2005) due to lack of receptors for

Müllerian-inhibiting substance. These mice become pseudo-hermaphrodites and are frequently infertile because sperm passage is blocked by the presence of the female reproductive system (Parr and McMahon 1998). *Wnt7A*-null female mice have abnormal differentiation of the oviduct and uterus. They do not develop uterine glands and have aberrant uterine smooth muscle organization (Miller and Sassoon 1998).

Wnt5A is also critically involved in female reproductive tract development (Mericskay *et al.* 2004). *Wnt5A*-null mice have a coiled and shortened uterus and poorly defined cervix and vagina (Mericskay *et al.* 2004). Genetic analysis revealed that Wnt5A acts in concert with Wnt7A (Mericskay *et al.* 2004). It has been proposed that highly regionalized repression of Wnt7A is required to allow endometrial luminal epithelium to change fate and form glands, and that Wnt5A may control this down-regulation. Alternatively, it is possible that Wnt7A repression is not directly linked with Wnt5A and that Wnt5A is simply required for subsequent development of the stroma (Mericskay *et al.* 2004). In contrast, mice with a dominant active hedgehog signalling pathway show increased Wnt5A expression in the oviduct and uterus. These mice are infertile with abnormal reproductive tract development (Migone *et al.* 2012).

Finally, Wnt4 is also an important player in development of the Müllerian duct. In *Wnt4* conditionally-ablated mice, *Wnt7A* knockout mice and *Wnt5A* ablated uteri all failed to develop endometrial glands (Miller and Sassoon 1998; Mericskay *et al.* 2004; Franco *et al.* 2011). To sum up, Wnt4 is critical in initial Müllerian duct formation, Wnt7A is required for the differentiation of the oviduct and uterus, and Wnt5A is similarly required for functional uterus, cervix and vagina formation. All three ligands are essential for endometrial gland formation. Hence, these Wnt ligands might be acting in a coordinated fashion among uterine compartments to promote glandular development (Wetendorf and DeMayo 2012).

Wnt9B is a central organizing signal regulating diverse components of the mammalian urogenital system (Carroll *et al.* 2005). In the absence of Wnt9B, Müllerian duct development stops prematurely. Wnt9B acts upstream of Wnt4 in this process (Carroll *et al.* 2005). Conditional deletion of *β-catenin* in the Müllerian duct mesenchyme results in abnormal uteri after birth and a lack of uterine smooth muscle, underscoring the importance of canonical Wnt signalling for the development of Müllerian duct derivatives (Arango *et al.* 2005). In contrast, Tanwar et al. showed that conditional

activation of *β-catenin* by Amhr2-Cre results in the formation of large tumorous growths and multiple haemorrhagic sites on the surface of the uterus in mice (Tanwar *et al.* 2009). In a recent follow-up study, these authors showed that Wnt/β-catenin signalling was induced by deletion of *APC* through the same Amhr2-Cre system. These mice exhibited defects in the myometrium and also displayed endometrial hyperplasia and cancer, combined with abnormal estrogen signalling (Tanwar *et al.* 2011b). The effects of constitutive *β-catenin* activation within the mesenchyme of the developing Müllerian duct have also been examined (Stewart *et al.* 2013). β-catenin stabilization in the mesenchyme caused alterations within the epithelium, including reduced proliferation, delayed uterine gland formation, and induction of an epithelial-mesenchymal transition (EMT) event. This EMT event is observed before birth and is complete within 5 d after birth (Stewart *et al.* 2013). In a separate experiment, it has been shown that abnormal activation of β-catenin contributes to adenomyosis development, which is the presence of endometrial glands and stroma within the myometrium, through the induction of EMT (Oh *et al.* 2013).

From these findings it is evident that functional Wnt signalling is required for normal endometrial development. Restricted access to human samples and a lack of mechanistic studies have precluded investigations on the role of Wnt components in human uterine development. On a positive note, however, new mouse models have allowed us to investigate and clarify the roles of various Wnt components in the uterine development of mammals.

1.5.2 Wnt signalling in different uterine components, compartments and cell types

The lining of the uterine cavity, the endometrium, is the part of the uterus where implantation takes place. This layer experiences morphologic and functional changes that are closely associated with the cyclic release of sex hormones (i.e. estrogen and progesterone). The endometrium consists of a single layer of columnar epithelium resting on the connective tissue layer (stroma). Endometrium contains glands that synthesize, transport and secrete a complex array of proteins and related substances that are essential for survival and development of the conceptus. In humans, endometrial development begins before birth and is completed during puberty (Gray *et al.* 2001).

Various studies have investigated the expression and significance of Wnt ligands and other signalling components in human endometrium (Bui *et al.* 1997; Tulac *et al.* 2003),

and presented evidence of Wnt signalling in diverse biological roles in different endometrial cell types. *Wnt2, Wnt3, Wnt4, Wnt5A, Wnt7B, Wnt8B, FZD6* and *LRP6* mRNAs have all been identified in endometrial samples and in endometrial epithelial and/or stromal cells (Bui *et al.* 1997; Tulac *et al.* 2003) **(Table 1.1)**. *Wnt7A* was found to be expressed exclusively in luminal epithelial cells, whereas *sFRP4, DKK1* and *FZD* were all found in uterine glands and stroma (Tulac *et al.* 2003). Transcriptional profiling of epithelial cells from pre- *vs.* post-menopausal endometria has identified the Wnt signalling pathway as one of the most differentially expressed gene families (Nguyen *et al.* 2012), and an intact canonical Wnt signalling cascade is present within the endometrial epithelial compartment in both pre- and post- menopausal endometrium (Nguyen *et al.* 2012). Eleven Wnt genes (*Wnt2, 2b, 4, 5a, 5b, 7a, 8a, 9b, 10b, 11* and *16*) and their antagonists (*sFRP1, 2, 5, Dkk1, Dkk2* and *WIF1*) were detected in equine endometrium (Atli *et al.* 2011) (**Table 1.1**).

Table 1.1 Expression of Wnt signalling related components in different pregnancy-associated tissues. *Abbreviations*: M, mouse; H, human; P, primate; E, equine.

Findings	References
Blastocyst	
Murine blastocyst: *Wnt1, Wnt3, Wnt4, Wnt5A, Wnt5B, Wnt6, Wnt7A, Wnt7B, Wnt10B, Wnt11* and *Wnt13* expression confirmed.	(Mohamed *et al.* 2004)[M]
Uterine development	
Epithelial cells from the coelomic wall: Wnt4.	(Kobayashi *et al.* 2004; Carroll *et al.* 2005)[M]
Müllerian duct: Wnt5A. Müllerian duct mesenchyme: Wnt7A.	(Miller and Sassoon 1998; Mericskay *et al.* 2004)[M]
Wolffian duct (in the presence of Müllerian duct): Wnt9B.	(Carroll *et al.* 2005)[M]
Endometrium	
Endometrial epithelial and stromal cells: *Wnt2, Wnt3, Wnt4, Wnt5A, Wnt7B, Wnt8B; FZD6; LRP6; ROR2; sFRP4* and *DKK1*. Luminal epithelium: Wnt7A. Endometrial stromal cells: ROR2 (increased during pregnancy).	(Bui *et al.* 1997; Tulac *et al.* 2003; Hatta *et al.* 2010)[H, H, M]
Changes during early/mid-luteal transition: *Wnt10B* and *DKK1* expression increased; LRP6, sFRP1 and sFRP4 decreased.	(Carson *et al.* 2002; Borthwick *et al.* 2003)[H]
Equine endometrium during the estrous cycle and early pregnancy: *Wnt2, Wnt2B, Wnt4, Wnt5A, Wnt5B, Wnt7A, Wnt8A, Wnt9B, Wnt10B, Wnt11, Wnt16, sFRP1, sFRP2, sFRP5, Dkk1, Dkk2* and *WIF1*.	(Atli *et al.* 2011)[E]

Murine uterus during implantation: W*nt4, Wnt5A, Wnt7A, Wnt7B, Wnt11, Wnt16; Fzd2, Fzd4* and *Fzd6*. Human uterus during implantation: DKK1 and sFRP4 expression up-regulated and down-regulated, respectively.	(Kao *et al.* 2002; Hayashi *et al.* 2009)[H, M]
Endometrium of RIF-IVF patients: Up-regulation of DKK1 and down-regulation of sFRP1 and LEF1.	(Koler *et al.* 2009)[H]
Endometrium of RIF-IVF patients: Down-regulation of sFRP4.	(Revel *et al.* 2011)[H]
Changes in estrus vs proestrus uterus: upregulation of *Wnt2, Wnt2B, Wnt4, Wnt5B, Wnt6, Wnt7A, Wnt9A, Wnt9B, Wnt11, Fzd1, Fzd2, Fzd3, Fzd7, Fzd9, Fzd10, sFRP1, sFRP2, sFRP5, WIF1* and many other Wnt signalling components.	(Yip *et al.* 2013)[M]
22 Wnt-associated genes were modulated in post-menopausal compared with pre-menopausal (proliferative and secretory) endometrial epithelial cells, including *Wnt10A, FZD2, FZD9, LRP5, Axin2 a*nd *GSK3*.	(Nguyen *et al.* 2012)[H]
Trophoblast and placenta	
Upregulation of canonical Wnt signalling by BMP4 during human embryonic stem cells (hESCs) differentiation of down the trophoblast lineage. Upregulation of Wnt target genes: *SOX2, SOX14, MYC, CD44,* and *MMP7,* receptors *FZD3* & *FZD5* and antagonist *sFRP2*.	(Marchand *et al.* 2011)[H]
Expression of TCF3/4 and β-catenin markedly increased in invading trophoblasts. sFRP4 expression predominantly in the villous syncytiotrophoblast, invasive cytotrophoblast and amnion.	(Pollheimer *et al.* 2006; White *et al.* 2009)[H, P]
14 out of 19 Wnt ligands and 8 out of 10 Fz receptors expressed in the	(Sonderegger *et*

placenta. Wnt3A, Wnt8A, Wnt8B, Wnt9A, and Wnt16, receptors FZD8, and FZD9 were absent.	*al.* 2007)[H]
Reproductive pathologies	
Levels of Wnt2, Wnt3, Wnt4, and Wnt5A are reduced in endometrial carcinoma compared with the normal endometrium.	(Bui *et al.* 1997)[H]
Wnt10B expression is elevated in endometrial cancer tissues compared to hyperplastic and normal samples.	(Yi *et al.* 2009; Chen *et al.* 2013)[H]
Wnt10A expression in endometrioid cancer tissues is higher compared to other types of cancerous samples.	
DKK1 expression in endometrial cancer is significantly lower than benign endometrium.	
Wnt7A expression markedly elevated in cells from endometriotic lesions which are capable of migrating to the eutropic endometrium.	(Wu *et al.* 2006; Santamaria *et al.* 2012)[H]
17 other Wnt related genes are also upregulated, including *Wnt2, Wnt3A, Wnt7A, Wnt8A and Wnt10A, FZD1, FZD8* and *FZD10*.	
In preeclamptic placenta, β-catenin and Wnt2 levels are down-regulated and DKK1 and sFRP4 up-regulated compared to normal placental controls in 3rd trimester.	(Zhang *et al.* 2013b; Zhang *et al.* 2013c)[H]

1.5.3 Changes in Wnt signalling in the uterine cycle

The uterine or menstrual cycle describes a series of changes that occur in the uterus, which are essential for the production of ova and for the preparation of the uterus for pregnancy. In humans, the cycle is classified into proliferative (follicular) and secretory (luteal) phases. During the proliferative phase, the lining of the uterus grows or proliferates and the ovarian follicles start secreting increasing amounts of estrogen. Estrogens initiate the formation of the proliferative endometrium. During the secretory phase, the corpus luteum produces progesterone and the endometrium becomes receptive to implantation of the blastocyst and supportive of early pregnancy. It has

been proposed that, during the proliferative phase of the menstrual cycle, estrogens induce Wnt/β-catenin signalling; on the other hand, during the secretory phase, progesterone takes over and induces differentiation, and inhibits canonical Wnt signalling in addition to its other activities. Subsequent studies have supported this general hypothesis (Wang *et al.* 2010; van der Horst *et al.* 2012). In the gastrointestinal tract, proliferating epithelial cells display evidence of canonical Wnt signalling; however, differentiated (non-proliferating) cells exhibit reduced Wnt/β-catenin signalling (Clevers 2006). The Wnt signalling pathway is found to be one of the most significantly enriched signalling clusters in the estrus *vs.* proestrus uterus of the mouse, with 9 members of the Wnt family and six *Fzd* genes being up-regulated (Yip *et al.* 2013) **(Table 1.1).** Similarly, activation of Wnt signalling is a characteristic of the cyclic endometrium in the human uterus (Ruiz-Alonso *et al.* 2012). In human endometrium, clear nuclear localization of β-catenin has been observed during the proliferative phase, while nuclear accumulation of β-catenin has been reported to decrease during the secretory phase (Nei *et al.* 1999).

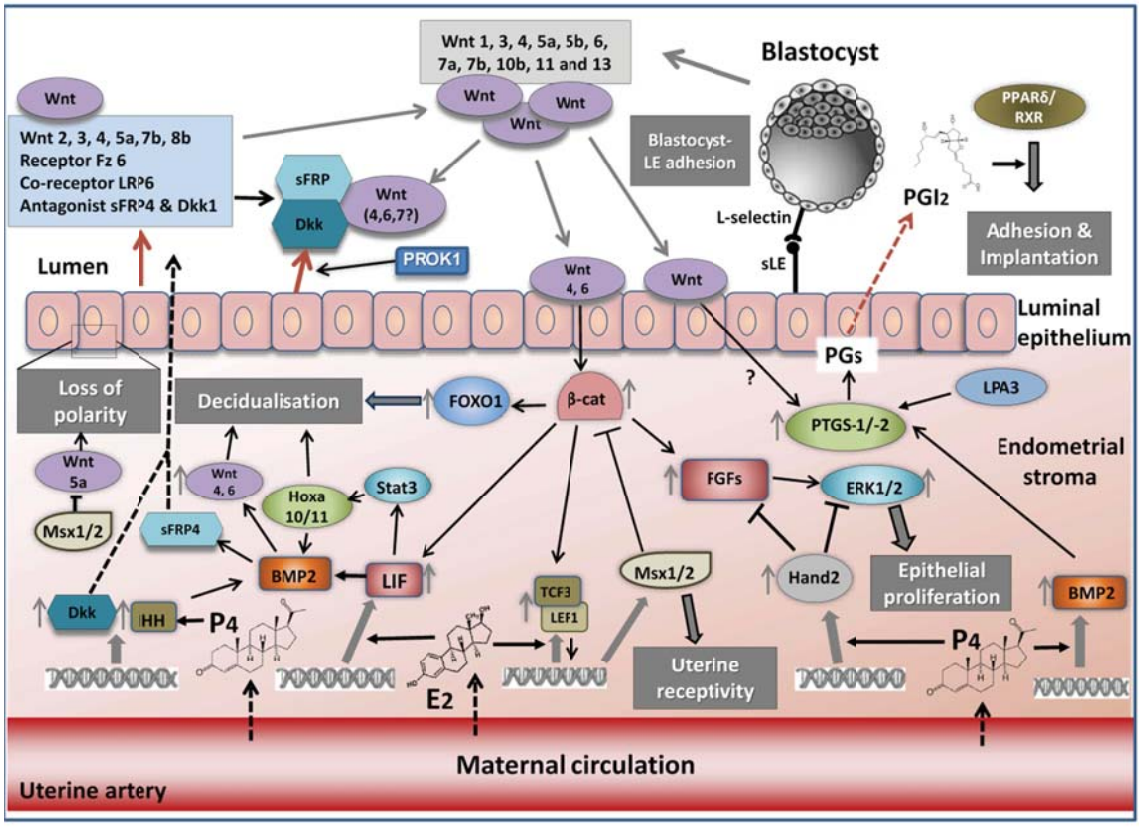

Figure 1.4: Potential relationship between Wnt signalling and processes involved with implantation. This is a hybrid schematic, with data from both human and mouse studies to show the connections of Wnt signalling with other signalling networks. The components of Wnt signalling and a wide array of other factors contribute to the success of implantation in an autocrine, juxtacrine and paracrine manner. The dashed arrows indicate translocation across membranes. *Abbreviations*: β-cat, β-catenin; BMP2, bone morphogenetic protein 2; Dkk, dickkopf; E2, estradiol; ER, Estrogen receptor; ERK, extracellular-signal-regulated kinases; FGF, fibroblast growth factor; FOXO1, forkhead box protein O1; LEF1, lymphoid enhancer-binding factor 1; Hoxa, homeobox A; IHH, indian hedgehog homolog; LIF, leukocyte inhibitory factor; LPA3, lysophosphatidic acid receptor 3; sLE, L-selectin ligands; Msx, msh homeobox; PGI2, prostacyclin; PPARδ, Peroxisome proliferator-activated receptor δ; PROK1, prokineticin 1; PTGS, prostaglandin G/H synthase; P4, progesterone; RXR, retinoid X receptor; sFRP, secreted frizzled related proteins; Stat3, signal transducer and activator of transcription 3; TCF3, t-cell factor 3.

Estrogen has been demonstrated to induce Wnt4, Wnt5A and Fzd2 expression in the mouse uterus and promote recruitment of nuclear β-catenin in endometrial epithelium (Hou *et al.* 2004). Inhibition of canonical Wnt signalling inhibits estrogen-dependent activation of β-catenin and epithelial cell proliferation, which suggests hormone mediated uterine cell growth involves Wnt signalling (Hou *et al.* 2004). In addition, estrogen also upregulates expression of the β-catenin-dependent transcription factors

TCF-3 and lymphoid enhancer-binding factor 1 (LEF-1). The hormone also facilitates physical interaction of estrogen receptor (ER) α with activated LEF-1/TCF-3 and recruits Wnt transcription factors to Wnt/estrogen dependent target genes (Ray *et al.* 2008). Therefore, the cross-talk between estrogen and Wnts appears to be integral for endometrial function - at least in mice (**Figure 1.4**).

Progesterone is a complex modulator of estrogen-mediated activation of the canonical Wnt signalling pathway (Rider *et al.* 2006). Progesterone down-regulates Gsk3 levels in rat uteri, a possible prerequisite for estrogen-mediated activation of the canonical Wnt signalling pathway (Rider *et al.* 2006). In humans, while estrogen treatment induces Wnt signalling pathway components in endometrium (Wang *et al.* 2009), progesterone upregulates DKK1 expression in human endometrial stromal cells (Tulac *et al.* 2006), while progesterone receptor knock-down reduces its expression (Cloke *et al.* 2008). This indicates that estrogen treatment promotes Wnt activation, while progesterone-dependent induction of DKK1 expression inhibits Wnt signalling (Wang *et al.* 2009). The evidence, therefore, supports the hypothesis that canonical Wnt signalling, under the influence of estrogens, is activated during the proliferative phase, but in the secretory phase, canonical Wnt signalling is inhibited, possibly through the actions of DKK1 when progesterone takes over. However, further investigation is necessary to elucidate the mechanisms and details of this signalling cross-talk (**Figure 1.4**).

Microarray analysis of global endometrial gene expression during the menstrual cycle has revealed the pattern of expression of Wnt pathway components and also their potential regulation by steroid hormones. Proliferative endometrium is characterized by elevated Wnt3 levels, whereas DKK1 levels increase in the mid-secretory phase and also in *in vitro* decidualised cells of endometrial stroma (Kao *et al.* 2002; Tulac *et al.* 2003). Carson et al showed that the expression of *DKK1* and *Wnt10B* mRNAs increased sharply between early and mid-luteal phases (Carson *et al.* 2002) **(Table 1.1)**. In contrast, expression of the secreted inhibitors sFRP1 and sFRP4 was found to be down-regulated in endometrial samples at the time of luteinizing hormone (LH) surge (Kao *et al.* 2002; Borthwick *et al.* 2003). Although there are minor differences in expression of Wnt components in the endometrium from species to species, most studies show a consistent picture in terms of cycle-associated expression profiles. The changing expression pattern observed during different stages of the menstrual cycle is consistent with an important regulatory role of estrogen and progesterone.

1.5.4 Wnt signalling in endometrial receptivity and decidualisation

It is generally accepted that successful implantation is dependent on blastocyst quality and timely synchronization between uterine receptivity and the developmental stages of the embryo. Uterine receptivity is defined as a restricted time-related period when the uterus is receptive to blastocyst attachment and implantation, primarily coordinated by the ovarian sex hormones, estrogen and progesterone (Cha *et al.* 2012). Recent evidence also indicates preimplantation blastocyst release factors to influence endometrial receptivity (Cuman *et al.* 2013). Uterine sensitivity to implantation is classified into pre-receptive, receptive and non-receptive (refractory) phases. The local concentration of estrogen within a very narrow range determines the duration of the window of receptivity. In humans, the uterus becomes receptive only if exposed to a small amount of estrogen after 24-48 h of progesterone priming (Wang and Dey 2006; Cha *et al.* 2012). During the secretory phase, the uterus is considered to be pre-receptive for the first approximately 7 d following ovulation (day 0). The uterus then becomes receptive during the mid-secretory phase spanning 7-10 d after ovulation. The non-receptive phase comprises the rest of the secretory phase (Wang and Dey 2006; Cha *et al.* 2012). If pregnancy occurs, the endometrium goes through a process called decidualisation. Stromal cells surrounding the implanting blastocyst undergo extensive proliferation and differentiation into specialized cell types called decidual cells (Cha *et al.* 2012).

Different Wnt signalling components are expressed in the mouse uterus. Among the Wnt signalling components, different Fzd receptors (*Fzd2, Fzd4* and *Fzd6*) and Wnt ligands (*Wnt4, Wnt5A, Wnt7A, Wnt7B, Wnt11* and *Wnt16*) are expressed before and after implantation (Hayashi *et al.* 2009). They are also expressed during stromal cell differentiation (Hayashi *et al.* 2009). The expression of most of these genes is greatest at the time of the receptive endometrium (day 5 after fertilisation). In ovariectomised mice, upon implantation and decidualisation, expression of Wnts and Fzds undergoes dynamic changes regulated by steroid hormones (Hou *et al.* 2004; Hayashi *et al.* 2009) **(Table 1.1)**. The expression of *Wnt7A, Wnt7B, Wnt11* and *Wnt16* increases in implantation sites compared to the non-implantation sites. Upon stromal cell differentiation, their expression decreases (Hayashi *et al.* 2009). In porcine endometrium, expression of *Wnt4, Wnt5A, Wnt7A, β-catenin* and *E-cadherin* proteins show a spatially differential distribution in pre-implantation endometrium. Of these, Wnt7A, Wnt5A and E-cadherin gene expression significantly changed before

implantation (Kiewisz *et al.* 2011). The above mentioned studies reveal some species specific variations in Wnt components expression patterns; however, a precisely regulated Wnt signalling pathway is essential for endometrial receptivity and normal implantation (Chen *et al.* 2009).

In mice, conditional deletion of *β-catenin* in the mesenchymal compartment of the uterus results in a complete failure of decidualisation and infertility, suggesting that endometrial stromal β-catenin is essential for steroid-driven mesenchymal-epithelial cross-talk and decidualisation (Zhang *et al.* 2012). Further support for the important role of Wnt/β-catenin signalling in decidualisation comes from observations that mice with conditional deletion of *β-catenin* or over-expression of stabilizing β-catenin show defects in decidualisation (Jeong *et al.* 2008). Constitutive expression of β-catenin causes hyperplasia and subfertility, whereas, *β-catenin* ablation causes metaplasia and infertility (Jeong *et al.* 2008). Therefore, appropriate and timely regulation of Wnt/β-catenin signalling is a requirement for normal decidualisation.

As mentioned above, different Wnt ligands are essential for normal mammalian uterine development, and their role in endometrial receptivity and decidualisation has been investigated in different studies. In the mouse, on the morning of day 4 of pregnancy, no *Wnt4* is detectable in the pregnant uterus. However, expression begins to increase in the stroma surrounding the embryo at midnight of day 4. The levels of *Wnt4* continue to increase the following day. By day 7, robust expression of *Wnt4* is detectable throughout the whole decidua (Paria *et al.* 2001). *Wnt4* expression has also been found to increase sharply in stromal cells around the implanting blastocyst, with *Wnt7B* expression also increased in the luminal epithelium, suggesting their specific roles during implantation (Hayashi *et al.* 2009). This pattern of *Wnt4* expression coincides with that of an important growth factor, *bone morphogenetic protein 2 (BMP2)* (Paria *et al.* 2001). Wnt4 is a target gene for BMP2 regulation during progesterone-induced stromal cell decidualisation of the uterus in both the mouse and human (Li *et al.* 2007). Indeed, the silencing of Wnt4 expression with siRNA impairs the decidualisation process in both species (Li *et al.* 2007). Bmp2 has also been shown to be required for normal decidualisation in the mouse (Lee *et al.* 2007) (**Figure 1.4**). The deletion of *Bmp2* disrupts expression of *Wnt4* and *Wnt6* and deregulates the expression of *sFRP4* and prostaglandin G/H synthase-2 *(PTGS-2/cyclooxygenase-2)* (Lee *et al.* 2007). BMP2 appears to be an important link between Wnt signalling and PTGS-2 activity, both

pivotal in uterine function and decidualisation. It is possible that PTGS-2 is directly up-regulated by Wnt signalling, as has been demonstrated in cancer cells (Araki *et al.* 2003; Nuñez *et al.* 2011). PTGS-2-derived prostacyclin acts in implantation by activating uterine peroxisome proliferator-activated receptor-δ (PPARδ) and retinoid X receptor (RXR) (Lim *et al.* 1999) (**Figure 1.4**).

BMP2 is also an important down-stream mediator of homeobox gene activity. Homeobox A 10 (*Hoxa10*)$^{-/-}$ mice exhibit defects in decidualisation, downregulation of *sFRP4* and aberrant msh homeobox (Msx)-1 and Wnt4 signalling (Daikoku *et al.* 2004). Leukaemia inhibitory factor (Lif) deficiency results in similar changes and it has been previously shown that both Bmp2 and Hoxa10 expression in murine stroma is dependent on Lif (Fouladi-Nashta *et al.* 2005). These results suggests that BMP2 may be acting downstream of Hoxa10 and Lif (Lee *et al.* 2007), which is a major mediator of estrogen action acting via signal transducer and activator of transcription 3 (Stat3) (Niwa *et al.* 1998) (**Figure 1.4)**.

Wnt4 also appears to act downstream of BMP2, via β-catenin, to regulate human endometrial stromal cell differentiation (Li *et al.* 2013). *Wnt4* conditional knock-down affects stromal cell survival, differentiation and responsiveness to progestagens in the mouse (Franco *et al.* 2011). In these animals, implantation fails because of the inability of the embryo to invade into the luminal epithelium. Wnt4 also acts in decidualisation, probably by modifying survival mechanisms through forkhead box protein O1 (FOXO1) localization (Franco *et al.* 2011; Wetendorf and DeMayo 2012; Li *et al.* 2013). When FOXO1 is nuclear, it regulates the transcription of its target genes which include multiple pro-apoptotic genes (Huang and Tindall 2007). In uterine stromal cells in response to progesterone (P4) treatment, FOXO1 is shuttled to the cytoplasm (Brosens and Gellersen 2006; Labied *et al.* 2006). In the uteri of *Wnt4* conditional knock down mice, FOXO1 remains in the nucleus during decidualisation when it should be in the cytoplasmic (when compared with control) (Franco *et al.* 2011) (**Figure 1.4**).

Wnt6 has been found to be critical for stromal cell proliferation in a study utilizing *Wnt6*-mutant mice. Deficiency in Wnt6 resulted in impaired stromal cell proliferation with minimal effects on differentiation (Wang *et al.* 2012). In an *in vitro* study, it was reported that *Wnt4* and *FZD2* expression are down-regulated in primary decidualised endometrial stromal cells in the presence of trophoblast conditioned media, suggesting a

paracrine mode of regulation of decidualisation by trophoblasts through Wnt signalling (Hess *et al.* 2007). The opposite pattern of Wnt4 expression found in this study may be due to species-specific difference between mice and humans, or noted endocrine influences in the *in-vivo* studies **(Figure 1.4)**. Collectively, these studies support the concept that there are differential molecular mechanisms regulating stromal cell proliferation as opposed to decidual transformation, and that different Wnt ligands are critical for both phases.

It has been reported that Wnt5A induces cell polarity by formation of an E-cadherin/β-catenin complex via the non-canonical Wnt signalling pathway in a melanoma cell line (Witze *et al.* 2008). In a murine study, where muscle segment homeobox gene (Msh) family members *Msx1* and *Msx2* were deleted, loss of *Msx1* and *Msx2* raised Wnt5A levels in both uterine epithelial and stromal cells and inhibited blastocyst implantation through reduced uterine receptivity (Daikoku *et al.* 2011) (**Figure 1.4**). In a separate experiment, conditional deletion of *Msx1* and *Msx2* resulted in up-regulation of several Wnt ligands, including Wnt4, Wnt5A, Wnt7A and Wnt7B, in the epithelium and stromal cells of the pre-implantation uterus (Nallasamy *et al.* 2012). In these cells, the increased β-catenin levels stimulated the expression of a subset of fibroblast growth factors (FGFs). Collectively, these studies suggest that Msx1/2, Wnts and FGFs are all involved in a signalling network in the uterus in preparation for the implanting blastocyst (Nallasamy *et al.* 2012) (**Figure 1.4**). However, the mechanistic details remain to be elucidated.

Recently, DKK1 expression has been reported to be induced by prokineticin1 signalling in endometrial epithelial cells and in first trimester decidua explants (Macdonald *et al.* 2011). This study demonstrated important roles for both prokineticin 1 and DKK1 in endometrial receptivity and early pregnancy **(Figure 1.4).**

1.5.5 Wnts and implantation

Implantation is a dynamic process in which the blastocyst apposes, attaches, and invades into the endometrium to establish the placenta. A plethora of regulatory molecules has been identified that play functional roles in this process. These regulatory molecules include both secreted and adhesion/contact factors, such as, hormones, cytokines, neuropeptides, adhesion molecules and proteases (Minas *et al.* 2005; Dimitriadis *et al.* 2010; Cha *et al.* 2012; Sinderen *et al.* 2013; Zhang *et al.* 2013a).

Considering the fact that Wnt signalling plays a critical role in embryogenesis, organogenesis and metastasis, it is not surprising that Wnt signalling is also implicated in implantation. The study that first confirmed that Wnts are a key family of regulators of implantation utilized the TCF/Lef-LacZ reporter mouse (Mohamed *et al.* 2005). This study demonstrated that canonical Wnt signalling in the uterus is induced at the prospective site of embryo attachment immediately before implantation. This transient, but timely, activation requires the presence of a blastocyst and pre-implantation estrogen secretion (Mohamed *et al.* 2005) (**Figure 1.4**). Intrauterine delivery of Wnt7A protein, a Wnt ligand expressed by the active blastocyst (Mohamed *et al.* 2004; Mohamed *et al.* 2005), can induce uterine Wnt/β-catenin signalling, whereas administration of sFRP2 along with Wnt7A inhibits the signalling pathway and impairs implantation (Mohamed *et al.* 2005). Wnt7A has also been shown in sheep to be transiently expressed by uterine luminal epithelium during the peri-implantation period, inducing a variety of Wnt related effects in placental trophoblast cells (Hayashi *et al.* 2007). In primates, Wnt7A has a role in postmenstrual endometrial regeneration, after which its expression is down-regulated by progesterone, possibly to accommodate secretory transformation and preparation for implantation (Fan *et al.* 2012). Studies using a variety of *in vitro* models add weight to the notion that Wnt signalling is critical in human implantation. For example, DKK1 treatment to JAR cell spheroids reduces their capability to attach to endometrial-like Ishikawa cells (Liu *et al.* 2010b).

Non-pregnant mice express non-canonical Wnt receptors such as Ror2 in endometrial epithelial and stromal cells (Hatta *et al.* 2010). During pregnancy, Ror2 expression in endometrial stromal cells is increased and expression also appears in uterine natural killer cells, suggesting a possible role for Ror2 in implantation and trophoblast invasion (Hatta *et al.* 2010). The reported increase in expression of both Dkk1 and the non-canonical receptor Ror2 may indicate a shift from canonical to non-canonical Wnt signalling after successful implantation, as Dkk1 has been hypothesized to specifically inhibit canonical Wnt signalling (Semënov *et al.* 2001; Kawano and Kypta 2003). Some recent findings have also implicated a shift from canonical to non-canonical signalling after successful implantation (Semënov *et al.* 2001; Kawano and Kypta 2003), although these data have yet to be confirmed experimentally. To date the available evidence indicates that implanting embryos actively participate in the implantation process;

however, it remains to be determined whether embryonic Wnt ligands activate Wnt/β-catenin signalling or whether other signalling molecules act as a master regulator.

In summary, the regulation of uterine receptivity, decidualisation, and implantation all appear to involve a plethora of endogenous Wnts, receptors and associated factors, as well as the fine regulation of canonical and non-canonical pathways to maintain the balance of proliferation, differentiation, cell migration and invasion.

1.6 Wnt signalling in placentation

1.6.1 Wnt signalling in trophoblast differentiation and invasion

Trophoblasts, comprising the outer layer of the blastocyst, are a unique and multifunctional cell type that display manifold basic and fundamental functions during the development and operation of the placenta. The maintenance of trophoblast function is dependent on a variety of extra- and intracellular signals. Trophoblasts are broadly divided in three main types: extravillous trophoblast (EVT), cytotrophoblast and syncytiotrophoblast. After implantation, further embryonic development depends on the rapid formation of the placenta, which is achieved through trophoblast differentiation and invasion (Red-Horse *et al.* 2004; Cartwright *et al.* 2010; Menkhorst *et al.* 2014). Cytotrophoblast progenitors follow two different differentiation pathways. Some fuse to form multinucleated syncytiotrophoblasts, which encase the floating villi of the placenta. Other cytotrophoblasts follow an invasive pathway and differentiate into the EVT (Cartwright *et al.* 2010) (**Figure 1.5**).

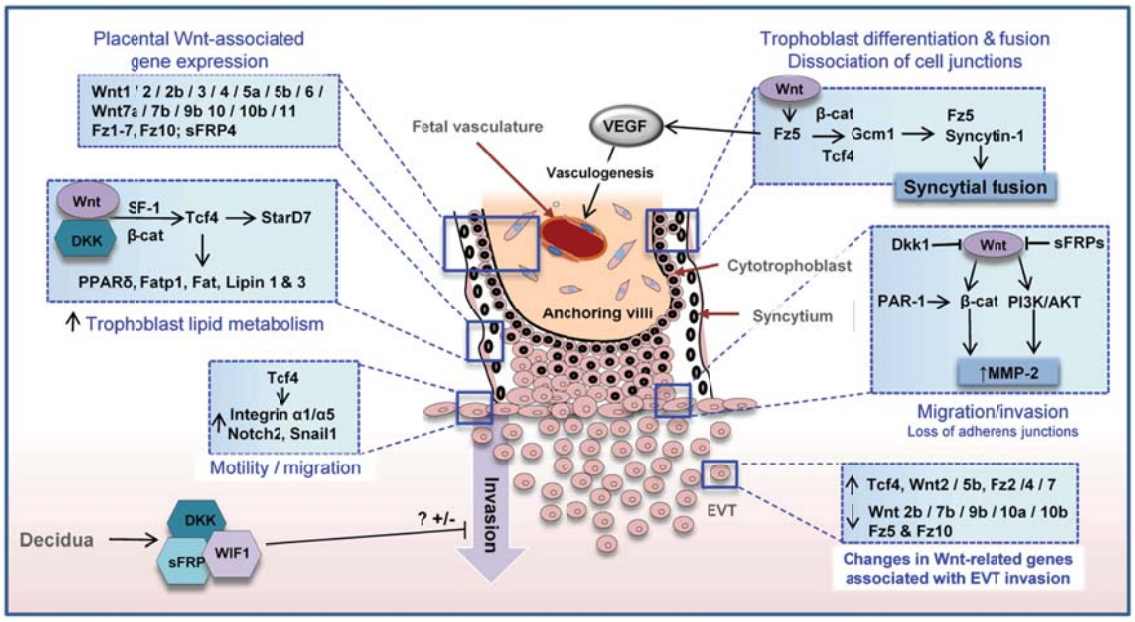

Figure 1.5: Role of the Wnt signalling in trophoblast differentiation, migration and invasion. This cartoon, representing findings from human and mouse studies, depicts the expression profile and major functions of Wnt signalling proteins during trophoblast differentiation, migration and invasion. Wnt components from both trophoblast and decidua actively interact in a paracrine/autocrine/juxtacrine manner to regulate multiple aspects of placentation. A*bbreviations*: AKT, protein kinase B; Dkk1, dickkopf1; EVT, extravillous trophoblast; Fatp1, fatty acid transport protein 1; Fat, fatty acid translocation; FZD, frizzled receptors; Gcm1, glial cells missing homolog 1; MMP2, matrix metallopeptidase 2; PAR-1, protease activated receptor 1; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PPARδ, peroxisome proliferator-activated receptor δ; SF-1, steroidogenic factor 1; sFRP, secreted frizzled related proteins; StarD7, steroidogenic acute regulatory protein-related lipid transfer domain containing 7; Tcf, t-cell factor; VEGF, vascular endothelial growth factor; WIF1, Wnt inhibitory factor-1.

Trophoblast invasion is very similar to cancer cell invasion, with multiple signalling pathways being shared between these two processes, the Wnt signalling pathway being one of them (Fitzgerald *et al.* 2010; Sonderegger *et al.* 2010). Fourteen out of 19 Wnt ligands and 8 out of 10 FZD receptors were reported to be expressed in the human placenta in an expression study utilizing whole human placenta and different trophoblast model systems (Sonderegger *et al.* 2007). *Wnt1, Wnt7B, Wnt10A* and *Wnt10B* were highly expressed in the first trimester (**Figure 1.5**); however, these ligands were largely absent in full term trophoblast (Sonderegger *et al.* 2007), indicating that they might be particularly important in early pregnancy. Five Wnt ligands, Wnt3A, Wnt8A, Wnt8B, Wnt9A and Wnt16, as well as receptors FZD8 and FZD9, were absent from all placental cell types (Sonderegger *et al.* 2007). In a primate placenta expression

study, the antagonist sFRP4 was found to be expressed predominantly in the villous syncytiotrophoblast and the invasive cytotrophoblast, as well as in the amnion (the inner fetal membrane) (White *et al.* 2009). In mouse embryonic stem cells, Cdx2, a critical transcription factor of early trophoblast development, is induced by Wnt3A in a manner that is dependent on LEF-1, a Wnt-related transcription factor (He *et al.* 2008). Recently, it was reported that regulation of canonical Wnt signalling was observed in human embryonic stem cells that had differentiated down the trophoblast lineage. These cells were cultured in the presence of BMP4 (Marchand *et al.* 2011).

Apart from these findings implicating Wnt signalling in early trophoblast lineage differentiation, there is more convincing evidence of active Wnt signalling in later differentiation of invasive trophoblasts (Pollheimer *et al.* 2006) **(Table 1.1).** A substantial amount of nuclear β-catenin expression was observed in invasive trophoblasts during both *in vivo* and *in vitro* differentiation from chorionic villous explant cultures (Pollheimer *et al.* 2006). Using a Matrigel invasion assay, it was demonstrated that Wnt3A stimulated both trophoblast invasion and migration, which could be blocked by the application of Dkk1, suggesting that abnormal activation of the Wnt/β-catenin signalling pathway may contribute to trophoblastic hyperplasia and local invasion (Pollheimer *et al.* 2006) (**Figure 1.5**). In villous explant cultures, Wnt3A increased trophoblast outgrowth and activated the canonical pathway as well as AKT phosphorylation. DKK1 treatment did not inhibit AKT phosphorylation, suggesting that the event is independent of canonical LRP/FZD involvement (Sonderegger *et al.* 2010a). In addition, the proximal invasion zone of anchoring villi showed transient loss of adherens junction components, such as β-catenin and E-cadherin (Zhou *et al.* 1997).

The role of Dkk1 in trophoblast cell invasion during placentation remains unclear. In mice, Dkk1 is found to be expressed in maternal decidual tissue, but expression is minimal in ectoplacental cones. Dkk1 secreted by decidual cells has been demonstrated to induce trophoblast cell invasion (Peng *et al.* 2008). Other ligands may also contribute to β-catenin/TCF-mediated transcription in trophoblasts. The protease activated receptor-1 (PAR-1) has been shown to stabilise β-catenin and promote trophoblast invasion (Grisaru‐Granovsky *et al.* 2009) (**Figure 1.5**).

Immunohistochemical data suggests that levels of the Wnt dependent transcription factors TCF3 and TCF4 are strongly increased in invading trophoblasts (Pollheimer *et*

al. 2006). TCF4 is almost exclusively expressed in non-proliferating cyclin-dependent kinase inhibitor 1C (p57/KIP2)-positive trophoblasts (Pollheimer *et al.* 2006) and, in a recent study, it has been shown that the Wnt/TCF4-dependent signaling could play a role in EVT differentiation, promoting motility and expression of promigratory genes (Meinhardt *et al.* 2014). These findings suggest TCF4 could be an important transcription factor committing and differentiating the EVT phenotype. The Wnt inhibitor *sFRP2* was found to be methylated in first trimester trophoblasts consistent with reduced expression and, therefore, possible enhanced activation of Wnt signalling (Novakovic *et al.* 2008). Collectively, these studies indicated that Wnt signalling is involved in trophoblast adhesion and invasion of maternal uterine tissue.

The crucial role played by Wnt signalling in normal development of the murine placenta has been demonstrated in several *in vivo* studies. *Wnt2* deletion caused oedema, decreased numbers of capillaries, and fibrinoid deposition in the murine placenta. As a result, the pups were underweight and half of them died perinatally (Monkley *et al.* 1996). Similarly, *Wnt7B* deletion resulted in placental abnormalities and pups did not survive past mid-gestation (Parr *et al.* 2001). In mice, homozygous deletion of *R-spondin3*, a secreted Wnt agonist, causes fetal death around embryonic day (ED) E10 due to failure of the interaction between chorion and allantois (Aoki *et al.* 2007). A similar deficiency in chorion-allantois fusion and consequent severe placental formation defects were observed in mice with *TCF-1* and *LEF-1* deletions (Galceran *et al.* 1999). Lu *et al* recently showed that in mice, FZD5 was necessary for the expression of glial cells missing homolog 1 (Gcm1), an important transcription factor for placental labyrinth development and syncytial fusion (including syncytin-1 expression), while Gcm1 up-regulates FZD5 expression specifically at branching sites of the chorion (Lu *et al.* 2013) (**Figure 1.5**).

The angiogenic actions of FZD5 are, at least in part, mediated by induction of expression of vascular endothelial growth factor (VEGF) in chorion trophoblast cells and the dissociation of cell junctions (Lu *et al.* 2013). The findings corroborate earlier studies which showed that the Gcm-1/syncytin-1 pathway is a direct target of β-catenin/TCF4 signalling (Matsuura *et al.* 2011) and that FZD5-null mice die around ED10 due to defects in placental vascularisation (Ishikawa *et al.* 2001). Therefore, the importance of Wnt signalling in the placenta is particularly evident in vascularisation,

chorion-allantois fusion and labyrinth function. Further experimentation is necessary to determine the mechanistic details of these interactions.

1.6.2 Other aspects of placental function involving Wnt signalling

In addition to their role in trophoblast differentiation and invasion, Wnts have been implicated in the regulation of other aspects of trophoblast cell biology. Steroidogenic acute regulatory protein-related lipid transfer domain containing 7 (StarD7), is a member of the StAR family. StAR proteins are involved in intracellular transport and metabolism of lipids. In the human placental choriocarcinoma cell line JEG-3, it was shown that StarD7 expression is a direct target gene of TCF/β-catenin (Rena *et al.* 2009). In a follow up study, it was demonstrated that β-catenin could function as a bridge between steroidogenic factor 1 (SF-1, a nuclear receptor) and TCF4, forming a ternary complex that stimulates StarD7 expression. The SF-1 and β-catenin pathway convergence on StarD7 expression and may have important implications in phospholipid uptake and transport, contributing to normal trophoblast development (Rena *et al.* 2011) (**Figure 1.5**). It was also reported that a decrease in Dkk1 levels contributed to lipid accumulation in the placenta. It was suggested that Dkk1 regulates certain aspects of placental lipid metabolism through the Wnt signalling pathway (Strakovsky and Pan 2011). Therefore, Wnt signalling components appear to have important roles in placental lipid uptake, transport and metabolism (**Figure 1.5**).

The Wnt signalling pathway may also have important roles in remodelling at the maternal-fetal interface. To satisfy the nutrient and oxygen demands of the developing fetus, the uterine vasculature requires dramatic and orchestrated reorganization. The dysregulation of the Wnt signalling has been extensively studied in multiple diseases, including some angiogenic disorders. Wnt signalling activation is a major stimulator in pathological angiogenesis and thus, tightly controlled vasculature reorganization at the placenta most probably involves Wnt antagonists (Zhang and Ma 2010).

1.6.3 Applications of Wnt components to placental disorders

Therapeutic applications based on the administration of sFRP4 and other Wnt antagonists have been explored in a number of different studies (Muley *et al.* 2010; Ramachandran *et al.* 2011; Cho *et al.* 2012; Longman *et al.* 2012; Saran *et al.* 2012; Warrier *et al.* 2014). sFRP4 has been demonstrated to inhibit angiogenesis by decreasing proliferation, migration and tube formation of endothelial cells (Muley *et al.* 2010). It was also reported that both the cysteine-rich and netrin-like domains of sFRP4 have anti-angiogenic properties (Longman *et al.* 2012). sFRP4 has been shown to be able to sensitize glioma cells and stem cells to chemotherapeutics and could be utilized to destroy cancer stem cells of a glioma cell line (Warrier *et al.* 2014). WIF1 is down-regulated in human primary cervical tumors and cell lines. WIF1 re-expression induces significant apoptosis and inhibits cervical cancer cell proliferation and invasion both *in vitro* and *in vivo* (Ramachandran *et al.* 2011). DKK1 has been shown to inhibit the survival and migration of human papillary thyroid cancer cells by regulating Wnt/β-catenin signalling and E-cadherin expression (Cho *et al.* 2012). In a recent study it was demonstrated that sFRP4 can act as a predictive marker of chemosensitivity in ovarian cancer and have therapeutic potential when combined with other drugs (Saran *et al.* 2012). Therefore, the use of Wnt antagonists might have similar applications in the treatment of pathological conditions involving uterine vascular reorganization.

While our understanding of the role of Wnt signalling in the trophoblast and placenta is beginning to unfold, we have very limited knowledge of its significance in the first few weeks of placental development in the human due to the difficulty in obtaining human tissue samples from this time. To date, animal models and *in vitro* cell culture have provided all the available information. We need to develop and implement new approaches, such as xenograft models, trophoblast stem cell models and cell co-culture models, in order to make further progress in our comprehension of the role of Wnt signalling during the early stages of pregnancy.

1.7 Roles of prostaglandins in early pregnancy

Prostaglandins (PGs) are lipid autacoids, widely produced in mammalian cells from arachidonic acid which is released from membrane phospholipids by the action of

phospholipase A_2 enzyme (Kennedy *et al.* 2007; Salleh 2014). Arachidonic acid is converted to PGH_2 by the action of cyclooxygenase (COX) isoenzymes (Ricciotti and FitzGerald 2011). There are two COX isoenzymes in mammals, COX1 and COX2. COX1 and COX2 are encoded by genes *Ptgs1* and *Ptgs2* respectively. *Ptgs1* is constitutively expressed, whereas *Ptgs2* is inducible. PGH_2 is converted to the biologically active prostaglandins by specific synthases, which act through G-protein-coupled cell-surface receptors (Kennedy *et al.* 2007; Salleh 2014).

PGs play a key role in the generation of the inflammatory response. The processes of implantation and trophoblast invasion are considered comparable to proinflammatory responses. Indeed, it has been shown that PGs play a role in ovulation, endometrial vascularization and decidualisation (Chakraborty *et al.* 1996; Kennedy *et al.* 2007; Salleh 2014). In humans, COX1 is expressed in the luminal and glandular epithelia and COX2 is expressed in the luminal epithelia and perivascular cells during implantation window period in human (Marions and Danielsson 1999). Female mice with deletion of *COX2* have multiple reproductive deficits and are infertile. *COX1* deleted mice have normal fertility but show parturition defects (Lim *et al.* 1997).

Hormones and paracrine factors are reported to be capable of controlling PG biosynthesis in the reproductive tract (Kennedy *et al.* 2007; Salleh 2014). Mifepristone, a progesterone receptor antagonist, treatment decreased expression of *COX1* and *COX2* in glandular and luminal epithelia respectively suggesting progesterone could influence *COX* expression (Marions and Danielsson 1999). In mice, uterine *COX1* and *COX2* genes are possibly regulated by ovarian steroids and implanting blastocyst respectively (Chakraborty *et al.* 1996). The most important event in early pregnancy influenced by prostaglandins is decidualization. Increased level of cyclic cyclic adenosine monophosphate (cAMP) is required to maintain the decidualization process (Logan *et al.* 2013). PGE_2 has been shown to have synergistic effect with estradiol in decidualization (Frank *et al.* 1994). PGE_2 also increase intracellular cAMP level and stimulates alkaline phosphatase activity (ALP) in rat (Yee and Kennedy 1993). Decidual cells are demonstrated to synthesize and secrete prostaglandins, as well as prostaglandin receptors (Arosh *et al.* 2003; Kang *et al.* 2006). Cyclooxygenases and prostaglandin E synthase expression are reported to increase in decidua cells suggesting that prostaglandins are required in stromal cells decidualization (Shaw *et al.* 1994; Alfaidy *et al.* 2003). In mice, uterine epidermal growth factor (EGF) and COX2

expression dysregulation during peri-implantation resulted in implantation failure (Song *et al.* 2000). In mice, 8-cell, morula and blastocyst stages all synthesize PGE_2, however, prostacyclin (PGI_2) is the most abundant prostaglandin in mouse blastocyst (Pakrasi and Jain 2008). COX1, COX2 and PGI synthase (PGIS) are also reported to be expressed in inner cell mass and trophoectoderm of mouse blastocyst (Huang *et al.* 2004). Cyclooxygenases, PGIS, prostacyclins and PGE_2 all have important roles in preimplantation embryo development in mouse (Tan *et al.* 2005). The role of prostaglandins in trophoblast invasion is largely unknown; however, some recent evidence indicates its possible role in this process. PGE_2 and its receptor EP_2 agonist have been demonstrated to increase expression of cell adhesion proteins focal adhesion kinase and integrins, as well as increasing overall adhesiveness of HTR-8/SVneo trophoblast cell line to the extra-cellular matrix (ECM) via MEK/MAPK signalling pathway (Waclawik *et al.* 2013). The expression of PGE_2 receptors PE_2 in trophoblast has also been reported, which can be stimulated by PGE_2 in an autocrine or paracrine manner (Waclawik *et al.* 2013). COX2 and PGE_2 synthase have also been detected in HTR-8/SVneo cell line (Dominguez‐Lopez *et al.* 2012). The decidua derived factors including prostaglandins increase trophoblast cell invasiveness modulating different tissue inhibitors of metalloproteinases (TIMPs) and other adhesion proteins (Godbole *et al.* 2011). In brief, prostaglandins especially PGE_2 has possible important role on blastocyst/trophoblast during implantation and subsequent placentation, however, the detail mechanisms and likely interactions with other signalling networks are largely unknown. Influence of decidua derived factor on trophoblasts prostaglandins production and possible prostaglandin signalling reinforcement on trophoblast in an autocrine-paracrine manner remains an interesting prospect to be investigated.

1.8 Wnt signalling in reproductive pathologies

1.8.1 Endometrial, ovarian and chorionic carcinoma

Wnt signalling has been demonstrated to be induced by estrogen in endometrium (Wang *et al.* 2009). Interestingly, nuclear β-catenin, the central component of the canonical Wnt signalling pathway, is detectable in about 30% of estrogen-associated cancers (Moreno-Bueno *et al.* 2002; Cloke *et al.* 2008), whereas Dkk1 levels are reduced in

endometrial carcinoma (Yi *et al.* 2009) **(Table 1.1)**. However, treatment with recombinant Dkk1 also downregulated the invasiveness of endometrial carcinoma cells *in vitro* suggesting aberrant elevation of estrogen-induced Wnt signalling is associated with endometrial malignancy (Yi *et al.* 2009). An expression study of endometrial cancer tissues revealed significantly elevated levels of *Wnt10B* compared with hyperplastic and normal tissues (Chen *et al.* 2013). This activation of the Wnt signalling pathway is often accompanied by the loss of phosphatase and tensin homolog (PTEN) in endometrial cancers. A recent study has reported synergistic actions between the Wnt/β-catenin and PTEN signalling pathways in endometrial cancer onset and progression. However, the specific nature of their function and interactions remain unknown (Zee *et al.* 2013). Mutations in the WNT and PI3K pathways are common in the human ovarian endometrioid adenocarcinomas (OEAs). It has been shown that mice with a gain-of-function mutation in β-catenin followed by nuclear β-catenin accumulation in the ovarian surface epithelium (OSE) cells develop undifferentiated tumors with both mesenchymal and epithelial characteristics. Homozygous deletion of PTEN in the same OSE resulted in development of significantly more aggressive tumors (Tanwar *et al.* 2011a).

Abnormal inactivation of Wnt inhibitors can also lead to malignancy, as demonstrated by the finding that *APC* and *sFRP2* are hypermethylated in choriocarcinomas (Novakovic *et al.* 2008; Wong *et al.* 2008). Similarly, JEG-3 and JAR choriocarcinoma cells are deficient in of Dkk1 expression, while overexpression of Dkk1 can induce apoptosis and growth arrest through JNK induction independent of Wnt signalling (Peng *et al.* 2006). Invasiveness of these choriocarcinoma cell lines was promoted by recombinant Dkk1 in co-cultivation experiments in the presence of decidual cells, whereas Dkk1 antibodies and antisense oligonucleotides reduced invasiveness (Peng *et al.* 2006). Overexpression of sFRP4 also decreases cancer cell proliferation in Ishikawa cell with sFRP4 directly binding with Wnt7A to inhibit activation of Wnt/β-catenin signalling (Carmon and Loose 2008). In a different study, upregulation of nuclear β-catenin was detected in complete hydatidiform mole placentae (Pollheimer *et al.* 2006). To sum up, abnormally elevated levels of Wnt/β-catenin are associated with endometrial or trophoblastic malignancy. Continuous Wnt/β-catenin signalling in the endometrium may be an early step in endometrial carcinogenesis.

In contrast, the expression of Wnt ligands such as *Wnt2, Wnt3,Wnt4* and *Wnt5A* is generally reduced in endometrial carcinoma compared with the normal endometrium (Bui *et al.* 1997) **(Table 1.1)**. sFRP4 expression has been reported to be elevated in estrogen-dependent endometrial and breast cancer, suggesting its involvement in endometrial cell proliferation (Abu-Jawdeh *et al.* 1999). These findings highlight the complexity of Wnt signalling and underscore the different roles played by the Wnt components under different circumstances.

1.8.2 Endometriotic lesions

Recently it has been shown that cells from endometriotic lesions are capable of migrating to the eutopic endometrium. Global gene expression analysis in these cells revealed up-regulation of Wnt7A, as well as 17 other genes associated with the Wnt signalling pathway (Santamaria *et al.* 2012). The endometrium of women treated with the anti-progestin RU486, which causes endometrial breakdown, expresses elevated levels of *sFRP1, Wnt5A, FZD4, FZD6, FZD9, FZD10, β-catenin* and *Axin-2* (Catalano *et al.* 2007), suggesting a role for Wnt signalling in endometrial repair. Elevated levels of expression of *Wnt7A, Wnt2* and *FZD1* have also been observed in endometriotic tissues (Wu *et al.* 2006; Gaetje *et al.* 2007). The increased expression of Wnt7A in endometriotic tissues is an interesting finding because Wnt7A has been demonstrated to induce Hoxa10 expression, which is strongly implicated in the development of endometriosis (Zanatta *et al.* 2010). Moreover, abnormal activation of the Wnt signalling pathway with persistent expression of nuclear β-catenin was found in mid-secretory endometrium in endometriotic patients who were infertile (Matsuzaki *et al.* 2010). Similarly, mRNA expression of antagonists DKK1 and sFRP1 mRNA is reduced in endometriotic compared to normal fibroblasts, possibly indicating over-activation of Wnt/β-catenin signalling (Aghajanova *et al.* 2010).

In contrast, higher levels of *sFRP1* expression were reported in endometriotic lesions (Cheng *et al.* 2008). It has been suggested that angiogenesis is one of the key processes effecting development of endometriosis; sFRP1 induces angiogenesis in several distinct models increasing cultured endothelial cell migration and tube formation (Dufourcq *et al.* 2002). Therefore, it is possible that sFRP1 is involved in endometriosis development by inducing angiogenesis within the lesion. These findings of aberrant over-expression

of the Wnt components might be associated with a persistent proliferative potential and impaired decidualisation capability in endometriosis. Over-activation of the Wnt/β-catenin signalling pathway may be caused by enhanced estrogen signalling relative to inhibited progesterone signalling in ectopic endometrium, thereby promoting the growth of endometrial tissue outside its natural environment (van der Horst *et al.* 2012).

1.8.3 Implantation failure

Repeated implantation failure (RIF) is a severe obstacle in human assisted reproduction treatment. In a global gene profiling study, RIF patients showed significantly lower expression levels of *sFRP1* and *LEF1* in endometria compared with fertile women (Koler *et al.* 2009). Interestingly, *DKK1* expression was found to be up-regulated (Koler *et al.* 2009) (**Table 1.1**). Other genes were found to have altered expression, including those belonging to the cell cycle and cellular adhesion pathways. In a follow-up study it was demonstrated Wnt signalling is one of the pathways regulated by several differentially expressed miRNAs. Consistent with the expression pattern in secretory endometrium, *sFRP4* was found to be lowered in RIF-IVF patients (Revel *et al.* 2011) (**Table 1.1**). In a recent genome-wide miRNA profiling study in women with recurrent miscarriages, it has been reported that Wnt signalling is among the main targets of the most differentially expressed miRNAs (Dong *et al.* 2014). The physiological process most likely to be affected by the aberrant miRNA expression is cell adhesion.

Collectively, these studies suggest that both miRNAs and the molecular pathways they target are differentially expressed in the secretory endometrium of RIF patients compared to fertile women. Multiple miRNAs act in concert to regulate a variety of physiological functions essential for implantation, including cell adhesion, angiogenesis and cell cycle regulation. The Wnt signalling pathway appears to play a vital role in this process. It remains to be seen whether future studies can lead to the development of diagnostic biomarkers or potential drug targets to help in the diagnosis or treatment of RIF.

1.8.4 Trophoblast diseases

In the preeclamptic placenta, a decrease in β-catenin and increase in Dkk1 expression has been observed compared to normal placental controls during the third trimester of

pregnancy. Both β-catenin and Dkk1 were expressed predominantly in the syncytiotrophoblast and the extravillous trophoblast (EVT) (Zhang *et al.* 2013b). In another study, expression of Wnt2 and sFRP4 were shown to be decreased and increased, respectively, in the third trimester placenta of women with severe preeclampsia (Zhang *et al.* 2013c). An elevated level of Dkk1 was reported in decidua tissues and in the serum of the patients with unexplained recurrent spontaneous miscarriage (Bao *et al.* 2013). Wnt2 was predominantly expressed in the villous syncytiotrophoblast and the EVTs. In the preeclamptic placenta, invasion of EVTs into the maternal decidua and myometrium is shallow and limited, and endovascular invasion is nearly absent. Therefore, aberrant down regulation of Wnt/β-catenin signalling by the elevated levels of secreted Wnt antagonists may have functional/pathophysiological associations with preeclampsia. More investigation is necessary to determine whether aberrant Wnt/β-catenin signalling is a cause or effect of these pathological conditions, as well as defining the mechanistic details and diagnostic implications.

1.9 Summary

Wnt signalling cascades play fundamental roles in embryonic development, tissue homeostasis and progression of cancer. They affect numerous key cellular processes such as primary axis formation, axon guidance, cell motility, proliferation and differentiation (Logan and Nusse 2004; Clevers 2006; Cadigan and Peifer 2009; Clevers and Nusse 2012; Niehrs 2012). In human reproduction, the role of Wnt signalling in the initiation of pregnancy, decidualisation, regulation of endometrial function and trophoblast differentiation is crucial. Wnt signalling components have been found in cancers of reproductive tissues, in endometriosis and in other pregnancy-related complications. However, the Wnt signalling pathway is very diverse and frequently interacts with itself and other signalling pathways (e.g. prostaglandins) with difficult-to-predict outcomes. With 19 ligands, 10 receptors, several secreted agonists and antagonists, and above all, multiple signalling transduction cascades, it is a very complex process to untangle (Logan and Nusse 2004; Clevers 2006; Cadigan and Peifer 2009; Clevers and Nusse 2012; Niehrs 2012). Especially, our knowledge on the

reciprocal effects of fetal and maternal cells on Wnt signalling is rather limited. A limited number of Wnt family knockout mice studies have revealed reproductive-related phenotypes, which is probably due to the fact that genome-wide deletion of many Wnt family-related genes result in embryonic lethality (Aoki and Taketo 2008). Hence, despite recent progress, a considerable amount of further work is necessary to fully assess the role of Wnt signalling related components in pregnancy and reproduction

Fertility rates are declining worldwide due to societal changes (e.g. delayed parenthood) and potential biological causes (environmental toxins, obesity, dietary deficiencies and reduced semen quality) (Skakkebæk *et al.* 2006; Baird *et al.* 2010). The increasing incidence of infertility in women due to impaired initiation, implantation and uterine function is also a critical health concern. All of the above issues have increased the demand for fertility treatment. To address this and also to take preventive measures and therapies to reduce the incidence and morbidity of gestational diseases, we need to understand the roles and regulation of Wnt signalling in implantation, placentation and pregnancy. Future studies will serve to illuminate the mechanisms by which Wnt signalling regulates uterine function, implantation and placentation, and also how Wnt signalling itself is regulated. This advancement in knowledge will give us a framework in which to explore new diagnostic and therapeutic approaches to reproduction related diseases.

1.10 Thesis hypotheses, aims and objectives

Understanding the critical events underpinning successful implantation and placentation has been a challenge for reproductive and developmental biologists. However, this knowledge is necessary to both reduce infertility and prevent pregnancy related complications, and also to develop novel contraceptive approaches to restrict world population growth.

In light of the interest in Wnt signalling in various aspects of implantation and placentation, and the scarcity of knowledge regarding their precise importance and role, the **following aims** are proposed:

AIM 1: Characterize the mRNA and protein expression of various Wnt signalling proteins (with an emphasis on Wnt antagonists) in term placental and decidual cells in vitro.

AIM 2: Determine the effects of cross-talk between trophoblast and decidua cells on expression of Wnt signalling proteins during the phase of trophoblast attachment to decidua.

AIM 3: Determine the effects of selected Wnt antagonists on selected aspects of decidual and trophoblast cellular function.

My **specific hypotheses** are as follows:

1. Reciprocal Wnt signalling between maternal and fetal cells during gestation is important for implantation, placentation and trophoblast differentiation.
2. Secreted Wnt antagonists play important roles in modifying Wnt signalling in endometrial and trophoblastic tissues.
3. Secreted components of the Wnt signalling play important role in interacting with other cell signalling pathways.

Chapter 2: Materials and methods.

2.1 Materials

Reagent	Supplier
Anti-active β-catenin antibody	Merck-Millipore, Billerica, MA, USA.
Antibiotic/ Antimycotic solution (100X)	Life technologies, Grand island, NY, USA
Anti-COX1 monoclonal antibody	Cayman chemical, Ann arbor, MI, USA
Anti-cytokeratin 7 antibody	Dako, glostrup, Denmark
Anti-DKK1 antibody	Santa Cruz biotechnology, Dallas, Texas, USA
Anti-MSX-1 antibody	Cell signalling Tech Inc, MA, USA
Anti-sFRP4 antibody	Abcam, Cambridge, UK
Anti-Total β-catenin antibody	Cell signalling Tech Inc, MA, USA
Anti-vimentin antibody	Dako, glostrup, Denmark
Anti-β-actin antibody	Abcam, Cambridge, UK
Bioscript reverse transcriptase	Bioline, London, UK
Bovine serum albumin (BSA)	Bovogen, Vic, Australia
Collagenase type I	Life technologies, Grand island, NY, USA
Complete protease inhibitors	Sigma-Aldrich, St Louis, MO, USA
Dispase-II	Life technologies, Grand island, NY, USA
DNAseI	Roche diagnostics, Germany
DNAseI (RNase-free)	Applied Biosystems, USA
Dulbecco's modified Eagle's medium/Nutrient mixture F-12 (DMEM/F-12)	Sigma-Aldrich, St Louis, MO, USA
Dulbecco's phosphate buffered saline (D-PBS)	Invitrogen, Carlsbad, CA, USA (?)
DyLight™ 488 goat anti-mouse IgG	Biolegend, San diego, USA

DyLight^{TM} 594 goat anti-mouse IgG	Biolegend, San diego, USA
ECL select	GE healthcare, Uppsala, Sweden
Fetal calf serum	Bovogen, VIC, Australia
Epidermal growth factor	Invitrogen, Carlsbad, CA, USA
Hoechst 33258	Sigma-Aldrich, St Louis, MO, USA
Antibiotic/antimycotic solution (100X)	Invitrogen, Carlsbad, CA, USA
HRP conjugated anti-mouse/ rabbit antibody	Sigma-Aldrich, St Louis, MO, USA
Human WIF1 and DKK1 ELISA kit	R&D systems, Minneapolis, MN, USA
Immolase DNA polymerase	Bioline, London, UK
Insulin transferrin sodium selenite cocktail (100X)	Life technologies, Grand island, NY, USA
KiCqStart SYBR green qPCR ready mix	Sigma-Aldrich, St Louis, MO, USA
Medium 199 with Earle's salts and L-glutamine	Life technologies, Grand island, NY, USA
Corning transwell inserts	Sigma-Aldrich, MO, USA
Mini-protean TGX precast gels	Biorad, Hercules, CA, USA
Percoll	GE healthcare, Uppsala, Sweden
Ponceau S	Sigma-Aldrich, St Louis, MO, USA
Prostaglandin E_2 EIA kit monoclonal	Cayman chemical, Ann arbor, MI, USA
Recombinant human sFRP4	R&D systems, Minneapolis, MN, USA
Recombinant mouse EGF	Invitrogen, Carlsbad, CA, USA
RNeasy Mini Kit	QIAGEN, Venlo, Limburg, Netherlands
RT² HT First Strand Kits	QIAGEN, Venlo, Limburg, Netherlands
RT^2 SYBR green ROX fast mastermix	QIAGEN, Venlo, Limburg, Netherlands
sFRP4 ELISA kit	USCN life science Inc, Wuhan, PRC
Wnt Signaling Pathway PCR Array	QIAGEN, Venlo, Limburg, Netherlands
Recombinant human DKK1	Merck Millipore, Billerica, MA, USA
HRP conjugated anti-mouse/rabbit antibody	Sigma-Aldrich, St Louis, MO, USA

2.2 Methods

2.2.1 Trophoblast cell isolation

Placentas were obtained with informed consent from women after delivery by Caesarean section at term as approved by the Human Research Ethics Committee of the Women and Newborn Health Service, Perth WA. All placentas were from elective Caesarean sections at term. The selected for placenta collection were Cytotrophoblast cells were extracted from term placenta as previously described with modifications (Aye *et al.* 2010). It has been ensured that the placenta is obtained within 30 minutes of delivery. Placenta was transferred to the laminar flow cabinet (Thermo Fisher scientific, Waltham, MA, USA) and was placed cord-down. Surface of the placenta was washed with D-PBS to remove blood. Chunks of villous tissues (2-5 mm square) were cut away using scissor, avoiding connective tissue, sinews, blood vessels and discoloured areas where possible. Around 80-100g of villous tissue was dissected free of membranes and blood vessels were removed from lobe to lobe. These tissues were extensively washed in D-PBS to remove as much blood as possible. The washed tissues were transferred to a 500 ml sterile bottle and were digested in 0.25% *w/v* (2.4 U/ml) dispase-II (Invitrogen, Carlsbad, CA, USA) dissolved in 250 ml HBSS (**Table 2.1**). Digestion was continued for 1 h at 37˚C on a gentle stir (approximately 560 rpm). Suspension became very murky and took on a dark hue due to the liberation of red blood cells. DNAseI (Roche diagnostics, Germany) (800 μg) was added 15 min prior to the end of digestion. Tissue digests liberated primary trophoblasts which were then filtered through 70 μm cell filters and were collected in 50-ml Falcon tubes and centrifuged at 300 g for 10 min. Supernatant was washed in D-PBS once. Erythrocytes were removed by incubation of a cell pellet in red cell lysis buffer for 10 min (**Table 2.2**). After red cell lysis the cells were collected in FCS (Bovogen, VIC, Australia) after centrifugation at 300 g for 10 min. Cells were washed with media and then suspended in 5% Percoll (GE healthcare, Uppsala, Sweden). Trophoblast cells were purified by centrifugation at 1200 g in slow mode (brakes were removed on the centrifuge machine) for 20 min on a discontinuous Percoll gradient (20–60%). Before loading to the Percoll gradient, the cells were again filtered through 70 μm cell filters. Percoll gradient was freshly made each time from 20%, 30%, 35%, 40%, 45%, 50% and 60% Percoll solutions. Gradient was made by carefully overlaying solutions with a glass Pasteur pipette from the densest (60%) to the

rarest (20%). Cells which migrated between the 20 and 40% Percoll bands were collected. Cells were further washed in media and suspended in 10 ml media. 10 µl cell suspension was mixed with 90 µl trypan blue. This mixture was placed in a hemocytometer to count the number of cells in the central gridded square (25 small squares) on the slide under a microscope (Motic, Hong Kong). The formula used to calculate the total number of isolated cells was:

Count (in 25 small squares) × 10 (trypan blue dilution) × 10000 (µl to ml) × total volume = total cells isolated.

Table 2.1: Components for HBSS

Components	Concentration
NaCl	0.137 M
KCl	5.4 mM
Na_2HPO_4	0.25 mM
KH_2PO_4	0.44 mM
$NaHCO_3$	4.2 mM

Table 2.2: Components for red cell lysis buffer

Components	Concentration
NH_4Cl	50 mM
$NaHCO_3$	10 mM
EDTA	0.1 mM

2.2.2 Decidua cell isolation

Primary decidual cells were extracted from term placenta as previously published with modifications (Keelan *et al.* 2010). It has been ensured that the placenta is obtained within 30 min of delivery. Placenta was then transferred to the laminar flow cabinet (Thermo Fisher scientific, USA) and was placed cord-down. Placenta surface was washed with D-PBS to remove blood. Membranes were cut from the placenta in pieces and transferred to a new petri dish. The blood clots and vessels were removed by washing in D-PBS (Sigma-Aldrich, St Louis, MO). Decidua was then scraped from the underlying chorioamnion using a glass slide. Scraped decidua was then minced using

two scalpels into fine pieces (about 2 mm^2). The collected decidual tissue was transferred to a 500 ml sterile bottle and was digested with dispase-II (Invitrogen, USA) dissolved in 100 ml HBSS (**Table 2.1**). Digestion with dispase-II (0.25% *w/v*) continued for 1.5-2 h at 37˚C on a gentle stir (approximately 560 rpm). DNaseI (400µg; Roche diagnostics) was added for the final 15-30 min of incubation. Tissue digests were then filtered through 70 µm cell filters and were collected in 50-ml Falcon tubes and centrifuged at 300 g for 10 min. Supernatant was washed in D-PBS once. Erythrocytes were removed by incubation of a cell pellet in red cell lysis buffer (**Table 2.2**) for 8 min. After red cell lysis the cells were collected in FCS after centrifugation at 300 g for 10 min. After another wash with media to get rid of lysis buffer, the decidua cells were purified by centrifugation at 1200 g for 20 min on a discontinuous Percoll (GE healthcare) gradient 40%/60%. Before loading to the Percoll gradient, the cells were again filtered through 70 µm cell filters. Percoll gradient was freshly made each time from 40% and 60% Percoll sulutions. Gradient was made by carefully overlaying 40% solution with a glass Pasteur pipette over 60% solution. Cells lying above the 40% layer were recovered by aspiration, washed in media and recovered by centrifugation. The number of cells was counted using the formula described above.

2.2.3 Cell culture and treatments

Collected trophoblast cells were seeded at 1×10^6 cells/ml (2.1×10^5 cells/cm^2) and cultured in M199 (Life technologies, NY, USA) media. Collected decidual cells were seeded at 5×10^5 cells/ml (1.05×10^5 cells/cm^2) and cultured in F12/DMEM (Sigma-Aldrich, MO, USA) media. Both types of media were supplemented with 10% fetal calf serum (Bovogen, VIC, Australia), 10 ng/ml epidermal growth factor (Invitrogen, Carlsbad, CA, USA), 5 ng/ml insulin, 10 ng/ml transferrin, 0.2 nM sodium selenite (Life technologies, NY, USA), and penicillin/streptomycin (100 U/ml; Invitrogen, Carlsbad, CA, USA) in a 95% air/5% CO_2 humidified atmosphere at 37°C. Cells were washed twice with D-PBS after an overnight incubation and media was replaced. The isolated primary trophoblast and decidua cells were grown in a co-culture setting using 6 and 24-well plates (BD biosciences, NJ, USA) containing 24 mm or 6.5 mm diameter Transwell inserts (Sigma-Aldrich, MO, USA) comprising of a 0.4 µm polyester (PET) microporous membrane which allows mixing of media and secreted factors between compartments. Trophoblast cells were seeded in the upper (inner) compartment to attach to the apical surface of the membrane, while decidual cells were placed in the lower

(outer) compartment to attach to the plastic surface of the plate. For comparison, single cell type cultures were also prepared containing either decidual cells with empty inserts, or trophoblast-coated inserts within un-seeded plates. Cells were cultured for up to 4 d with media changes on every other day. In the treatment studies, cells were treated with 30 ng/ml of DKK1 (Merck-Millipore, MA, USA) and 300 pg/ml of sFRP4 (R&D systems, MN, USA) in fresh media. Treatment concentrations were selected based on our measurement of the concentrations of these antagonists in co-culture and from published studies demonstrating effective concentrations. Treatment commenced on day 1 of culture and continued for 1 and 3 d. For analysis of PGE_2 production, cells were incubated for 4 h with/without treatments in serum-free media supplemented with 1% bovine serum albumin (BSA). Cells were lysed by incubation in 1 N NaOH overnight and assayed for total protein by bicinchinonic acid assay (BCA) calibrated against BSA (Redinbaugh and Turley 1986).

Table 2.3: Cell seeding concentrations

Cell type	Plate/insert type	Cell concentration
Trophoblast	6-well plate (9.5 cm^2/well)	1×10^6 cells/ml
	24-well plate (1.9 cm^2/well)	
	96-well plate (0.32 cm^2/well)	
	Transwell® insert 6-well (4.67 cm^2/well)	
	Transwell® insert 24-well (0.33 cm^2/well)	
Decidua	6-well plate (9.5 cm^2/well)	5×10^5 cells/ml
	24-well plate (1.9 cm^2/well)	
	96-well plate (0.32 cm^2/well)	
	Transwell® insert 6-well (4.67 cm^2/well)	
	Transwell® insert 24-well (0.33 cm^2/well)	

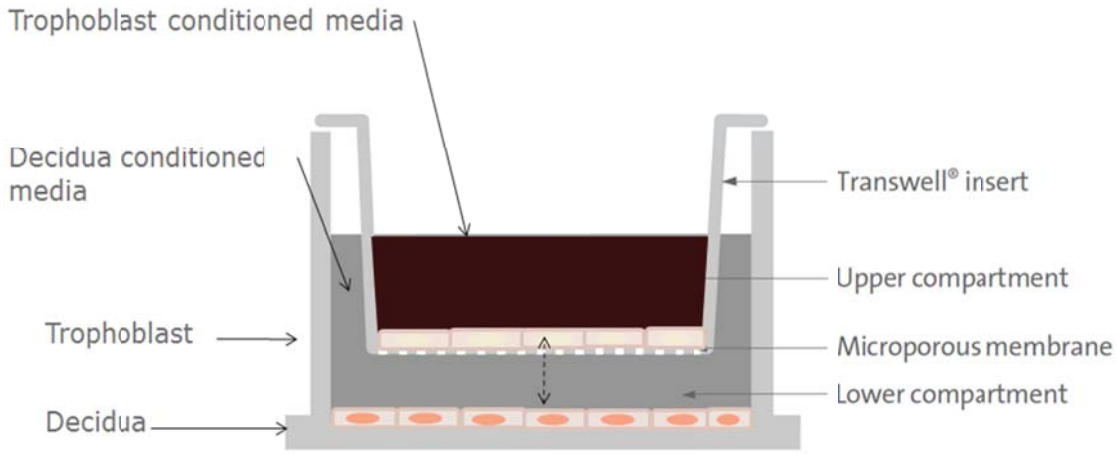

Figure 2.1: The co-culture setting. Transwell inserts are used to co-culture trophoblast and decidua cells. Dotted arrow represents movement and mixing of media between two compartments.

2.2.4 Immunoflourescence

The purity of the isolated trophoblast and decidua cells were established by immunofluorescence staining. Isolated trophoblast and decidua cells were washed twice after 24 h incubation in 96-well plates. Cells were then fixed using 1:1 methanol:acetone. Trophoblast and decidua cells were incubated with anti-cytokeratin-7 (1:100; Dako, glostrup, Denmark) and anti-vimentin (1:200; Dako) antibodies respectively for 1 h at room temperature on an orbital shaker at mild rotating speed. After 3X washing with PBS-T, trophoblast and decidua cells were incubated with DyLight™® 488 (1:100; Biolegend, San diego, USA) and DyLight™® 594 (1:100; Biolegend) goat anti-mouse IgG respectively for 1 h at room temperature on an orbital shaker. After 3X washing with PBS-T, cells were incubated with Hoechst 33258 (5µg/ml; Sigma-Aldrich) for 5 min at room temperature with shaking. After again 3X wash with PBS-T the cells were taken for microscopic imaging. Cytokeratin-7 and vimentin positive cells in 5 fields at 200 × magnifications were counted using an Eclipse T*i* inverted fluorescent microscope (Nikon, Tokyo, Japan). Around 500-700 cells were counted for each types (n=5) and percentages of the cytokeratin-7 and vimentin positive cells were calculated to determine cell purity (≥ 95% for each).

2.2.5 RNA extraction

After 3 d of culture total RNA was extracted from trophoblast and decidua cells from 2 wells of a 6-well plate and 3 x 24 mm diameter transwell inserts, grown either separately or in co-culture (n=3), using the RNeasy kit (QIAGEN, Venlo, Limburg, Netherlands) according to the manufacturer's protocols. In brief, cells were lysed open by the lysis buffer included in the kit. After addition of 70% ethanol the cell-lysed mix was centrifuged on a cell column. RNA remained bound with the column and flow-through was discarded. After a series of washes with different buffer the RNA was eluted in RNAse free water. Around 30 µg of RNA was extracted in 30 µl RNAse free water. RNA quality and quantity were checked by measuring the 260:280 nm ratio on a Nanodrop ND-1000 (Thermo Fisher scientific). It was ensured that all the samples are relatively pure (260/280 = between 1.8 to 2).

2.2.6 Wnt PCR array

Using a Reaction Ready First-Strand cDNA Synthesis kit (QIAGEN), 1 µg total RNA was reverse transcribed for 15 min at 42˚ according to the manufacturer's protocol for the real-time PCR reaction (n=3); the resulting cDNA was kept on ice. Real-time PCR procedures were carried out according to the manufacturer's protocol (QIAGEN). Briefly, cDNA was diluted and added to the RT^2 SYBR Green Master Mix containing SYBR green and references dyes (QIAGEN) for the Wnt PCR array® in Rotor-disc 100 format. The mixtures were then aliquoted into rotor-disc 100 PCR array plates using high-precision PCR setup bench top machine QIAgility (QIAGEN). This disc format contained primer assays for 84 pathway focused genes and 5 housekeeping genes. In addition, one well contained a genomic DNA control (GDC), 3 well contained reverse-transcription controls (RTC) and 3 wells contained a positive PCR control (PPC). Before loading the rotor-disc 100 to the PCR machine, it was sealed with a film using rotor-disc heat sealer (QIAGEN). Thermal cycling was carried out according to the manufacturer's protocol which follows: 95˚ for 10 min for activation of HotStart DNA polymerase, followed by 40 cycles of denaturation at 95˚ for 15 sec each and finally primer extension at 60˚ for 30 sec) on a Rotorgene Q (QIAGEN) thermocycler. The fluorescent SYBR green signal was detected immediately after the extension step of each cycle, and the cycle at which the product was first detectable was recorded as the cycle threshold. Raw data were acquired and threshold cycle (C_T) value for each

gene/well from each sample was determined using the Rotor-Gene Q series software (QIAGEN) according to the array manufacturer's protocol. The data were imported into and later analysed by the excel-based PCR array data analysis template provided by QIAGEN available at (http://www.sabiosciences.com/pcrarraydataanalysis.php). For normalization of trophoblast gene expression, ribosomal protein L13a (RPL13A) and β-actin (ACTB) were used as the house-keeping genes. For decidual cells, expression was normalized against β-2-microglobulin (B2M) and ribosomal protein L13a as the house-keeping genes. Arithmetic means of the C_t values of the house-keeping genes were used for normalization. For any target gene C_T values greater than 33 were considered as not detected. GDC and RTC wells were checked to ensure there were no genomic DNA contamination and no inhibition of the reverse-transcription reaction respectively. PPC wells were checked to ensure that there was no significant variability from sample to sample PCR reactions. Melting curve analysis was performed to verify PCR specificity for each product. Relative gene expressions of all the genes were calculated using $\Delta\Delta C_T$ method. The Wnt PCR array includes 15 Wnt ligands, 8 FZD receptors, co-receptors, -regulators of the Wnt signalling pathway, competitive Wnt-binding antagonists, intracellular signalling molecules, target genes involved in growth regulation and proliferation, and genes involved in protein modification downstream of Wnt signalling, including genes involved in kinase and phosphatase activity and ubiquitination (**Appendix 1-3**).

2.2.7 Reverse transcription for qRT-PCR

Total RNA was extracted from trophoblast and decidual cells as described above. RNA was converted to cDNA using the Bioscript enzyme (Bioline, London, UK) with random hexamer priming according to the manufacturer's protocol. 500 ng of RNA was used for cDNA conversion for each sample. RNA was first made up to 10 µl with DEPC water. 4 µl random hexamer was added to each sample and incubated at 70˚C for 5 min. After that dNTP, buffer and reverse-transcription enzyme were added to the mix (**Table 2.4**). Following thermal condition was used using PTC-100 thermal cycler (Biorad, Hercules, CA, USA): 5 min at 25˚C, 60 min at 42˚C, 10 min at 70˚C and finally hold at 4˚C.

Table 2.4: Components for reverse transcription reaction

Components	Amount for 1 reaction (µl)
10 mM dNTPs	1
5X Bioscript® Reaction Buffer	4
Bioscript® enzyme	1

2.2.8 Quantitative real-time polymerase chain reaction (qRT-PCR)

Analysis of WIF1, sFRP1, sFRP4, FZD5, FZD4, DKK1, WNT5A, PTGS1, PTGS2, MSX1, MSX2, HOXA10 and SDHA mRNA expression by qPCR was performed in duplicates with 0.2 – 1 µg of cDNA using KiCqStart® SYBR Green qPCR Ready mix (Sigma-Aldrich). All primers were predesigned and *in silico* prevalidated by Sigma-Aldrich (**Table 2.6, Appendix figure I**). Around 300 nM of each primer was used for PCR reaction. Each cDNA sample was made up to 7 µl with water. The master mix was mixed with the primers and template in 20 µl reaction volume (**Table 2.5**). KiCqStart® SYBR green qPCR ready mix contains optimized concentrations of $MgCl_2$, dNTPs (dATP, dCTP, dGTP, dTTP), KiCqStart® Taq DNA Polymerase, SYBR Green dye and stabilizers. PCR amplification and detection was performed on a Rotorgene Q instrument (QIAGEN) using the initial denaturing condition of 95˚C for 3 min, followed by 40 cycles of 95˚C for 15 sec, 55˚-60˚C for 15 sec and 60˚ for 1 min. Full activation of KiCqStart® Taq DNA polymerase occurs within 1 second at 95˚C. The fluorescent SYBR green signal was detected immediately after the extension step of each cycle, and the cycle at which the product was first detectable was recorded as the cycle threshold. Raw data were acquired and threshold cycle (C_T) value for each well from each sample was determined using the Rotor-Gene Q series software (QIAGEN). Expression of mRNA was quantified using the comparative threshold cycle (C_t) method for relative quantification ($2^{-\Delta\Delta Ct}$), normalised to SDHA.

Table 2.5: Components of qRT-PCR reaction

Components	Amount for 1 reaction (µl)
KiCqStart SYBR green qPCR ready mix	10
Forward primer	1.5
Reverse primer	1.5

Table 2.6: Gene specific primers for qRT-PCR.

Primer Name	Unigene	Reference sequence	Tm ˚C	Sequence (5′3′)	Product size (bp)
WNT5A	Hs.696364	NM_003392	56	**Forward**: ATTAATTCTGGCTCCACTTG **Reverse:** GGTTATTCATACCTAGCGAC	186
FZD4	Hs.19545	NM_012193	60	**Forward**: GCAGTTCTTCCTTTGTTCTG **Reverse:** AGGCAAATCCAAATTCCTTC	134
FZD5	Hs.17631	NM_003468	56	**Forward**: AACTTGAGTGTAGGCATTTG **Reverse:** ATACTAGGTTTCTTCCCACC	153
DKK1	Hs.40499	NM_012242	58	**Forward**: GGACAAGAAGGTTCTGTTTG **Reverse:** CTTCTTTCAGGACAGGTTTAC	103
sFRP1	Hs.713546	NM_003012	58	**Forward**: ATTCTCTGCAACACTTTGAG **Reverse:** AATCTACCATCCTTTCCTCC	135
sFRP4	Hs.658169	NM_003014	58	**Forward:** AGGCTATGCTTCAGTTTTTC **Reverse:** TTTTCCACCAGCTTTAACTC	93
WIF1	Hs.284122	NM_007191	59	**Forward**: AGTTGTTCAAGTTGGTTTCC **Reverse:** TAGCATTTTGAGGTGTTTGG	119
MSX1	Hs.424414	NM_002448	58	**Forward**: CAACAAAACATTTGCTCTGG **Reverse:** AATTGTGTTTCTGGTACTGG	95
MSX2	Hs.89404	NM_002449	57	**Forward**: AAGACATATGAGCCCTACC **Reverse:** CTGTTTCTGACGGAACTTG	118
PTGS1	Hs.201978	NM_000962	60	**Forward**: AAAGGGAAACAGGCTAAATG **Reverse:** ATCCACCAAATCAAAACTCC	101
PTGS2	Hs.196384	NM_000963	57	**Forward**: AAGCAGGCTAATACTGATAGG **Reverse:** TGTTGAAAAGTAGTTCTGG	113
PTGES	Hs.146688	NM_004878	58	**Forward**: CAAAAACATCACTCCCTCTC **Reverse:** AAAAGTCTGCATTCTTAGCC	113
HOXA10	Hs.110637	NM_018951	60	**Forward**: CTCCCCTTCATAACAGAAAAAG **Reverse:** AATTGCCTTGACACATTTCC	153
SDHA	Hs.440475	NM_004168	60	**Forward**: TGGGAACAAGAGGGCATCTG **Reverse:** CCACCACTGCATCAAATTCATG	86

2.2.9 Protein extraction

Cells were washed twice with D-PBS and then lysed in radioimmunoprecipitation assay (RIPA) buffer (**Table 2.7**). Protease inhibitor cocktail (Sigma-Aldrich) was added to the RIPA buffer fresh before each use. Cell lysis mix was homogenized by a sonicator (Hielscher, Germany) on ice for uniform lysis and to get rid of viscosity imparted by DNA.

2.2.10 Protein quantification by bicinchinonic assay

Protein concentration was quantified by bicinchinonic assay (BCA) calibrated to bovine serum albumin (BSA). RIPA buffer was first diluted 1:10 using NaOH and a series of BSA standards were made for a 7 point standard curve, using 2-fold serial dilutions starting with 2000 µg/ml. Protein samples were also diluted 1:10 before loading. 200 µl of BCA reagent was added on 25 µl of standards and protein samples in duplicates in a 96 well plate. The plate was placed on an orbital shaker for 1 h at room temperature. After incubation the plate was taken to a VersaMax plate reader (Molecular devices, CA, USA). Protein sample concentrations were measured using the SOFTMAX proV software ((Molecular devices, CA, USA) following "BCA Protein Assay" protocol. The plate was read at 562 nm and the results were adjusted according to the dilution factor 10.

Table 2.7 Components of RIPA buffer

Components	Concentration
Tris HCl, pH 7.5	50 mM
NaCl	150 mM
EDTA	2 mM
Sodium deoxycholate	0.5%
SDS	0.1%
Triton X100	1%
Protease-inhibitor cocktail	1%

2.2.11 Immunoblotting

Approximately 40 µg of proteins from each sample was mixed with 5X Laemmli sample buffer (0.5 ml 0.5 M Tris HCl pH 6.8, 0.8 ml Glycerol, 1.6 ml 10% SDS, 0.4 ml 2-mercaptoethanol, 4.0 ml 0.1% bromophenol blue and 0.8 ml deionized water). Each sample was made up to 42 µl by addition of RIPA buffer and was loaded in a 10% pre-cast Tris glycine extended (TGX) polyacrylamide gradient gel (Biorad, Hercules, CA, USA). Precision Plus® protein dual color standards (Biorad, Hercules, CA, USA) was used as marker. The gels were electrophoresed in mini-protean tetra cell systems (Biorad) with generic running buffer (**Table 2.8**). Gels were run in 150 V for 50 to 60 min and then proteins were transferred from gel to a PVDF membrane in an Xcell SureLock^{TM} Mini-Cell transfer module (Invitrogen). For transfer, generic transfer buffer was used (**Table 2.9**). Transfer module was run for 120 min at 20 V. PVDF membranes were checked with Ponceau S (Sigma-Aldrich) solution for proper transfer of proteins. PVDF membranes were then blocked in 5% skimmed milk powder dissolved in TBS-T buffer (Tris-buffered saline and Tween 20) for 1 h and later incubated overnight at 4˚C with anti-active β-catenin (1:1000; Merck-millipore), COX1 (1:1000; Cayman chemical, Ann arbor, MI, USA), DKK1 (1:200; R&D systems), MSX1 (1:1000; Cell signalling Tech Inc, MA, USA), sFRP4 (1:1000; Abcam, Cambridge, UK), total β-catenin (1:1000; Cell signalling Tech Inc) or β-actin (1:2500; Abcam) for 1 h. All antibodies were diluted down to working concentration with 5% skimmed powder dissolved in TBS-T buffer. Membranes were then incubated with horseradish peroxidise-conjugated goat anti-rabbit or anti-mouse antibody (1:2500; Sigma-Aldrich) for 1 h. In between all incubations membranes were washed thrice with TBS-T, 10 min each time. After that the membranes were visualized by chemiluminescence using ECL select® (GE Healthcare, Uppsala, Sweden). Provided ECL select solution A and B were mixed in 1:1 ratio and spread on top of the membrane in adequate volume to cover its surface for 5 min. Images were photographed and quantified using an ImageQuant 350 digital imaging and quantification system (GE Healthcare).

Table 2.8: Components of electrode (running) buffer

Components	Concentration
Tris-HCl	25 mM
Glycine	200 mM
SDS	0.1%

Table 2.9: Components of transfer buffer

Components	Concentration
Tris-HCl	25 mM
Glycine	200 mM
Methanol	20%

2.2.12 The enzyme-linked immunosorbent assay (ELISA) and enzyme immunoassay (EIA)

To measure DKK1 and WIF1, in cell extract, ELISA Duo set kits from R&D systems (MN, USA) were used according to the manufacturer's protocol. Proteins from cell extracts were collected after 3 d after culture in co-culture setting. The DKK1 assay was calibrated against a highly purified *Sf* 21–expressed recombinant human DKK1 produced at R&D systems. The WIF1 assay was calibrated against a highly purified NS0-expressed recombinant human WIF1 produced at R&D systems. Samples were analysed in duplicates, diluted 1:10 for DKK1 and 1:2 for WIF1 measurement in media. First, a high binding ELISA plate (Greiner bio-one, Kremsmünster, Austria) was incubated overnight with provided capture antibody dissolved in PBS at room temperature on orbital shaker. The plate was then incubated with blocking solution (1% BSA in PBS) for 1h, followed by 2 h of incubation with standards (7 point standard curve using 2-fold serial dilutions starting with 4000 pg/ml for DKK1 and 1500 pg/ml for WIF1) and samples in 1% BSA solution. The plate was then incubated with detection antibody for 2 h followed by 20 min of incubation with streptavidin-HRP. All the incubations were done in room temperature on orbital shaker and in between the incubations, plates were washed 3X with PBS-T using Asys Atlantis microplate washer (Biochrom, Cambridge, UK). Colour was developed using TMB reagent [0.1 mg/ml TMB and 0.02% H_2O_2 in citrate phosphate buffer (26.5 mM citric acid, 50 mM Na_2HPO_4, pH 5.5)] and stopped by addition of 3% H_2SO_4; absorbance at 450 nm was

measured on a VersaMax plate reader (Molecular Devices, CA, USA) and curve fitting and data extrapolation were carried out using the on-board software (SOFTMAX proV, Molecular Devices). The DKK1 assay had a calibration range of 60-4000 pg/ml and a limit of detection of 30 pg/ml. The WIF1 assay had a calibration range of 15-2000 pg/ml and a limit of detection of 15 pg/ml.

For determination of cellular sFRP4 concentrations, an ELISA kit was employed from Uscn life science inc. (Wuhan, PRC) and used according to the manufacturer's protocol. Standards (7 point standard curve using 2-fold serial dilutions starting with 4000 pg/ml) and samples were incubated for 2 h at 37˚C. The wells were then incubated with supplied detection reagent A. After 1 hr incubation, the wells were then incubated with supplied detection reagent B for 30 min at 37˚C. In between each incubation steps, the wells were washed three to five times with supplied wash buffer using Asys Atlantis microplate washer (Biochrom). Substrate solution from the kit was loaded on the plate, incubated for 25 min at 37˚C. The plates were protected from direct light followed by addition of supplied stop solution after optimal colour development. absorbance at 450 nm was measured on a VersaMax plate reader (Molecular Devices, CA, USA) and curve fitting and data extrapolation were carried out using the on-board software (SOFTMAX proV, Molecular Devices). The assay had a calibration range of 60-4000 pg/ml and a limit of detection of 25 pg/ml. All samples were diluted 1:5 in media. The intra-assay precision of ELISAs was <10%.

Prostaglandin E2 was measured by enzyme immunoassay (EIA) using kits from Cayman Chemicals (MI, USA). It was a competitive assay kit based on the competition between PGE_2 and a PGE_2-acetylcholinesterase (AChE) conjugate (PGE_2 tracer) for a limited amount of PGE_2 monoclonal antibody. Media (serum-free) was collected from cultured cells treated with DKK1 and sFRP4 on day 1 and 3 after 4 h incubation. All the samples were collected from triplicate wells. EIAs were performed following the manufacturer's instructions on samples diluted 1:5 prior to assay in assay buffer provided by the manufacturer (Cayman Chemicals). Supplied EIA buffer was used to make standards, 1000 pg/ml, 500 pg/ml, 166.67 pg/ml, 55.56 pg/ml, 18.5 pg/ml and 6.2 pg/ml. Standards, samples and in case of maximum binding well (B_0), EIA buffer was loaded in high binding ELISA plates (Greiner bio-one). A couple of wells were kept as blank. Next, prostaglandin E_2 AChE tracer was loaded in each well followed by

prostaglandin E_2 monoclonal antibody. Each plate was covered with plastic film and incubated for 18 h at 4˚C. The wells were emptied and rinsed 5X with supplied wash buffer followed by incubation with Ellman's reagent for 90 min at room temperature. Absorbance at 405 to 420 nm was measured on a VersaMax plate reader (Molecular devices, CA, USA); concentrations of PGE_2 in media samples were extrapolated from the standard curve (range: 6.2-1000 pg/ml).

Total protein concentration was quantified in each well by bicinchinonic assay (BCA) using SoftMax Pro V software. The intra-assay precision of ELISAs was <15%. Inter-assay variability was <17%.

2.2.13 Statistics

All studies were performed multiple times-: PCR array (n=3), quantitative real-time RT-PCR (n=8), immunostaining (n=4), immunoblot (n=4), PGE_2 EIA (n=3) and ELISA (n=4). Data are represented as mean ± SD or mean ± interquartile range, as stated in figure legends. Descriptive statistics were derived for each data set, normalized to control where appropriate, and the mean data combined for collective analysis. Graphs were plotted and statistics were performed using Microsoft Excel (Redmond, USA) or InStat (GraphPad Software, CA, USA). The distribution of data was tested by Kolmogorov and Smirnov's method. Paired Student's-tests and nonparametric Mann-Whitney U tests were performed according to whether data were normally distributed. $P<0.05$ was considered to be significant.

Chapter 3: Characterization of the mRNA expression of various Wnt signalling proteins in term placental and decidual tissues cells *in vitro* by PCR array.

3.1 Introduction

Human reproduction is a complex and highly regulated process that requires successful completion of several discrete events. The implantation of the blastocyst into the maternal uterus is one of these crucial events. This delicate process involves a complex sequence of signalling events, mediated by a large number of molecular regulators (Minas *et al.* 2005; Dimitriadis *et al.* 2010; Zhang *et al.* 2013a). Accumulating evidence suggests that, among a range of identified signalling pathways, the Wnt signalling pathway plays a particularly important role in implantation, decidualization and placentation (Chen *et al.* 2009; Sonderegger and Pollheimer 2010; Nayeem *et al.* 2014). The invasive nature of implantation is often compared with cancer, so it is not surprising that Wnts are involved in regulating a variety of processes and pathways common to both.

Various studies have investigated the expression and significance of Wnt ligands and other signalling components in human endometrium. *Wnt2, 3, 4, 5a, 7b, 8b, FZD6, LRP6, DKK1* and *sFRP4* mRNAs have all been identified in endometrial samples and in endometrial epithelial/stromal cells (Bui *et al.* 1997; Tulac *et al.* 2003), *Wnt7A* was found to be expressed exclusively in luminal epithelial cells, whereas *sFRP4, DKK1* and FZD were all found in uterine glands and stroma (Tulac *et al.* 2003). Expression of 22 Wnt associated genes were modulated in post-menopausal compared with pre-menopausal endometrial epithelial cells, including *Wnt10A, FZD2, 9, LRP5, Axin2* and *GSK3* (Nguyen *et al.* 2012). During implantation the expression of DKK1 and sFRP4 expression up-regulated and down-regulated respectively (Kao *et al.* 2002). Few studies have also looked for the Wnt signalling in trophoblast and in placenta. 14 Wnt ligands (out of 19) and 8 FZD receptors (out of 10) were detected in the placenta (Sonderegger *et al.* 2007). Expression of TCF3/4 and β-catenin markedly increased in invading trophoblast (Pollheimer *et al.* 2006). There are various studies investigated murine tissues for the Wnt signalling, both in fetal (Miller and Sassoon 1998; Kobayashi *et al.*

2004; Mericskay *et al.* 2004; Mohamed *et al.* 2004; Carroll *et al.* 2005) and in maternal cells (Hayashi *et al.* 2009; Yip *et al.* 2013).

Wnt signalling has been implicated in reproductive pathologies in many occasions (Sonderegger and Pollheimer 2010; Nayeem *et al.* 2014). Abnormally elevated levels of Wnt/β-catenin (Pollheimer *et al.* 2006; Chen *et al.* 2013) and/or reduced level of Wnt antagonists (Peng *et al.* 2006; Carmon and Loose 2008; Novakovic *et al.* 2008; Wong *et al.* 2008; Yi *et al.* 2009) are associated with endometrial or trophoblastic malignancy. Continuous Wnt/β-catenin signalling in the endometrium may be an early step in endometrial carcinogenesis. Interestingly, Opposite expression pattern was reported in some studies as well (Bui *et al.* 1997; Abu-Jawdeh *et al.* 1999) highlighting the complexity of Wnt signalling and underscore the different roles played by the Wnt components under different circumstances. Elevated levels of Wnt components (Wu *et al.* 2006; Catalano *et al.* 2007; Gaetje *et al.* 2007; Santamaria *et al.* 2012) and decreased expression of DKK1 and sSFRP1 have also been demonstrated in endometrial lesions (Aghajanova *et al.* 2010). Collectively these studies suggest a tightly regulated pattern of Wnt components expression is essential for a successful pregnancy and maintaining a healthy reproductive system.

With 19 ligands, 10 receptors, several secreted modulators and many intracellular components; Wnt signalling is a very complex network. There are many unanswered questions about their role in early pregnancy, such as their cross-talk with other pathways, role of non-canonical pathway, individual roles of different factors, especially Wnt antagonists and the extent of their changing expression pattern. To proceed in this interesting area of research we need a complete gene expression picture of all known Wnt components. To our knowledge, no study so far looked at specifically at all the Wnt related genes in both decidualized endometrial cells and in trophoblast cells from term placenta. The objective of this study was to characterize the expression of all the known Wnt components in decidualized endometrial cells and in trophoblast cells. We hypothesize that the expression data when combined with their differential expression data (chapter 4) will be a powerful tool for functional analysis of individual Wnt components.

3.2 Results

3.2.1 Purity of trophoblast and decidua cells extracted form term placenta

Immunohistochemistry of extracted trophoblast and decidua cells with anti-cytokeratin 7 and anti-vimentin respectively revealed that both types of cells were ≥95%. Cytokeratin-7 (green) and vimentin (red) are trophoblast and decidua cell markers respectively. **(Figure 3.1)**.

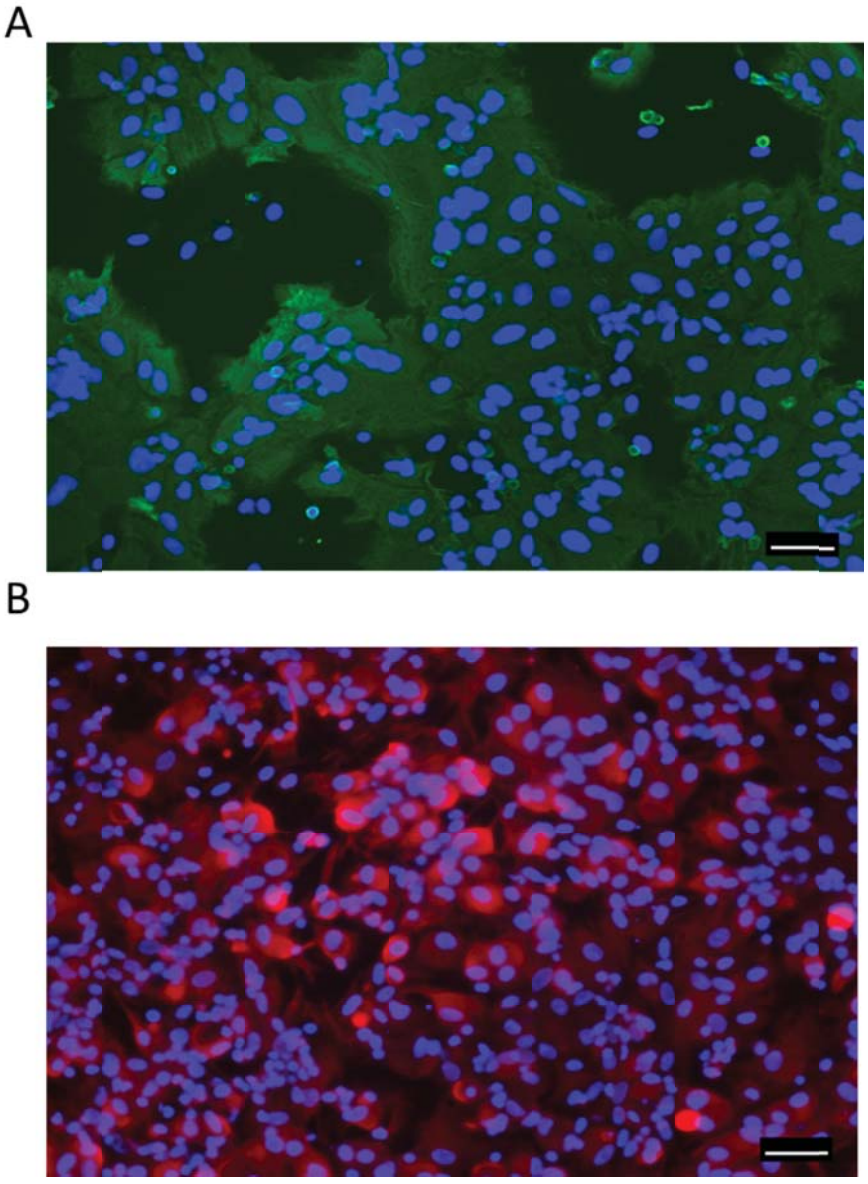

Figure 3.1: Immunoflourescence analysis of (A) Trophoblast and (B) decidual cells. Fixed trophoblast cells incubated with anti-cytokeratin 7 antibody followed by DyLightTM 488 secondary antibody (green) and fixed decidual cells incubated with anti-vimentin antibody followed by DyLightTM 594 (red). All cells were counterstained

with Hoechst 33258. Eclipse T*i* inverted fluorescent microscope (Nikon) was used in 5 fields at 200 × magnifications. Scale bar indicates 100 μm.

3.2.2 Expression of the Wnt signalling components in trophoblast

The PCR array analysis revealed that most of the Wnt signalling pathway genes on the array are expressed in trophoblast cells (**Figure 3.2A, Appendix Table I**). Among the 84 genes tested on the array, only five [Fibroblast growth factor 4 (FGF4), follicle stimulating hormone, beta polypeptide (FSHB) and T, brachyury homolog, mouse (T), Wnt3A and Wnt8A] were not detected. With the exception of two genes, all Wnt ligands were detectable, 13 in total. All eight of the included FZD receptors were detectable (**Figure 3.2A, Appendix Table I**). Among the most abundantly expressed Wnt-related genes were several ligands (Wnt4, Wnt5A, Wnt5B, Wnt6), canonical signal components (β-catenin, GSK3β, PP2R1A, DVL, CtBP2), negative regulators (DKK1) and target genes (cyclin D1, FOSL1, JUN) (**Table 3.1**). Wnt6 and Wnt4 were next most expressed Wnt genes (**Figure 3.2A, Appendix Table I**). Decidua cells express Wnt5A and Wnt5B most abundantly. Wnt2 and Wnt6 are next most expressed Wnt ligands.

3.2.3 Expression of the Wnt signalling components in decidua

Decidual cells show a very similar expression pattern of Wnt signalling components with 80 out of 84 genes detectable. All represented Wnt ligands were detected, except Wnt3A and Wnt8A, while all FZD receptors were detectable (**Figure 3.2B**). Among the other genes, only FGF4, and FSHβ were undetectable (**Appendix Table II**). The same genes that were highly expressed in trophoblast cells were also highly expressed in decidua (**Table 3.1**). Decidua cells express Wnt5A and Wnt5B most abundantly. Wnt2 and Wnt6 are next most expressed Wnt ligands. FZD8 and FZD4 were the most abundantly expressed FZD receptors in both trophoblast and decidua. DKK1 was the most abundantly expressed Wnt antagonist, followed by sFRP4 and sFRP1; WIF1 expression was low in both cell types.

3.2.4 Differentially expressed genes

DAAM1, LEF1, sFRP4 and Wnt2B were the only genes that were expressed significantly different between trophoblast and decidua and all were four more highly expressed in decidual cells *vs*. trophoblast (**Table 3.2, Appendix Table III**).

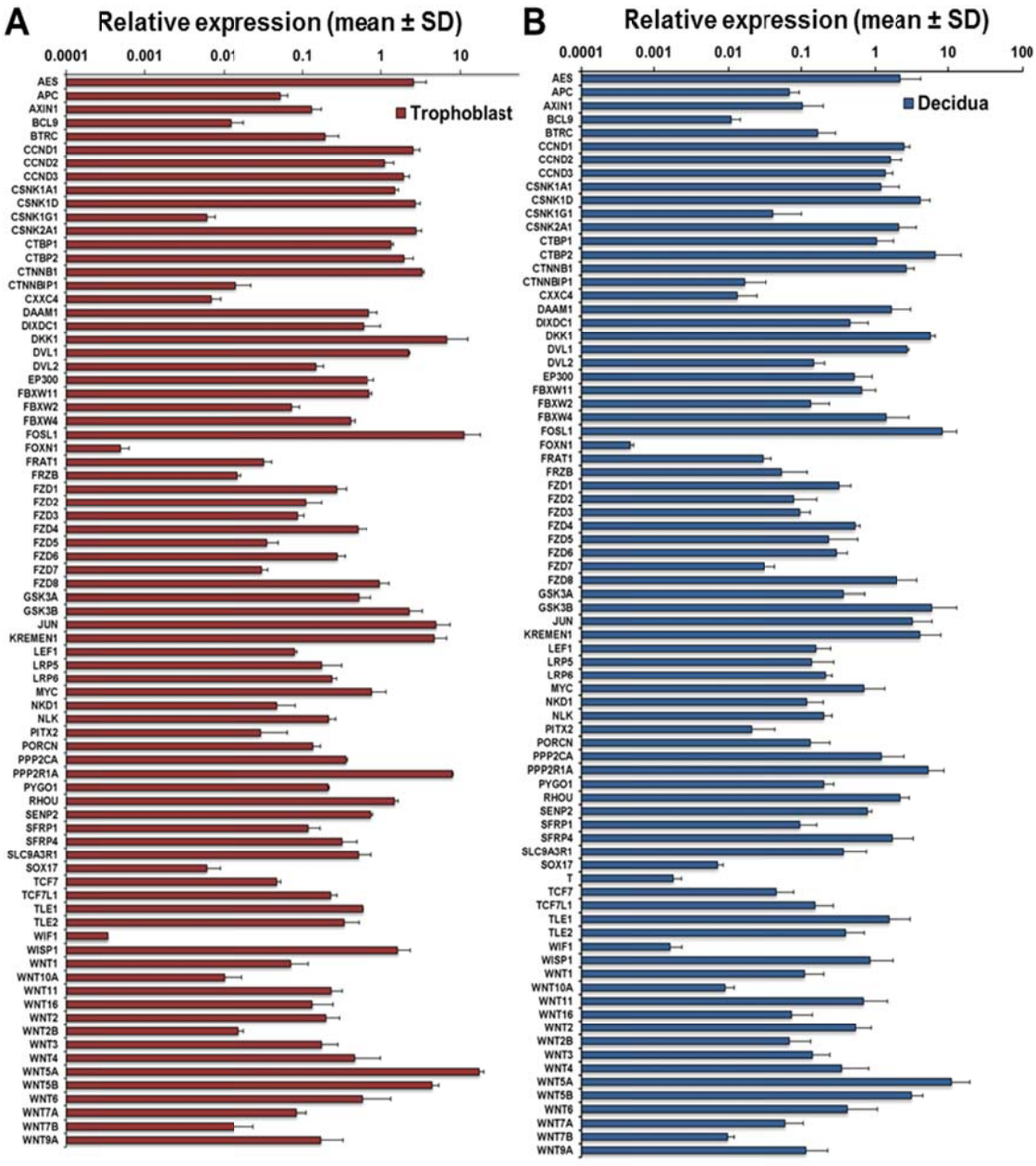

Figure 3.2: Relative mRNA expression levels of Wnt components in (A) trophoblast cells and (B) decidual cells as determined by Wnt specific PCR array. Cells were isolated from term placenta (n=3) and cultured for 3 d. Expression of target genes in trophoblast cells were normalised to the house-keeping genes ribosomal protein L13a (RPL13A) and β-actin. For decidua cells house-keeping genes β-2-microglobulin (B2M) and RPL13A were used for normalization. Gene expression is expressed on a log scale.

Table 3.1: Twenty most highly expressed genes in trophoblast and decidua cells.

Symbol	Gene name	Trophoblast 2^-ΔCt	Decidua 2^-ΔCt
WNT5A	Wingless-type MMTV integration site family, member 5A	0.290	0.224
FOSL1	FOS-like antigen 1	0.188	0.144
PPP2R1A	Protein phosphatase 2, regulatory subunit A, alpha	0.132	0.104
DKK1	Dickkopf homolog 1 (Xenopus laevis)	0.110	0.086
JUN	Jun proto-oncogene	0.084	0.068
KREMEN1	Kringle containing transmembrane protein 1	0.077	0.080
WNT5B	Wingless-type MMTV integration site family, member 5B	0.074	0.059
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa	0.056	0.048
CSNK2A1	Casein kinase 2, alpha 1 polypeptide	0.046	0.043
CSNK1D	Casein kinase 1, delta	0.045	0.058
AES	Amino-terminal enhancer of split	0.043	0.043
CCND1	Cyclin D1	0.043	0.043
GSK3B	Glycogen synthase kinase 3 beta	0.039	0.057
DVL1	Dishevelled, dsh homolog 1 (Drosophila)	0.038	0.044
CTBP2	C-terminal binding protein 2	0.033	0.059
CCND3	Cyclin D3	0.032	0.023
WISP1	WNT1 inducible signaling pathway protein 1	0.028	0.017
CSNK1A1	Casein kinase 1, alpha 1	0.025	0.024
RHOU	Ras homolog gene family, member U	0.025	0.032
CTBP1	C-terminal binding protein 1	0.023	0.020

Table 3.2: Genes that were expressed significantly different between trophoblast and decidua.

Symbol	Gene name	Trophoblast 2^-ΔCt	Decidua 2^-ΔCt	P value
DAAM1	Dishevelled associated activator of morphogenesis 1	0.012	0.0173	0.022
LEF1	Lymphoid enhancer-binding factor 1	0.001	0.002	0.036
SFRP4	Secreted frizzled-related protein 4	0.005	0.017	0.043
WNT2B	Wingless-type MMTV integration site family, member 2B	0.0003	0.0007	0.004

3.3 Discussion

Here, we report that trophoblast and decidual cells *in vitro* express a similar array of Wnt-related genes at similar relative levels. Wnt signalling is indeed a highly expressed pathway with almost all components detectable in both types of tissues. These findings indicate that the Wnt signalling pathway likely plays a particularly important role in certain aspects of implantation, decidualization and placentation.

Wnt signalling in pregnancy is of interest because of the wide ranging biological roles of Wnts and their involvement in many diseases, especially those involving cell invasion such as cancer. Many expression studies in different model organisms and in human have established Wnt signalling as one of the most expressed pathways in maternal uterine tissues (Chen *et al.* 2009; Sonderegger and Pollheimer 2010; Nayeem *et al.* 2014). Targeted analysis of Wnt ligands and receptors in trophoblast tissues have also showed their complex expression pattern (Sonderegger *et al.* 2007). However, previous studies either did not specifically target Wnts, or did not include all Wnt signalling components. Our data from the human Wnt-specific PCR array revealed that all major Wnt signalling components - including transcription factors, secreted factors and target genes - are expressed in both maternal (decidual) and placental (fetal) tissues *in vitro*. Hence, it is likely that both canonical and non-canonical Wnt signalling pathways are active in both cell types.

Due to difficulties in obtaining first trimester pregnancy tissues, expression studies are rarely carried out on trophoblast cells from early stages of pregnancy. Trophoblast cells isolated from term placenta can provide a good source of preliminary data, which was the approach utilised in this study. Here, we report the characterization of an extensive list of Wnt related components. A very interesting observation from this study is that there is an almost identical relative expression pattern of Wnt components between trophoblast and decidua cells. The same genes are among the most and least abundant genes expressed in both cell types. These similar expression patterns indicate identical tight control of the pathway, which is not surprising as these cells are extracted from a differentiated and mature tissue (term placenta). Although there are some expression studies of different Wnt-related genes in first trimester tissues (**Table 1.1**), an extensive analysis of all Wnt-related genes in early pregnancy is missing. It would be very useful

to complement our findings with data on the production of these factors at the placental-decidual interphase during first trimester.

Of the Wnt ligands on the arrays, only Wnt3A and Wnt8A expression was undetectable (both tissues), consistent with a variety of studies in different models suggesting that Wnt5A, 5B and 7A are the most abundant Wnts in the pregnant uterus (Nayeem *et al.* 2014). Importantly, Wnt5A, the most highly expressed Wnt ligand in both trophoblast and decidua cells, is considered primarily a non-canonical ligand (Kikuchi *et al.* 2012). This raises the possibility that in the placenta non-canonical Wnt signalling may be very important.

In addition to Wnt5A/B, the secreted Wnt antagonist DKK1 was one of the most highly expressed genes in both cell types, while sFRP1 and sFRP4 were also relatively abundantly expressed. DKK1 is considered to be capable of selectively inhibit Wnt canonical pathway by binding with co-receptor LRP5/6 (Kawano and Kypta 2003; Niehrs 2006). Transmembrane proteins KREMEN1 and KREMEN2 are high-affinity DKK1 receptors that functionally cooperate with DKK1 to block Wnt/β-catenin signalling (Mao *et al.* 2002). KREMEN1 is another highly expressed gene in both cell types underscoring their activity in placenta. In mice, it has been suggested that both Dkk1 and Kremen1 have important role in blastocyst activation and uterine receptivity during the window of implantation (Li *et al.* 2008). This combination indicates a shift to the non-canonical pathway. However, the abundant presence of beta-catenin and other Wnt ligands and FZD receptors indicate the other pathways are also likely active. In depth analysis of Wnt signalling intermediates will be necessary to dissect the divergence of the canonical and non-canonical signalling pathways in the placenta during placentation.

The expression data presented here is a starting point for later experiments to dissect autocrine and paracrine Wnt signalling between these two cell types. Our co-culture model (Chapter 4) was established to answer the questions that arose from this experiment and confirm the PCR array data at the mRNA and protein level. The major focus will be to explore the potential shift from canonical to non-canonical Wnt signalling, determine the major Wnt signalling effectors in trophoblast-decidua interactions, and ascertain whether there are other pathways and effectors associated with Wnt signalling.

Chapter 4: The effects of trophoblast and decidua cross-talk on mRNA and protein expression of Wnt signalling proteins.

4.1 Introduction

The implantation of the blastocyst into the maternal uterus is one of the crucial events of early pregnancy. Effective reciprocal interaction between the implantation-competent blastocyst and the receptive uterus is required for a successful implantation. Uterus and blastocyst both must undergo synchronous development and differentiation respectively for implantation (Cha *et al.* 2012). A series of complex interactions at the interface between the implanting blastocyst and the endometrial cells lead to the formation of decidua, a differentiated maternal tissue to supports embryo growth and early pregnancy (Ramathal *et al.* 2010). The intricate interplay between the blastocyst and endometrium during implantation shares similarities with the cell-cell communications displayed during organogenesis. However, the paracrine Wnt signalling between trophoblast and decidua remain largely incomplete, especially the expression pattern of secreted ligands and antagonists that mediate cell-to-cell communication between the two cell types.

Implantation is a two-way interaction between embryo and maternal endometrium leading to the establishment of a functional placenta and pregnancy (Menkhorst *et al.* 2012; Cuman *et al.* 2013; Fritz *et al.* 2014). With a few exceptions individual functions of different Wnts, FZDs and other secreted factors have not been elucidated. Their interactions with other signalling pathways are also largely unknown. For the mechanistic and functional analysis of these factors in early pregnancy events co-culture experiments can be a powerful tool. In a previously published co-culture study, the effect of trophoblast on endometrial stromal cells was investigated (Popovici *et al.* 2006). This study showed that *DKK1* and *Wnt-inducible protein (WISP)* were up-regulated (amongst other genes) when the two cell types were in proximity (Popovici *et al.* 2006). In another study, the effect of trophoblast conditioned media on global gene expression in decidualized endometrial stromal cells was investigated, reporting down-regulation of *WNT4* and *FZD* expression, amongst other genes (Hess *et al.* 2007). However, both of the studies mentioned above investigated the effect of trophoblast on decidua/endometrial cells and to the best of my knowledge there have been no studies looking specifically at placental Wnt signalling components in a co-culture setting.

Successful implantation is dependent on timely synchronization between uterine receptivity and the developmental stage of blastocyst. Canonical Wnt signalling has been shown to be induced at the prospective site of implantation before embryo attachment in mice (Mohamed *et al.* 2005). Intrauterine delivery of Wnt7A and sFRP2 induce and inhibit Wnt/β-catenin signalling, respectively (Mohamed *et al.* 2005). Different Wnt ligands and Fzd receptors are expressed before and after implantation in mouse uterus (Hayashi *et al.* 2009). The expression of Wnts and Fzds undergo dynamic changes regulated by steroid hormones in ovariectomised mice (Hou *et al.* 2004; Hayashi *et al.* 2009). Expression of some of the Wnt components show spatially differential distribution in pre-implantation porcine endometrium (Kiewisz *et al.* 2011). In mice, conditional deletion of β-catenin in the mesenchymal compartment of the uterus causes complete failure of decidualisation and intertility (Zhang *et al.* 2012). It has also been shown that over-expression and constitutive expression of β-catenin also cause a range of defects in decidualisation, hyperplasia and subfertility (Jeong *et al.* 2008). Non-canonical Wnt receptor Ror2 and DKK1 are increased in endometrial stromal cells suggesting a possible shift from canonical to non-canonical Wnt signalling after successful implantation (Hatta *et al.* 2010).

In the following study, several Wnt-associated proteins were also included for investigation. In mice, loss of Msx1 and Msx2 raises Wnt5a levels in both uterine epithelial and stromal cells and inhibits blastocyst implantation through reduced uterine receptivity (Daikoku *et al.* 2011). In a separate experiment, conditional deletion of Msx1 and Msx2 resulted in up-regulation of several Wnt ligands, including Wnt4, 5a, 7a and 7b, in the epithelium and stromal cells of the pre-implantation uterus (Nallasamy *et al.* 2012). In these cells, the increased β-catenin levels stimulated the expression of a subset of fibroblast growth factors (FGFs). Collectively, these studies suggest that Msx1/2, Wnts and FGFs are all involved in a signalling network in the uterus in preparation for the implanting blastocyst (Nallasamy *et al.* 2012). Homeobox A 10 $(Hoxa10)^{-/-}$ mice exhibit defects in decidualisation, downregulation of sFRP4 and aberrant Msx-1 and Wnt4 signalling (Daikoku *et al.* 2004). COX activity and Wnt signalling appears to be connected by BMP2 activity, both pivotal in uterine function and decidualization (Lee *et al.* 2007). COX2-derived prostacyclin acts in implantation by activating uterine peroxisome proliferator-activated receptor-δ (PPARδ) and retinoid X receptor (RXR) (Lim *et al.* 1999).

In summary, appropriate and timely regulation of Wnt/β-catenin signalling is a requirement for normal decidualisation (Nayeem *et al.* 2014). The aim of this study was to utilize a co-culture model to identify important Wnt regulatory components and their physiological function. The focus was on secreted Wnt signalling antagonists, as they are likely to be important in modulating paracrine signalling between maternal-fetal compartments.

4.2 Results

4.2.1 Effect of co-culture on expression of the Wnt signalling components in trophoblast and decidual cells

Relative expression levels of Wnt signalling components of trophoblast cells grown in co-culture settings were compared with control trophoblast cells (i.e. no decidua). Expression of CtBP2 and FZD5 in trophoblast cells was significantly ($p<0.05$) up-regulated when co-cultured with decidual cells (**Figure 4.1A, Table 4.1 and Appendix Table I**), with increases of 2- and 10-fold, respectively. However, most differentially expressed genes were down-regulated in trophoblast in response to the presence of decidual cells. Among them there were APC, CK1δ, CXXC4, cyclin D1, cyclin D2, FBXW11, FZD2, FZD3, FZD8, JUN, KREMEN1, LRP6, PPP2R1A, sFRP4, SOX17 and TCF7. The most responsive genes were CXXC4, KREMEN1, sFRP4 and TCF7, showing >90% inhibition (**Figure 4.1A, Table 4.1 and Appendix Table I**).

Compared to trophoblast cells co-cultured with decidual cells, fewer decidual genes were differentially expressed in the presence of co-cultured trophoblasts. Among those up-regulated were DAAM1 and TLE1 with increases of 3- and 4-fold respectively, while CXXC4 was down-regulated (>85% inhibition; **Figure 4.1B and Table 3**). Genes that were only differentially regulated in trophoblast, not in decidua, in response to co-culture were APC, CK1δ, CtBP2, cyclin D1, cyclin D2, FBXW11, FZD2, FZD3, FZD5, FZD8, JUN, KREMEN1, LRP6, PPP2R1A, sFRP4, SOX17 and TCF7. Conversely, DAAM1 and TLE1 expression were altered only in decidual cells in response to co-culture.

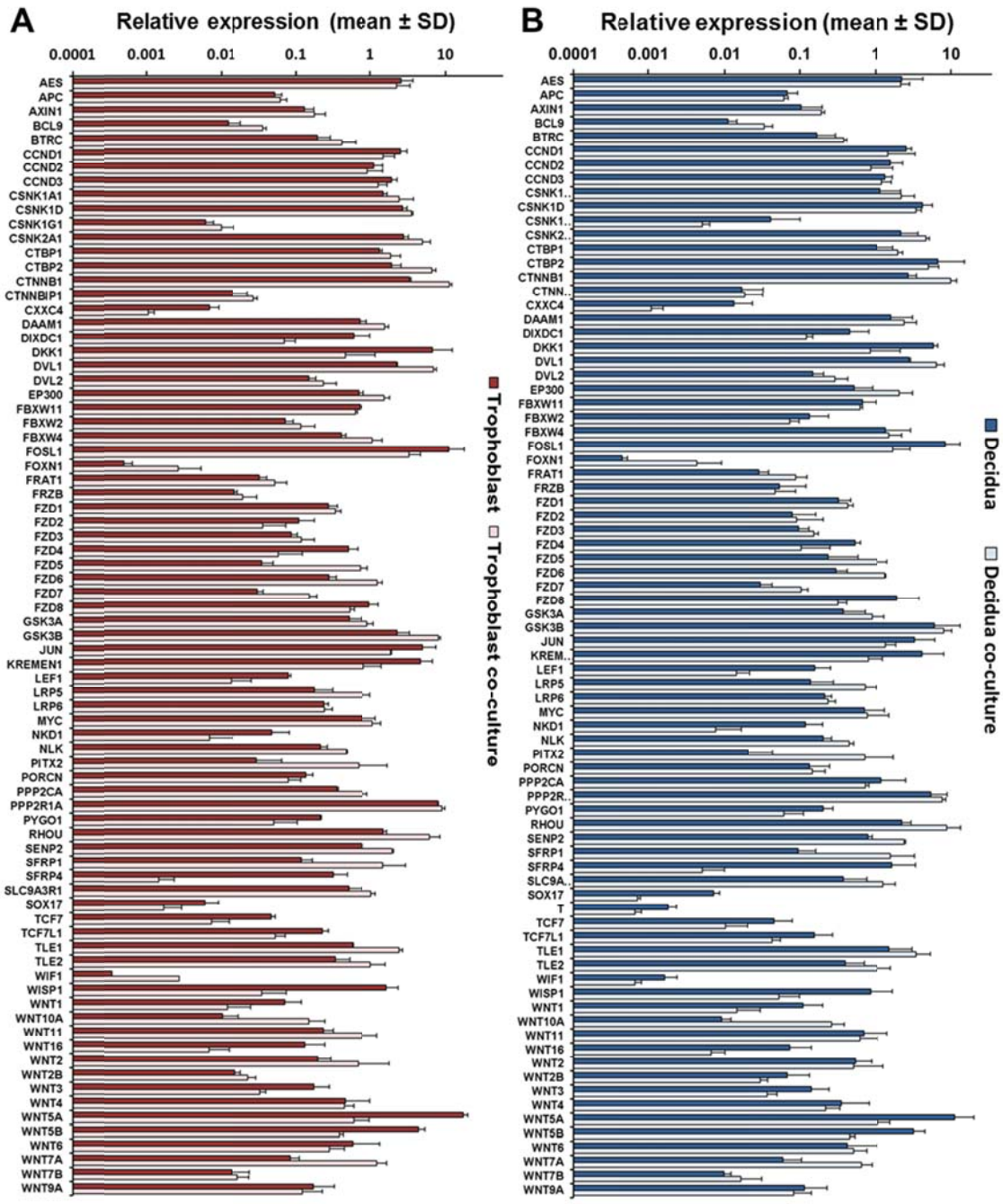

Figure 4.1: Relative mRNA expression levels of Wnt components in (A) trophoblast cells and (B) decidual cells in co-culture setting compared with control as determined by Wnt specific PCR array. Cells were isolated from term placenta (n=3) and cultured for 3 d. Expression of target genes in trophoblast cells were normalised to the house-keeping genes RPL13A and β-actin. For decidua cells house-keeping genes B2M and RPL13A were used for normalization. Gene expression is expressed on a log scale.

Table 4.1: Differentially expressed genes in co-cultures. Genes were selected based on their P value (<0.05) and and/or greater than 50% change in at least one cell type. Some genes were selected based on their large change in relative expression. Significant *P* values (≤0.05) are indicated in bold text.

Symbol	Gene name	Trophoblast		Decidua	
		Percent change	P value	Percent change	P value
SFRP4	Secreted frizzled-related protein 4	-99.8	**0.018**	-99.7	0.206
DKK1	Dickkopf homolog 1 (Xenopus laevis)	-99.4	0.209	-99.1	0.383
WNT5A	Wingless-type MMTV integration site family 5A	-99.2	0.245	-90.0	0.236
FZD4	Frizzled family receptor 4	-96.9	0.163	-94.5	0.316
TCF7	Transcription factor 7 (T-cell specific, HMG-box)	-94.4	**0.033**	-92.1	0.504
CXXC4	CXXC finger protein 4	-93.1	**0.030**	-85.6	**0.019**
KREMEN1	Kringle containing transmembrane protein 1	-93.1	**0.016**	-76.3	0.290
SOX17	SRY (sex determining region Y)-box 17	-88.6	**0.045**	-88.7	0.085
FZD2	Frizzled family receptor 2	-88.4	**0.044**	-15.3	0.916
JUN	Jun proto-oncogene	-82.1	**0.040**	-54.5	0.706
FZD8	Frizzled family receptor 8	-75.9	**0.018**	-75.7	0.104
CCND1	Cyclin D1	-74.8	**0.008**	-66.8	0.345
CCND2	Cyclin D2	-70.7	**0.045**	-38.7	0.332
DAAM1	Dishevelled associated activator of morphogenesis 1	-63.0	0.510	180	0.042
FBXW11	F-box and WD repeat domain containing 11	-61.4	**0.007**	55.0	0.817
LRP6	Low density lipoprotein receptor-related protein 6	-55.2	**0.0005**	24.0	0.872
PPP2R1A	Protein phosphatase 2, regulatory subunit A, alpha	-50.2	**0.020**	316	0.509
APC	Adenomatous polyposis coli	-47.9	**0.012**	-13.0	0.922
FZD3	Frizzled family receptor 3	-43.8	**0.009**	163	0.105
CSNK1D	Casein kinase 1, delta	-42.2	**0.015**	-29.6	0.784
WNT2B	Wingless-type MMTV integration site family 2B	-35.0	**0.005**	-44.8	0.275
WIF1	WNT inhibitory factor 1	56.0	0.423	-58.7	0.207
CTBP2	C-terminal binding protein 2	63.0	**0.033**	115	0.286
TLE1	Transducin-like enhancer of split 1	103	0.110	332	**0.004**
SFRP1	Secreted frizzled-related protein 1	402	0.130	3760	0.210
FZD5	Frizzled family receptor 5	877	0.052	2970	0.268

4.2.2 Validation of PCR array findings by qRT-PCR

To validate the data from the PCR arrays, relative expression levels of selected genes (primarily secreted ligands/antagonists and their receptors) were determined by qRT-PCR using a different set of primer pairs (**Table 2.6**). Total RNA was extracted after 3 d of culture (n=5-8 replicate experiments) and the expression of Wnt5A, FZD4, FZD5, DKK1, sFRP1, sFRP4 and WIF1 was validated by qRT-PCR, normalized to the housekeeping gene SDHA. In addition, the expression of some additional Wnt-related genes of potential importance in placentation was investigated: MSX1, MSX2, PTGS1, PTGS2, PTGES and HOXA10.

In trophoblasts with co-culture, in partial agreement with the array data, DKK1 and Wnt5A expression was significantly down-regulated ($p<0.005$), although the size of the response (~40-60%) was much more modest than suggested by the arrays. WIF1 expression, which was non-significantly increased in trophoblast co-cultures in the arrays, was also significantly up-regulated (84%) in the repeat analysis ($p<0.05$). On the other hand, in marked contrast to the PCR array findings, expression of FZD4 and sFRP4 (two genes shown to be markedly down-regulated on the arrays) was significantly up-regulated in trophoblast co-culture (136% and 55%, respectively; $p<0.005$) while FZD5 expression (increased on the arrays) was not significantly altered. Of the additional genes studied, expression of both PTGS1 and MSX1 was up-regulated in trophoblast co-culture by 94% and 220%, respectively (**Figure 4.2A**). In decidual cells, co-culture with trophoblast cells failed to induce any significant changes in mRNA expression in the replication studies when analysed by qRT-PCR (**Figure 4.2B**), including FZD5 and sFRP1 which were up-regulated ~30-fold in the PCR array data. No significant changes were observed in MSX2, PTGS2, PTGES or HOXA10 gene expression in co-culture setting in either cell type.

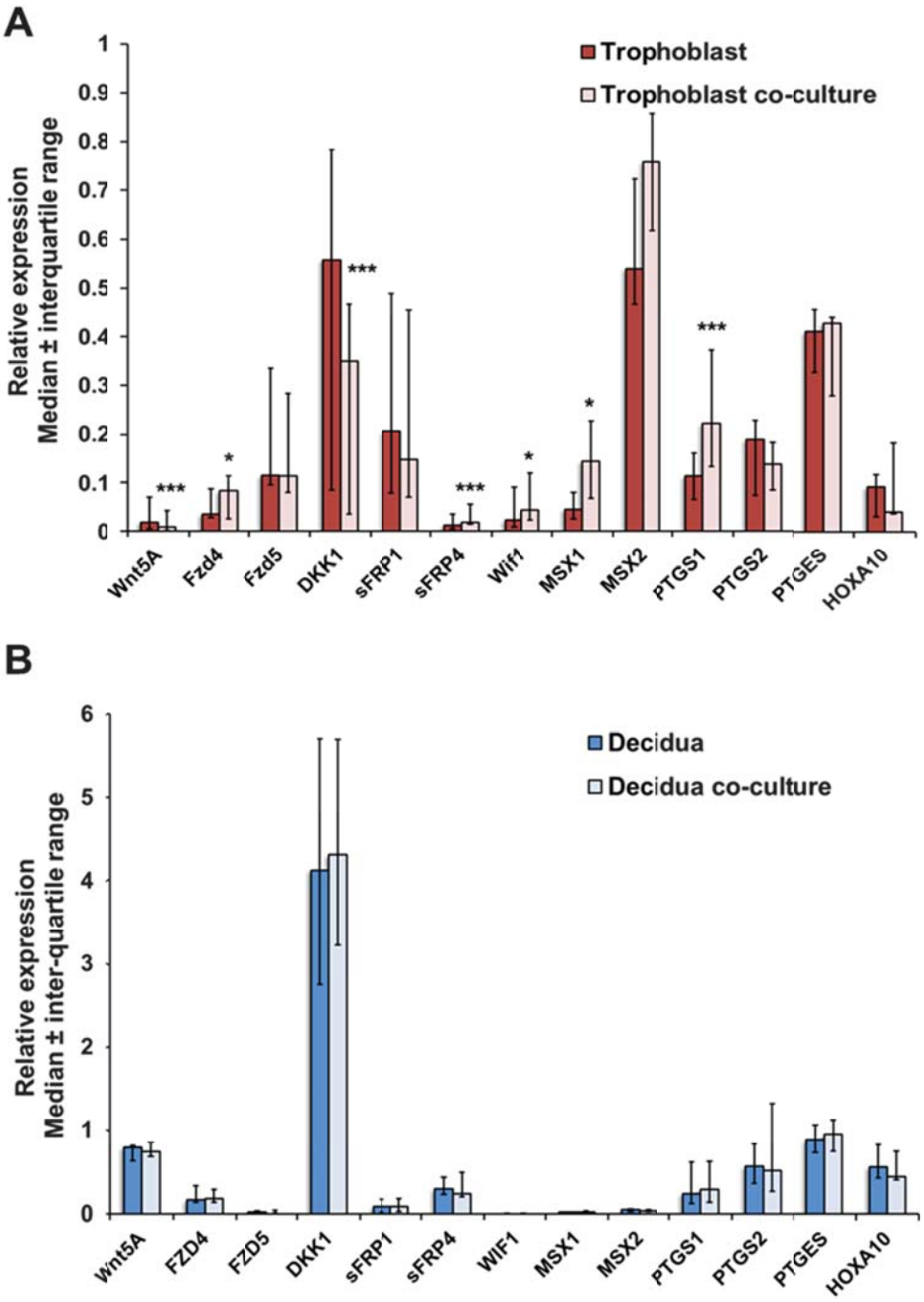

Figure 4.2: Relative mRNA expression levels of Wnt related genes in (A) trophoblast cells and in (B) decidual cells in co-culture setting compared with control as determined by qRT-PCR. Cells were isolated from term placenta (n=5-8) and cultured for 3 d prior to mRNA extraction. Expression of target genes in trophoblast cells were normalised to the house-keeping gene SDHA. Data are expressed as median and interquartile range. **P*<0.05, ****P*<0.005 by Mann-Whitney test. The PTGS2 and PTGES expression data in (A) were divided by 10 to allow expression on the same scale.

4.2.3 Changes in Wnt-related protein expression in response to co-culture

To determine whether the observed changes in gene expression were reflected at the protein level, the cellular abundance of selected Wnt-related proteins was analysed by immunoblotting after extraction of proteins from both trophoblast and decidual cells cultured for 3 d with or without co-culture (**Figure 4.3**). We also measured both total and active (unphosphorylated) β-catenin to assess whether co-culture and the associated changes in Wnt antagonists induced alterations in canonical Wnt signalling. β-catenin was readily detected in trophoblast extracts, but surprisingly was barely detectable in decidua (**Figure 4.3A**) despite its relatively abundant expression at the mRNA level. Active β-catenin was readily detected in trophoblast extracts but undetectable in decidua. The ratio of active:total β-catenin was significantly decreased in trophoblast with co-culture, consistent with the putative decrease in canonical signalling resulting from an increase in exposure to Wnt inhibitors. DKK1 was detected in both cell types, albeit across a range of sizes (~34-43 kDa), probably due to varying degrees of post translational modification. DKK1 levels were significantly increased ~6-fold in trophoblast cells with co-culture ($p<0.005$; **Figure 4.3B**) while sFRP4 abundance was also increased in decidua (but not in trophoblast, in contrast to the qPCR data) with co-culture ($p<0.05$; **Figure 4.3C)**; this ~50% increase in decidual sFRP4 protein levels was despite the lack of change in mRNA expression as shown in **Figure 4.2**. Interestingly, sFRP4 appeared as a single band in trophoblast cells, but as a doublet in decidual cells. The additional band is likely to be glycosylated sFRP4 (~55 kDa). COX1, appearing as a single 70 kDa band, was detected in both cell types and was also found to be significantly more abundant (~2.2-fold) in trophoblast with co-culture (**Figure 4.3B**). MSX1, on the other hand, was not detected in trophoblast cells. No significant changes in COX1, DKK1 and MSX1 were observed in decidual protein extracts with trophoblast co-culture. Overall, the immunoblotting data suggested that canonical Wnt signalling in trophoblast cells is inhibited in the presence of decidua, possibly via the increased effects of secreted Wnt antagonists sFRP4 and DKK1.

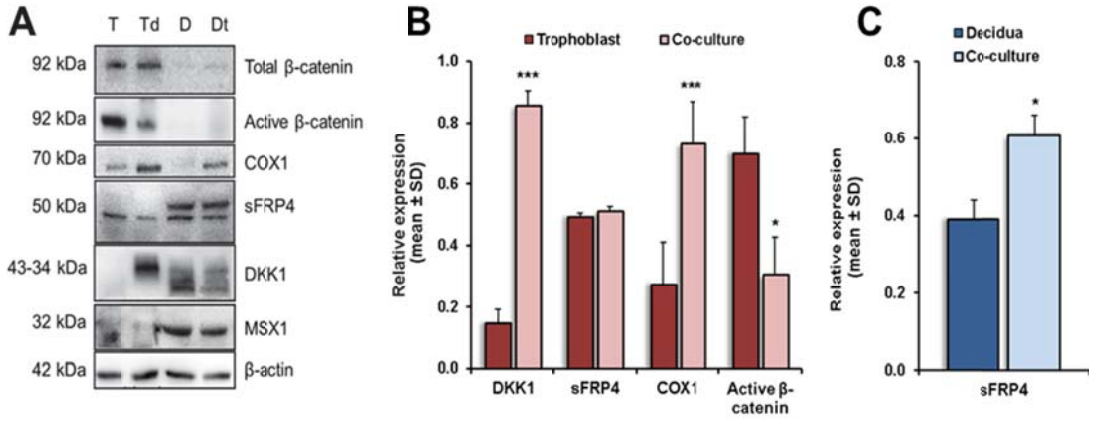

Figure 4.3: Secreted Wnt antagonist DKK1 is up-regulated in trophoblast cells in co-culture at protein level and in parallel the canonical Wnt signalling (total and active β-catenin ratio) was decreased. sFRP4 and COX1 increased in decidua and trophoblast cells in co-culture respectively. (A) Representative immunoblot of total β-catenin, active β-catenin, COX1, sFRP4, DKK1, MSX1 and β-actin (for normalization). (B) Quantitation of DKK1, sFRP4, active β-catenin and COX1 in trophoblast cells. Protein expression was quantified using densitometry and normalized to β-actin (n=7). Data are shown as mean ± SD from n=4-7 experiments. (C) Expression of sFRP4 in decidual cells with/without co-culture. Paired t-test was used to calculate statistical significance. T trophoblast, Td co-culture trophoblast, D decidua and Dt co-culture decidua. **P*<0.05, ***P*<0.01, ****P*<0.005.

4.2.4 Effects of co-culture on secretion of Wnt antagonist and prostaglandin synthesis in trophoblast cells

The mixing of media between inner and outer compartments of the Transwells can potentially confound attempt to measure changes in levels of trophoblast-secreted proteins in the media. Therefore, we attempted to confirm the immunoblotting findings by measuring the levels of sFRP4, DKK1 and WIF1 in cell extracts by ELISA, which is a more precise and sensitive technique. Protein expression levels of DKK1 and sFRP4 were increased ~4-fold in trophoblast cells in co-culture compared with control (p<0.05) **(Figure 4.4A)**. More modest rise ~2-fold increase in WIF1 levels were measured in decidual cells co-culture **(Figure 4.4B)**. However, cellular sFRP4 concentrations in decidua were not significantly altered in response to co-culture, in contrast to the immunoblotting data shown above. Co-culture also resulted in significantly increased levels (2-3-fold) of PGE_2 in media from trophoblast and decidual cells in co-culture

(**Figures 4.4A and B**); this is consistent with the data shown in Figure 4.3A/B of elevated PTGS1 protein levels in trophoblasts co-cultured with decidual cells.

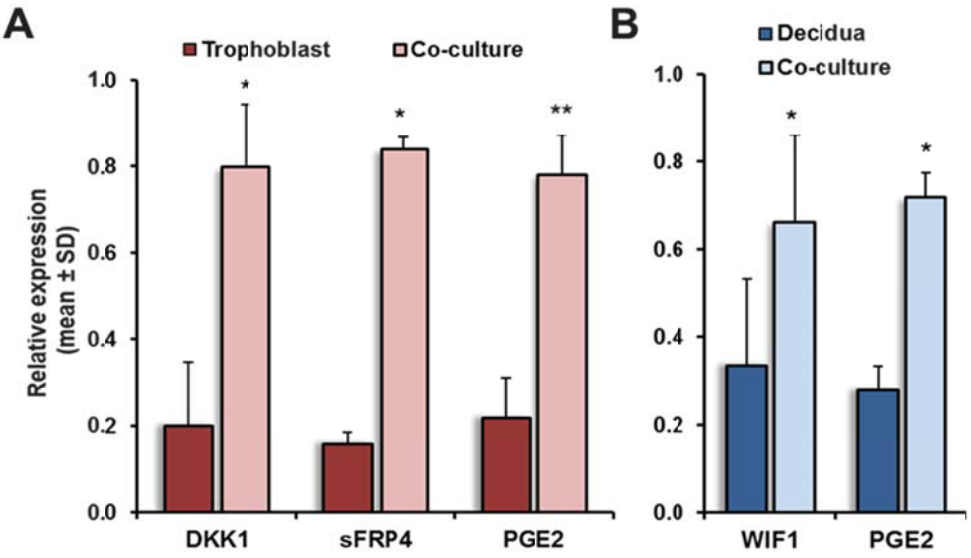

Figure 4.4: Effects of co-culture setting on production of Wnt antagonists and secretion of prostaglandins. Concentrations of DKK1, sFRP4, WIF1 and PGE_2 were measured by immunoassay in trophoblast-conditioned medium (A) or in decidua-conditioned medium (B) by immunoassay. Concentrations were normalized to the total protein loaded for each placenta sample (n=3 to 4). **P*<0.05, ***P*<0.01, ****P*<0.005 by Student's t-test.

4.3 Discussion

The main findings of this study are that cell-to-cell communication between decidual and trophoblast cells alters expression of Wnt-related genes (primarily in the placenta) and enhances the expression and production of Wnt antagonists. As a result of co-culture, increased concentrations of Wnt antagonists leads to down-regulation of canonical Wnt signalling in trophoblast. We have also shown that co-culture of decidual and trophoblast cells leads to up-regulation of COX1 expression and increased prostaglandin production.

Synchronized communication between decidualised endometrium and trophoblasts from blastocyst is essential for successful implantation. To elucidate the paracrine Wnt signalling between trophoblast and decidua we have employed two powerful techniques, co-culture and Wnt PCR array. The co-culture model employed in this study was established with the goal of exploring the effects of secreted factors from decidual and trophoblast cells on cellular function, with a particular interest in the Wnt pathway

and its secreted antagonists. In order to assess the effects of co-culture on Wnt expression we grew the cells in co-culture for three days, with daily media changes to ensure that the depletion of substrates and accumulation of metabolites was minimised. Trophoblast differentiation (syncytialisation) occurred during this period as expected. No specific assessment of syncytialisation was performed as the model is very robust and well-established in our laboratory. We then employed the Wnt PCR array to identify genes that were differentially expressed in co-culture. Wnt antagonists DKK1, sFRP4 and WIF1 were among the differentially expressed genes in co-culture validating our hypothesis of their possible importance in modulating paracrine signalling between fetal-maternal compartments. Decrease in active/total β-catenin in trophoblast co-culture is consistent with antagonists up-regulation and together it indicates down-regulation of canonical Wnt signalling in trophoblast cells in presence of decidua.

Interestingly, Wnt5A and the antagonists DKK1, sFRP4 and KREMEN1 were among the top genes identified as being responsive to co-culture (>93% reduction), although only sFRP4 and KREMEN1 were significantly down-regulated in this experiment. sFRP4 is an effective Wnt antagonist that directly binds with Wnt7A to inhibit activation of Wnt/β-catenin signalling (Carmon and Loose 2008). DKK1 is capable of selectively inhibiting the Wnt canonical pathway by binding with co-receptor LRP5/6 (Kawano and Kypta 2003; Niehrs 2006), while KREMEN1 is a high-affinity DKK1 receptor that functionally cooperates with DKK1 to block Wnt/β-catenin signalling (Mao *et al.* 2002). It has been suggested that in mice both Dkk1 and Kremen1 play an important role in blastocyst activation and uterine receptivity during the window of implantation (Li *et al.* 2008).

A number of other genes involved in Wnt signalling were significantly down-regulated by co-culture, including TCF7, JUN, LRP6, DAAM1, APC, PPP2R1A and casein kinase-1δ. These changes are all consistent with a decrease in Wnt signalling activity, despite the apparent reduction in Wnt antagonist expression. The majority of changes in expression were seen in trophoblast, perhaps reflecting the fact that the cells underwent differentiation during the culture period and hence were more profoundly affected by exposure to decidual factors. Alternatively, the decidua may produce more of the key factors that regulate Wnt gene expression than trophoblast. The expression levels of different Wnt components in decidua cells in presence of trophoblast cells remain high but largely unchanged.

To confirm these mRNA expression findings we repeated the co-culture experiments using a larger number of placental cultures and analysed the expression of selected genes by qRT-PCR and immunoblotting. Unexpectedly, we found that although the decrease in Wnt5A expression seen in the arrays was confirmed by qRT-PCR, the expression of several other genes in trophoblast was actually found to be significantly increased by co-culture, most notably sFRP4 and WIF1; DKK1 expression was only modestly reduced (-37% compared to -99% in the arrays). The reasons for the discrepancies are not clear, but could reflect variability between cultures derived from different placentas. Regardless, the mRNA expression, immunoblotting and immunoassay data all point to an elevation of sFRP4 and WIF1 production in one or both cell types with co-culture; DKK1 protein levels were also significantly increased, adding further weight to the conclusion that the array data were misleading.

The increased expression of Wnt antagonists observed in this study would be expected to result in reduced Wnt signalling in these cells. This would be consistent with some of the other data from the arrays showing down-regulation of several Wnt signalling genes. Importantly, evidence of reduced canonical Wnt signalling was also obtained from the analysis of levels of active β-catenin, which in trophoblasts decreased by around 50% with co-culture. Collectively, these findings suggest that the close proximity of trophoblast with decidua leads to enhanced production and action of Wnt antagonists and reduced Wnt signalling in trophoblast.

An additional, presumably down-stream, consequence of co-culture was the observed increase in PGE_2 production in both the decidual and trophoblast compartment. We measured mRNA expression of COX1/PTGS1 and COX2/PTGS2 and identified a significant increase in COX1 expression in trophoblast with co-culture, whereas COX2 expression was unaltered. The COX1 expression changes were also seen at the protein level by immunoblotting, adding weight to the possibility that this increase was responsible for the observed increase in PGE_2 levels. The addition of exogenous DKK1 and sFRP4 to trophoblast cultures increased both the production of PGE_2 and the expression of COX1 protein after 3 days of culture, consistent with the interpretation that the effects of co-culture on trophoblast prostaglandin production were, at least in part, mediated through the actions of these Wnt antagonists.

Trophoblasts are the outer layer of the blastocyst and after implantation are responsible for rapid formation of the placenta, achieved through differentiation and invasion (Red-Horse *et al.* 2004; Cartwright *et al.* 2010; Menkhorst *et al.* 2014). Trophoblast invasion is very similar to cancer cell invasion and naturally tightly controlled to ensure healthy placenta formation. Wnt signalling is active in both early and later stage of trophoblast differentiation (Pollheimer *et al.* 2006; Marchand *et al.* 2011). A substantial amount of nuclear β-catenin was observed in invasive trophoblast and in addition, Wnt3A was shown to stimulate both invasion and migration, which could be blocked by DKK1 (Pollheimer *et al.* 2006). Immunohistochemical data showed transcription factors TCF-3 and TCF-4 are strongly stimulated in invasive trophoblast and recently it has also been shown that Wnt/ TCF-4 dependent signalling could promote motility and expression of promigratory genes (Pollheimer *et al.* 2006; Meinhardt *et al.* 2014), suggesting abnormal Wnt/β-catenin signalling pathway may contribute to trophoblastic hyperplasia and local invasion. Our findings suggest that in the presence of decidua, DKK1 and sFRP4 production is stimulated and active β-catenin is down-regulated; this gives us further evidence that tight control of Wnt/β-catenin in trophoblast is important for normal placentation.

Prostaglandins (PGs) are lipid autacoids, widely produced in mammalian cells from arachidonic acid (Kennedy *et al.* 2007; Salleh 2014). Arachidonic acid is converted to PGH_2 by the action of cyclooxygenase (COX) isoenzymes (Ricciotti and FitzGerald 2011). There are two COX isoenzymes in mammals, COX1 and COX2. COX1 and COX2 are encoded by genes *PTGS1* and *PTGS2* respectively. *PTGS1* is constitutively expressed, whereas *PTGS2* is inducible. Prostaglandins have been shown to play a role in ovulation, endometrial vascularization and decidualisation (Chakraborty *et al.* 1996; Kennedy *et al.* 2007; Salleh 2014). In humans, COX1 is expressed in the luminal and glandular epithelia and COX2 is expressed in the luminal epithelia and perivascular cells during implantation window period in human (Marions and Danielsson 1999). Female mice with deletion of *COX2* have multiple reproductive deficits and are infertile. *COX1* deleted mice have normal fertility but show parturition defects (Lim *et al.* 1997).

The role of prostaglandins in trophoblast invasion is largely unknown. PGE_2 and other EP_2 agonists have been demonstrated to increase expression of cell adhesion proteins focal adhesion kinase and integrins and cell-to-ECM adhesion of HTR-8/SVneo

trophoblast cells (Waclawik *et al.* 2013). COX2 and PGE_2 synthase have also been detected in HTR-8/SVneo cells (Dominguez‐Lopez *et al.* 2012). Decidual factors including prostaglandins increase trophoblast cell invasiveness by modulating different tissue inhibitors of metalloproteinases (TIMPs) and other adhesion proteins (Godbole *et al.* 2011).

Numerous studies indicate associations between aberrant expression of Wnt antagonists and pathological conditions, including implantation failure, infertility and various trophoblast diseases (Sonderegger and Pollheimer 2010; Nayeem *et al.* 2014). Repeated implantation failure (RIF) patients showed significantly lowered expression of LEF1, sFRP1 and up-regulation of DKK1 in endometria compared with fertile women (Koler *et al.* 2009). Elevated levels of DKK1 have also been reported in decidual tissue and in the serum of patients with unexplained recurrent spontaneous miscarriage (Bao *et al.* 2013). Other experiments have shown that Wnt signalling is a target for the most differentially expressed miRNAs in women with recurrent miscarriages (Revel *et al.* 2011; Dong *et al.* 2014).

Compared with normal placenta, WNT2, β-catenin, DKK1 and sFRP4 were shown to be abnormally expressed in preeclamptic third trimester placenta (Zhang *et al.* 2013b; Zhang *et al.* 2013c). DKK1 levels are reduced in endometrial carcinoma and treatment with recombinant DKK1 also downregulated the invasiveness of endometrial carcinoma cells *in vitro* (Yi *et al.* 2009). APC and sFRP2 are hypermethylated in choriocarcinomas (Novakovic *et al.* 2008; Wong *et al.* 2008). JEG-3 and JAR choriocarcinoma cells are deficient in of DKK1 expression, while overexpression of DKK1 can induce apoptosis and growth arrest through JNK induction independent of Wnt signalling (Peng *et al.* 2006). Overexpression of sFRP4 also decreases cancer cell proliferation in Ishikawa cell with sFRP4 directly binding with Wnt7A to inhibit activation of Wnt/β-catenin signalling (Carmon and Loose 2008). In endometriotic fibroblasts, DKK1 and sFRP1 mRNA is reduced compared to normal fibroblasts (Aghajanova *et al.* 2010).

Wnt antagonists, in combination with other factors, likely keep Wnt signalling pathways under tight control to regulate trophoblast adhesion, proliferation, invasion and differentiation. Our results demonstrate that trophoblast production of Wnt antagonists sFRP4 and DKK1 is increased in the presence of decidua, resulting in down-regulation of Wnt/β-catenin and increased PGE_2 production. Direct links between these factors

may explain the observations. In the next chapter (Chapter 5) we investigated some possible connections.

Chapter 5: Functional analysis of the Wnt antagonists in tissue cross talk.

5.1 Introduction

Among a range of identified signalling pathways, the Wnt signalling pathway plays a particularly important role in implantation and placentation (Chen *et al.* 2009; Sonderegger and Pollheimer 2010; Nayeem *et al.* 2014). Various studies have investigated the expression and significance of Wnt ligands and other signalling components in human endometrium (Bui *et al.* 1997; Kao *et al.* 2002; Tulac *et al.* 2003; Nguyen *et al.* 2012). Few studies have also looked for the Wnt signalling in trophoblast and in placenta (Pollheimer *et al.* 2006; Sonderegger *et al.* 2007); however, not all Wnt components were not included in these expression analysis of trophoblast. We have previously conducted a comprehensive Wnt component expression analysis in both trophoblast and decidualized endometrial cells (Chapter 3). The Wnt signalling pathway was found to be highly expressed in both type of cells. We also characterized the altered expression of the Wnt-related genes due to cell-to-cell communication between decidual and trophoblast cells in a co-culture model (chapter 4). Nevertheless, with a few exceptions individual functions of different Wnt components in endometrial and placental differentiation have not been elucidated. Specific Wnt-FZD interactions, their downstream targets and their interactions with other signalling pathways in these tissues are open for investigation.

Prostaglandins such as PGE_2 have potentially important roles in implantation and subsequent placentation. We previously showed that in the presence of decidual cells trophoblast expression of COX1 and production PGE_2 was increased. However, the detailed mechanism of this response and likely interactions with other signalling networks have not been investigated.

Implantation is a two-way interaction between embryo and maternal endometrium involving spatiotemporally regulated endocrine, paracrine, autocrine and juxtacrine modulators, leading to the establishment of a functional placenta and pregnancy. Secreted Wnt factors, expecially the Wnt antagonists e.g. DKK1 and sFRP4, are implicated in this process by tightly regulating Wnt signalling. Aberrations of their

expression patterns have been observed in many pathological conditions (Nayeem *et al.* 2014). Using trophoblast-decidua co-culture and Wnt PCR array we identified DKK1 and sFRP4 as two most likely important players from the Wnt signalling in trophoblast cells (chapter 4). Production of both Wnt antagonists (DKK1 and sFRP4) and PGE_2 is increased in trophoblast cells in presence of decidua. It is possible that COX1 is directly up-regulated by Wnt signalling, as has been demonstrated in cancer cells with respect to COX2 (Araki *et al.* 2003; Nuñez *et al.* 2011). Pharmacological gain of function experiments show that Wnt/β-catenin synergizes with prostaglandin (PG) signalling to blastocyst competency to implantation (Xie *et al.* 2008). Nevertheless, the connection between the Wnt antagonists and PG production/secretion remained elusive. In this chapter, we have investigated possible relationship between them.

5.2 Results

5.2.1 Modulation of trophoblast prostaglandin production by Wnt antagonists

Based on our findings above, suggesting a functional link between co-culture and a) increased Wnt antagonist expression, b) reduced canonical Wnt signalling and c) increased trophoblast prostaglandin production, cells were treated individually with DKK1 (30 ng/ml) and sFRP4 (300 pg/ml) for 3 d to examine their effects on Wnt signalling proteins and PGE_2 production. Proteins from trophoblast cells were collected on day 1 and 3 of culture and analysed by immunoblotting (**Figure 5.1A**). This analysis showed that the addition of either of the two Wnt antagonist failed to significantly affect levels of activated β-catenin in trophoblast cultures after 1-3 d (**Figure 5.1 B**), although levels of COX-1 protein were significantly increased ($p<0.05$) following treatment with sFRP4 (**Figure 5.1C**).

Changes in prostaglandin secretion after DKK1 and sFRP4 treatment were measured by EIA in both decidual and trophoblast conditioned media (**Figure 5.1D**). Consistent with expectations, PGE_2 concentrations in media from trophoblast cultures were increased in response to both DKK1 and sFRP4 treatment after 3 d ($p<0.05$). However, PGE_2 production by decidua cells was not significantly affected by antagonist treatment (**Figure 5.1E)**, although there was a trend towards increased production with DKK1 exposure.

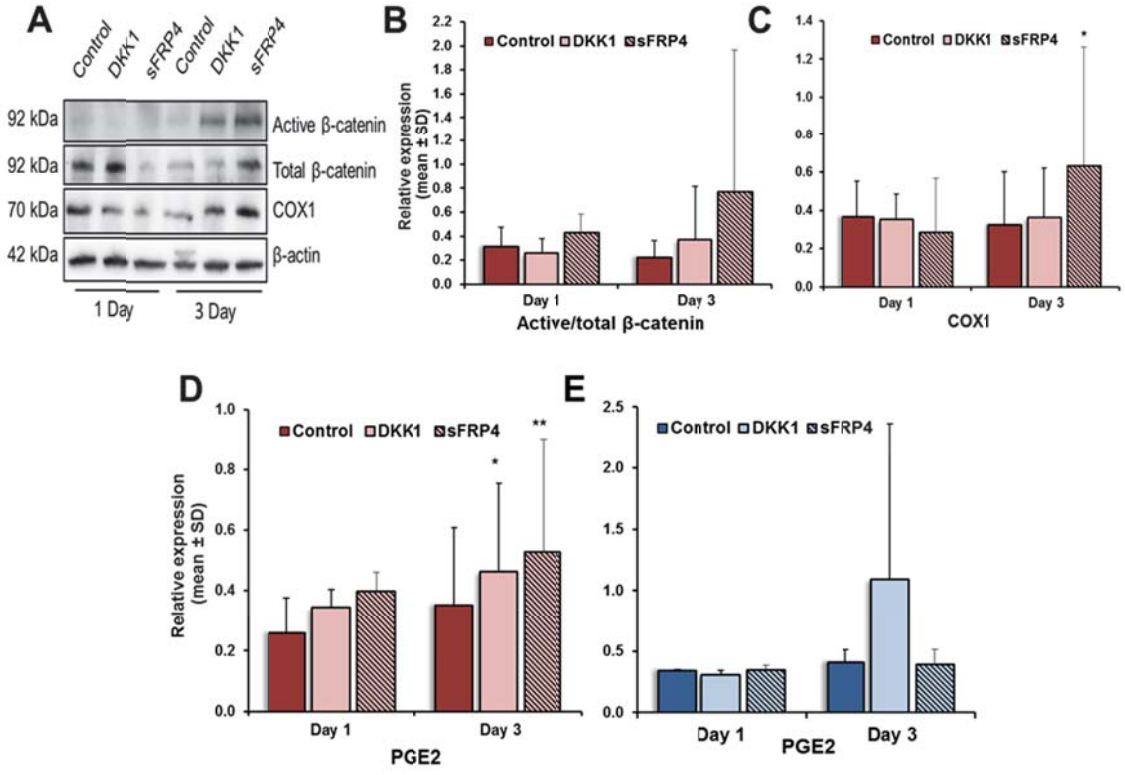

Figure 5.1: Trophoblast prostaglandin production is increased by Wnt antagonist sFRP4 and DKK1 treatment. A. Representative immunoblot of total β-catenin, active β-catenin and COX1. B. Relative expression of COX1 in trophoblast cells. C. Relative expression of active/total β-catenin in trophoblast cells. D and E. Relative secreted level of PGE_2 in trophoblast and decidua cells respectively. Protein expressions are quantified using densitometry and normalized using β-actin (n=3 to 4). Protein expressions analysis by PGE_2 was normalized to the total protein loaded for each placenta sample (n=3 to 4). Welch's t-test was used to calculate statistical significance. **P*<0.05, ***P*<0.01.

5.3 Discussion

We have previously demonstrated that members of the the Wnt signalling pathway are highly expressed in both trophoblast and decidualized endometrial cells from term placenta. Cell-to-cell communication between decidual and trophoblast cells alters expression of Wnt-related genes (primarily in the placenta) and enhances the expression and production of Wnt antagonists As a result of paracrine interactions generated in co-culture, increased concentrations of Wnt antagonists leads to down-regulation of canonical Wnt signalling in trophoblast. We have also shown that co-culture of decidual and trophoblast cells leads to up-regulation of COX1 expression and increased

prostaglandin production. Here, we have shown that trophoblast cells, when treated with DKK1 and sFRP4, have increased expression of COX1 and elevated production of PGE_2. The modulation of prostaglandins signalling by Wnt antagonists in trophoblasts is a novel finding, particularly as it appears to involve the regulation of COX1 expression and not COX2. However, whether or not these effects are mediated through Wnt/β-catenin remain inconclusive. The level of β-catenin remained unchanged after exposure to DKK1 and sFRP4. This might be because their inhibitory affect is time dependent and the time points selected were not optimal for assessing β-catenin activation. We have shown that most of the Wnt ligands are expressed in trophoblast and decidua. DKK1 and sFRP4 may block some ligands binding with their receptors but others may contribute in keeping β-catenin stabilized.

Both prostaglandins and Wnt signaling are active in the many pathological conditions, including cancers. Nevertheless, the mechanistic details regarding the links between these two key pathways has remained sketchy (Buchanan and DuBois 2006). Activation of COX/PGE_2 is a key step in gastric tumorigenesis, while enhanced Wnt/β-catenin signalling is also found in 30-50% gastric cancers (Oshima *et al.* 2009). It has also been shown that simultaneous activation of Wnt signalling and PGE_2 biosynthesis is responsible for the development of gastric adenocarcinoma (Oshima *et al.* 2006). In colon cancer cells, PGE_2 can stimulate proliferation by activating the β-catenin axis following ligation of the G protein–linked PGE_2 receptor, EP_2 (Castellone *et al.* 2005). The activation of this receptor in turn activates β-catenin signalling by inhibiting the phosphorylating destruction complex, independent of Wnt binding to the LRP/FZD receptor complex (Castellone *et al.* 2005).

Much of the information regarding the relationship between Wnt signalling and prostaglandin production and actions relates to COX2 rather than COX1. Familial adenomatous polyposis (FAP) is one of the best characterized inherited colon cancers. In both FAP patients and in mouse models of FAP, it has been suggested that the regulation of COX2 involves participation of APC, because induction of wild-type APC in genetically engineered HT-29 cells down-regulates COX2 protein expression; furthermore, COX2 protein levels are associated with the complexation of full-length APC with β-catenin (Eisinger *et al.* 2007). However, these studies do not provide direct evidence of β-catenin-mediated regulation of COX proteins.

A functional TCF4 binding element (TBE) in the COX2 promoter was identified and showed that COX2 gene at the transcriptional level was up-regulated by β-catenin and down-regulated by APC (Araki *et al.* 2003). In a recent study, the presence of a novel functional TCF/LEF-response element that responds directly to enhanced Wnt/β-catenin signalling in the promoter of COX2 was reported in gastric cancer cells (Nuñez *et al.* 2011). β-catenin accumulation contributes to the expression of COX2 in articular chondrocytes (Kim *et al.* 2002). Casein kinase-2 (CK2) has been shown to mediate up-regulation of COX2 and enhanced production of PGE_2 through activation of β-catenin dependent transcription in human colon and breast cancer cell lines and in embryonic kidney cells (Yefi *et al.* 2011). Treatment of human cholangiocarcinoma cells with ω3-polyunsaturated fatty acids blocks cell growth by down-regulating both Wnt/β-catenin and cyclooxygenase pathways, providing evidence that COX2–derived PGE_2 activates β-catenin in human cholangiocarcinoma cells (Lim *et al.* 2008). Activation of PI3-kinase and Wnt signaling is associated with COX2/PGE_2 production which in turn results in inhibition of apoptosis in colon cancer (Kaur and Sanyal 2010).

Our findings of up-regulation of PGE_2 production in decidual-trophoblast co-culture are consistent with the literature showing a role for prostaglandins in decidualisation and trophoblast invasion. However, the apparent decrease in Wnt signalling that we observed alongside the increase in PGE_2 production is unexpected and contrary to the findings of other studies. For example, Wnt3A has been shown to stimulate both trophoblast invasion and migration, which could be blocked by DKK1 (Pollheimer *et al.* 2006), while Wnt signalling is active in both early and later stages of trophoblast differentiation (Pollheimer *et al.* 2006; Marchand *et al.* 2011). Transcription factors TCF3 and 4 are activated in invasive trophoblast and TCF4 dependent signalling promotes motility and expression of promigratory genes (Pollheimer *et al.* 2006; Meinhardt *et al.* 2014). On the other hand, in mice it has been shown that Dkk1 secreted by decidual cells induces trophoblast cell invasion, consistent with a link between inhibition of Wnt signalling and invasive stimulation (Peng *et al.* 2008).

There are several imitations in this study that need to be acknowledged. Firstly, the cells used to prepare the cultures were all derived from term tissues and therefore may not necessarily reflect the expression profiles and responses of placental cells earlier in gestation. Secondly, the cells were cultured *in vitro* and the process of disruption, isolation and attachment to artificial substrates may alter gene expression and cellular

physiology. Thirdly, the co-culture model allows only slow mixing of media components between compartments and probably partially restricts the ready access of decidual secretions to trophoblast apical surfaces, thereby limiting the apparent effects of decidual factors on trophoblast function. For this reason we measured effects on gene expression after 3 days of culture. Finally, the presence of two sets of cells in one well may lead to increased depletion of nutrients and increased production of metabolites, which could perturb cellular function and gene expression over and above any effects of specific secreted ligands and inhibitors. We changed media regularly to obviate this concern.

Most studies on the links between Wnt signalling and prostaglandin production have focussed on COX2/PTGS2 in the area of cancer biology. All these studies show a positive relationship between Wnt signalling and prostaglandin production (Araki *et al.* 2003; Castellone *et al.* 2005; Nuñez *et al.* 2011), and to our knowledge there are no previous reports of an inverse association between β-catenin/Wnt signalling and PTGS1 expression/PGE_2 production. Interestingly, in prostate cancer cells high levels of COX2 expression and PGE_2 production enhances the release of Wnt antagonists (Liu *et al.* 2010a); this raises the possibility of a reciprocal relationship between Wnt antagonists and PGE_2 in decidual-trophoblast co-cultures, with elevated Wnt antagonist levels leading to increased PGE_2 production which in turn helps to stimulate Wnt antagonist production.

It has been suggested that abnormal/dysregulated Wnt/β-catenin signalling may contribute to trophoblastic hyperplasia and excessive invasion. Wnt antagonists and other Wnt components have been associated with implantation failure, infertility and various trophoblast diseases (Sonderegger and Pollheimer 2010; Nayeem *et al.* 2014). Our findings suggest that the enhanced secretion of Wnt inhibitors from decidual and trophoblast tissue when in close proximity may be a homeostatic mechanism to prevent excessive Wnt signalling and its negative physiological consequences.

Chapter 6: General discussion.

The principal findings of this study are that: 1) trophoblast and decidua express a similar array of Wnt-related genes at similar relative levels; 2) cell-to-cell communication between decidual and trophoblast cells alters expression of Wnt-related genes (primarily in the placenta) and enhances the expression and production of Wnt antagonists; 3) as a result of co-culture, increased concentrations of Wnt antagonists leads to down-regulation of canonical Wnt signalling in trophoblast; and 4) co-culture of decidual and trophoblast cells and/or exposure to Wnt antagonists (DKK1 and sFRP4) leads to up-regulation of PTGS1/COX1 expression and increased prostaglandin production. Collectively these data add strong support for a role of secreted Wnt antagonists (specifically, sFRP4, DKK1 and WIF1) as mediators of cell-cell communication at the maternal-placental interface, regulating placental prostaglandin production, immunomodulation and invasion/implantation.

However, some important considerations regarding the validity of the present findings relating to the strengths and weaknesses of the Transwell model should be considered. The co-culture model employed in this study was established with the goal of exploring the effects of secreted factors from decidual and trophoblast cells on cellular function, with a particular interest in the Wnt pathway and its secreted antagonists. The nature of the Transwell system allows gradual mixing of soluble components in the media between the upper and lower compartments. Presumably the apical surface of the trophoblast cells (grown on the membrane support of the inner chamber) receives signals predominantly from the inner compartment, while the basal aspect of the cells is more accessible to decidual factors present in the lower compartment. Hence, although the model has the advantage that it allows cells to respond to secreted factors released by other cells types present in the co-culture, it also suffers from the limitation that the rate of exposure is limited by diffusion issues and that measurements of production of substances by individual cultures is complicated by time-dependent dilution effects.

Previous studies both in humans and model organisms have established that components of the Wnt signalling pathway are expressed in both maternal intrauterine tissues and placental trophoblast cells (Chen *et al.* 2009; Sonderegger and Pollheimer 2010; Nayeem *et al.* 2014). Our data from the human Wnt-specific PCR array revealed that all

major Wnt signalling components are expressed in both maternal (decidual) and placental (fetal) tissues *in vitro*. Hence, both canonical and non-canonical Wnt signalling pathways are likely to be active in both cell types. Of the Wnt ligands, only Wnt3A and Wnt8A expression was undetectable (both tissues), consistent with a variety of studies in different models suggesting that Wnt5A, 5B and 7A are the most abundant Wnts in the pregnant uterus (Nayeem *et al.* 2014). Wnt5A, considered as primarily a non-canonical ligand, is the most highly expressed Wnt ligand in both trophoblast and decidua cells (Kikuchi *et al.* 2012). In addition to Wnt5A/B, DKK1 was one of the most highly expressed genes (in both cell types), while sFRP1 and sFRP4 were also relatively abundantly expressed. Collectively these observations suggest that the secreted Wnt antagonists play a key role in the paracrine communication between decidua and trophoblast and help to maintain appropriate levels of Wnt signalling in the two cell types.

In pilot studies we determined that although concentrations of secreted proteins in the inner and outer compartments were different, mixing of components between the two compartments was also evident, particularly at the longer incubation times (24 h). Inter-compartment mixing was much less significant prior to 4 h incubation. We grew the cells in co-culture for three days, with daily media changes to ensure that the depletion of substrates and accumulation of metabolites was minimised. However, it is possible that some of the responses seen in co-culture were the result of substrate utilisation or metabolism, rather than direct paracrine communication. Trophoblast differentiation (syncytialisation) occurred during this period as expected, and was thus modifiable by exposure to decidual factors.

We first employed the Wnt PCR array to identify genes that were differentially expressed in co-culture. The changes we observed are all consistent with a decrease in Wnt signalling activity, despite the apparent reduction in Wnt antagonist expression. The majority of changes in expression were seen in trophoblast, maybe because decidua produces more of the key factors that regulate Wnt gene expression than trophoblast. Another possibility is that the trophoblast cells underwent differentiation during the culture period and hence were more strongly affected by exposure to decidual factors.

To confirm the PCR array findings we repeated the co-culture experiments using a larger number of placental cultures and analysed the expression of selected genes by

qRT-PCR and immunoblotting. Interestingly, not all of the array findings were replicated, and overall we found that the close proximity of trophoblast with decidua leads to enhanced production and action of Wnt antagonists and reduced Wnt signalling in trophoblast. Another consequence of co-culture was elevated COX1 expression and PGE_2 production in both the decidual and trophoblast compartment which was replicated by treatment with exogenous DKK1 and sFRP4. This is consistent with the interpretation that the effects of co-culture on trophoblast prostaglandin production were, at least in part, mediated through the actions of these Wnt antagonists. PGs produced in the trophoblast most likely act in a synergy with secreted factors from decidua, including decidua-derived PGs, in an autocrine or paracrine manner to modulate trophoblast invasion and promote formation of a healthy, fully functional placenta (Kennedy *et al.* 2007).

The observed stimulation of PGE_2 production in trophoblasts by sFRP4 and DKK1 treatment raises the issues of the therapeutic potential of Wnt antagonists. Complete elucidation of their functions and mechanisms of action may lead to the development of novel diagnostic biomarkers or a range of therapeutics for treatment of placental disorders. Therapeutic applications based on the administration Wnt antagonists have been explored in a number of different studies (Muley *et al.* 2010; Ramachandran *et al.* 2011; Cho *et al.* 2012; Longman *et al.* 2012; Saran *et al.* 2012; Warrier *et al.* 2014). sFRP4 has been demonstrated to inhibit angiogenesis by decreasing proliferation, migration and tube formation of endothelial cells (Muley *et al.* 2010). Both the cysteine-rich and netrin-like domains of sFRP4 have been reported to exert anti-angiogenic properties (Longman *et al.* 2012). sFRP4 has also been shown to be able to sensitize glioma cells and stem cells to chemotherapeutics and could be utilized to destroy cancer stem cells of a glioma cell line (Warrier *et al.* 2014). In a recent study, it was demonstrated that sFRP4 can act as a predictive marker of chemosensitivity in ovarian cancer and have therapeutic potential when combined with other drugs (Saran *et al.* 2012). WIF1, on the other hand, is down-regulated in human primary cervical tumors and cell lines. WIF1 re-expression induces significant apoptosis and inhibits cervical cancer cell proliferation and invasion both *in vitro* and *in vivo* (Ramachandran *et al.* 2011). DKK1 has been shown to inhibit the survival and migration of human papillary thyroid cancer cells by regulating Wnt/β-catenin signalling and E-cadherin expression

(Cho *et al.* 2012). Therefore, the use of Wnt antagonists might have similar applications in the treatment of pathological conditions involving uterine vascular reorganization.

In conclusion, our findings suggest that cell-to-cell interactions between decidua and trophoblast stimulate secretion of Wnt antagonists, leading to increased placental prostaglandin production mediated through increased COX1 expression. Because of the important role played by prostaglandins in key aspects of implantation and placentation, and the evidence that placental pathology arises from dysregulation of Wnt signalling within the uterine cavity, the local secretion of Wnt antagonists may be a central mechanism through which normal Wnt-prostaglandin signalling is maintained for successful placental establishment, growth and function. While there are reports in the literature of Wnts and/or β-catenin modulating PGE_2 production and secretion and vice versa, to the best of our knowledge the role of Wnt antagonists on cyclooxygenase activity/PG production have not been investigated previously in any cell types or conditions. In our study, the exogenous addition of two Wnt antagonists, DKK1 and sFRP4, to trophoblast cultures increased both the production of PGE_2 and the expression of COX1 protein after 3 days of culture. These data are consistent with the interpretation that the effects of co-culture on trophoblast prostaglandin production were, at least in part, mediated through the actions of these Wnt antagonists.

Further studies are warranted to fully define the changes in Wnt signalling that occur in trophoblast in response to decidual paracrine factors, and delineate the specific contribution of the secreted antagonists to these changes. The present findings raise the possibility that aberrant secretion of Wnt antagonists may be part of the pathophysiology of placental disorders such as preeclampsia, and as such may be of interest in the development of disease biomarkers or therapeutic targets.

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Appendix 1: Complete PCR array results of trophoblast cells.

The following data presents the complete set of results from PCR array analysis of trophoblast cells. The average Ct values from both control and co-culture trophoblast cells and expression fold changes of all 84 Wnt related genes are presented in the table.

Table I: Complete PCR array results of trophoblast cells. Genes that were undetected are coloured red.

UniGene	Symbol	Description	AVG Ct without normalization		Fold change	P value	Comment
			Co-culture	control			
Hs.515053	AES	Amino-terminal enhancer of split	22.04333	20.47667	-4.75	0.239029	OK
Hs.158932	APC	Adenomatous polyposis coli	27.08333	26.03	-1.92	0.012418	OK
Hs.592082	AXIN1	Axin 1	25.66333	24.75667	-1.22	0.206999	OK
Hs.415209	BCL9	B-cell CLL/lymphoma 9	27.82667	28.28667	1.13	0.928113	OK
Hs.643802	BTRC	Beta-transducin repeat containing	24.48667	24.26	0.27	0.456467	OK
Hs.17631	FZD5	Frizzled family receptor 5	23.58333	26.66	9.77	0.052126	OK
Hs.523852	CCND1	Cyclin D1	22.56	20.43667	-3.97	0.007809	OK
Hs.376071	CCND2	Cyclin D2	23.45	21.65	-3.41	0.04484	OK
Hs.534307	CCND3	Cyclin D3	22.75333	20.82667	-4.00	0.091802	OK

Hs.529862	CSNK1A1	Casein kinase 1, alpha 1	21.96667	21.18667	-0.95	0.460335	OK
Hs.631725	CSNK1D	Casein kinase 1, delta	21.22667	20.33333	-1.73	0.015026	OK
Hs.646508	CSNK1G1	Casein kinase 1, gamma 1	29.83	29.16667	-0.95	0.236696	OK
Hs.644056	CSNK2A1	Casein kinase 2, alpha 1 polypeptide	20.76667	20.31	-1.25	0.002712	OK
Hs.208597	CTBP1	C-terminal binding protein 1	22.22667	21.34667	-1.95	0.070873	OK
Hs.501345	CTBP2	C-terminal binding protein 2	20.32	20.86333	1.63	0.033165	OK
Hs.476018	CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa	19.55667	20.03333	1.60	0.170408	OK
Hs.463759	CTNNBIP1	Catenin, beta interacting protein 1	28.25333	28.01667	0.06	0.599312	OK
Hs.12248	CXXC4	CXXC finger protein 4	32.9	29.01	-14.47	0.02994	OK
Hs.654934	DAAM1	Dishevelled associated activator of morphogenesis 1	22.41667	22.31333	0.37	0.509692	OK
Hs.655626	DIXDC1	DIX domain containing 1	26.95333	22.76333	-18.28	0.088056	OK
Hs.40499	DKK1	Dickkopf homolog 1 (Xenopus laevis)	26.26333	19.32667	-167.64	0.208521	OK
Hs.74375	DVL1	Dishevelled, dsh homolog 1 (Drosophila)	20.25667	20.6	1.44	0.182023	OK

Hs.118640	DVL2	Dishevelled, dsh homolog 2 (Drosophila)	25.31667	24.55667	-1.78	0.062631	OK
Hs.517517	EP300	E1A binding protein p300	22.44333	22.35667	0.38	0.513446	OK
Hs.484138	FBXW11	F-box and WD repeat domain containing 11	23.76333	22.25667	-2.59	0.006705	OK
Hs.494985	FBXW2	F-box and WD repeat domain containing 2	26.26667	25.58	-1.48	0.007319	OK
Hs.1755	FGF4	Fibroblast growth factor 4					No expression
Hs.283565	FOSL1	FOS-like antigen 1	21.42	18.56667	-8.29	0.100663	OK
Hs.663679	FOXN1	Forkhead box N1	30.17	33.08	2.01	0.678681	OK
Hs.126057	FRAT1	Frequently rearranged in advanced T-cell lymphomas	27.39667	26.74	-0.55	0.465742	OK
Hs.128453	FRZB	Frizzled-related protein	28.90667	27.79333	-2.19	0.054021	OK
Hs.36975	FSHB	Follicle stimulating hormone, beta polypeptide					No expression
Hs.94234	FZD1	Frizzled family receptor 1	24.66667	23.71333	-1.24	0.19271	OK
Hs.142912	FZD2	Frizzled family receptor 2	28.31667	25.13667	-8.62	0.044459	OK
Hs.40735	FZD3	Frizzled family receptor 3	26.26333	25.31	-1.78	0.008805	OK

Hs.19545	FZD4	Frizzled family receptor 4	27.71	22.84333	-32.62	0.163207	OK
Hs.591863	FZD6	Frizzled family receptor 6	22.74	23.68333	2.24	0.127166	OK
Hs.173859	FZD7	Frizzled family receptor 7	25.81667	26.8	2.31	0.109991	OK
Hs.302634	FZD8	Frizzled family receptor 8	24.02667	21.86333	-4.15	0.018417	OK
Hs.466828	GSK3A	Glycogen synthase kinase 3 alpha	23.22	22.86333	-0.08	0.571938	OK
Hs.445733	GSK3B	Glycogen synthase kinase 3 beta	20.04	20.69333	1.77	0.078411	OK
Hs.714791	JUN	Jun proto-oncogene	22.15	19.59667	-5.59	0.040045	OK
Hs.229335	KREMEN1	Kringle containing transmembrane protein 1	23.61333	19.62667	-14.55	0.015781	OK
Hs.555947	LEF1	Lymphoid enhancer-binding factor 1	29.65667	25.42333	-18.47	0.054018	OK
Hs.6347	LRP5	Low density lipoprotein receptor-related protein 5	23.44333	24.59333	2.28	0.525844	OK
Hs.584775	LRP6	Low density lipoprotein receptor-related protein 6	25.17667	23.87333	-2.23	0.000457	OK

Hs.202453	MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	22.99667	22.29333	-0.99	0.278911	OK
Hs.592059	NKD1	Naked cuticle homolog 1 (Drosophila)	31.57667	26.36667	-55.99	0.345938	OK
Hs.208759	NLK	Nemo-like kinase	24.15667	24.03667	0.36	0.466181	OK
Hs.643588	PITX2	Paired-like homeodomain 2	25.17	27.65667	6.50	0.056616	OK
Hs.386453	PORCN	Porcupine homolog (Drosophila)	26.81333	24.69333	-4.85	0.094807	OK
Hs.483408	PPP2CA	Protein phosphatase 2, catalytic subunit, alpha isozyme	23.43	23.28667	-0.33	0.231966	OK
Hs.467192	PPP2R1A	Protein phosphatase 2, regulatory subunit A, alpha	19.87667	18.78333	-2.01	0.020231	OK
Hs.256587	PYGO1	Pygopus homolog 1 (Drosophila)	27.91	24.02667	-17.65	0.169606	OK
Hs.647774	RHOU	Ras homolog gene family, member U	20.52333	21.19333	1.34	0.817889	OK

Hs.401388	SENP2	SUMO1/sentrin/SMT3 specific peptidase 2	22.07333	22.19333	1.21	0.150013	OK
Hs.713546	SFRP1	Secreted frizzled-related protein 1	22.99	24.94667	5.02	0.129941	OK
Hs.658169	SFRP4	Secreted frizzled-related protein 4	31.89	23.57333	-489.50	0.018324	OK
Hs.500822	FBXW4	F-box and WD repeat domain containing 4	23.00667	23.10333	0.61	0.756307	OK
Hs.728760	SLC9A3R1	Solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1	23.03	22.88	0.31	0.53965	OK
Hs.98367	SOX17	SRY (sex determining region Y)-box 17	35.495	29.30667	-8.80	0.044628	OK
Hs.389457	T	T, brachyury homolog (mouse)					No expression
Hs.573153	TCF7	Transcription factor 7 (T-cell specific, HMG-box)	30.41	26.16667	-17.88	0.03259	OK
Hs.516297	TCF7L1	Transcription factor 7-like 1 (T-cell specific, HMG-box)	27.36333	23.94333	-10.27	0.05304	OK
Hs.197320	TLE1	Transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)	21.77333	22.58667	2.03	0.109583	OK

Hs.332173	TLE2	Transducin-like enhancer of split 2 (E(sp1) homolog, Drosophila)	23.25667	23.50667	0.70	0.906744	OK
Hs.284122	WIF1	WNT inhibitory factor 1	31.75	#DIV/0!	1.56	0.42265	OK
Hs.492974	WISP1	WNT1 inducible signaling pathway protein 1	28.46333	21.17667	-213.30	0.144381	OK
Hs.248164	WNT1	Wingless-type MMTV integration site family, member 1	29.095	25.91333	-54.31	0.359328	OK
Hs.121540	WNT10A	Wingless-type MMTV integration site family, member 10A	26.06667	28.82333	21.98	0.380344	OK
Hs.108219	WNT11	Wingless-type MMTV integration site family, member 11	23.79333	23.96667	0.69	0.845154	OK
Hs.272375	WNT16	Wingless-type MMTV integration site family, member 16	30.60667	25.18333	-90.40	0.345506	OK
Hs.567356	WNT2	Wingless-type MMTV integration site family member 2	26.26333	24.22	-6.52	0.240938	OK
Hs.258575	WNT2B	Wingless-type MMTV integration site family, member 2B	28.52667	27.77667	-1.54	0.004966	OK

Hs.445884	WNT3	Wingless-type MMTV integration site family, member 3	27.97667	24.45667	-10.68	0.025608	OK
Hs.336930	WNT3A	Wingless-type MMTV integration site family, member 3A					No expression
Hs.25766	WNT4	Wingless-type MMTV integration site family, member 4	24.29333	23.62333	-1.71	0.529387	OK
Hs.696364	WNT5A	Wingless-type MMTV integration site family, member 5A	24.15667	17.66667	-122.76	0.245001	OK
Hs.306051	WNT5B	Wingless-type MMTV integration site family, member 5B	24.47667	19.64667	-25.99	0.012408	OK
Hs.29764	WNT6	Wingless-type MMTV integration site family, member 6	25.09333	23.88	-10.49	0.453934	OK
Hs.72290	WNT7A	Wingless-type MMTV integration site family, member 7A	22.84333	25.39	8.34	0.168838	OK
Hs.512714	WNT7B	Wingless-type MMTV integration site family, member 7B	29.02667	28.24333	-1.06	0.225841	OK

Hs.591274	WNT8A	Wingless-type MMTV integration site family, member 8A					No expression
Hs.149504	WNT9A	Wingless-type MMTV integration site family, member 9A	26.42333	24.70667	-3.85	0.229533	OK

Appendix 2: Complete PCR array results of decidua cells.

The following data presents the complete set of results from PCR array analysis of decidua cells. The average Ct values from both control and co-culture decidua cells and expression fold changes of all 84 Wnt related genes are presented in the table.

Table II: Complete PCR array results of decidua cells. Genes that were undetected are coloured red.

UniGene	Symbol	Description	AVG Ct without normalization		Fold change	P value	Comment
			Co-culture	Control			
Hs.515053	AES	Amino-terminal enhancer of split	21.69	22.60667	2.84	0.593383	OK
Hs.158932	APC	Adenomatous polyposis coli	26.81333	27.21	0.87	0.922536	OK
Hs.592082	AXIN1	Axin 1	25.17333	27.35667	16.78	0.427531	OK
Hs.415209	BCL9	B-cell CLL/lymphoma 9	27.70333	30.54333	5.23	0.116148	OK
Hs.643802	BTRC	Beta-transducin repeat containing	24.2	26.11	6.06	0.320157	OK
Hs.17631	FZD5	Frizzled family receptor 5	22.79	26.76667	30.70	0.267656	OK
Hs.523852	CCND1	Cyclin D1	23.05	21.92	-3.01	0.344638	OK

Hs.376071	CCND2	Cyclin D2	23.55	22.68667	-1.63	0.331727	OK
Hs.534307	CCND3	Cyclin D3	22.52	22.85333	0.64	0.881262	OK
Hs.529862	CSNK1A1	Casein kinase 1, alpha 1	21.73667	25.52	156.50	0.421132	OK
Hs.631725	CSNK1D	Casein kinase 1, delta	20.98667	21.24667	0.70	0.784247	OK
Hs.646508	CSNK1G1	Casein kinase 1, gamma 1	30.45333	29.02	-2.76	0.23924	OK
Hs.644056	CSNK2A1	Casein kinase 2, alpha 1 polypeptide	20.55333	22.53667	10.13	0.388298	OK
Hs.208597	CTBP1	C-terminal binding protein 1	21.77667	23.62	8.64	0.390805	OK
Hs.501345	CTBP2	C-terminal binding protein 2	20.51667	21.32667	2.15	0.286198	OK
Hs.476018	CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa	19.51	21.85	6.80	0.183125	OK
Hs.463759	CTNNBIP1	Catenin, beta interacting protein 1	29.09667	30.58	1.71	0.195771	OK
Hs.12248	CXXC4	CXXC finger protein 4	34.06333	29.86	-6.95	0.018546	OK
Hs.654934	DAAM1	Dishevelled associated activator of morphogenesis 1	21.57667	22.92333	2.80	0.042111	OK
Hs.655626	DIXDC1	DIX domain containing 1	25.84333	24.72667	-2.21	0.314919	OK

Hs.40499	DKK1	Dickkopf homolog 1 (Xenopus laevis)	25.20333	20.73667	-110.78	0.382565	OK
Hs.74375	DVL1	Dishevelled, dsh homolog 1 (Drosophila)	20.14667	21.76	4.09	0.243568	OK
Hs.118640	DVL2	Dishevelled, dsh homolog 2 (Drosophila)	24.7	26.10333	4.19	0.346029	OK
Hs.517517	EP300	E1A binding protein p300	21.82333	24.56	13.29	0.339331	OK
Hs.484138	FBXW11	F-box and WD repeat domain containing 11	23.47667	24.01333	1.55	0.816931	OK
Hs.494985	FBXW2	F-box and WD repeat domain containing 2	26.58667	26.45667	-0.32	0.343619	OK
Hs.1755	FGF4	Fibroblast growth factor 4					No expression
Hs.283565	FOSL1	FOS-like antigen 1	22.23667	20.35	-8.55	0.36762	OK
Hs.663679	FOXN1	Forkhead box N1	32.33	35	8.92	0.415442	OK
Hs.126057	FRAT1	Frequently rearranged in advanced T-cell lymphomas	26.35667	28.44	5.29	0.181053	OK

Hs.128453	FRZB	Frizzled-related protein	27.63333	28.20667	1.02	0.993959	OK
Hs.36975	FSHB	Follicle stimulating hormone, beta polypeptide					No expression
Hs.94234	FZD1	Frizzled family receptor 1	24.04333	24.99	2.10	0.026633	OK
Hs.142912	FZD2	Frizzled family receptor 2	27.41333	25.17	0.85	0.916268	OK
Hs.40735	FZD3	Frizzled family receptor 3	25.50667	26.71	2.63	0.104971	OK
Hs.19545	FZD4	Frizzled family receptor 4	27.21667	24.19333	-18.34	0.315653	OK
Hs.591863	FZD6	Frizzled family receptor 6	22.32	25.08333	9.43	0.227393	OK
Hs.173859	FZD7	Frizzled family receptor 7	26.07	28.42333	7.29	0.236321	OK
Hs.302634	FZD8	Frizzled family receptor 8	24.51	22.70667	-4.12	0.10357	OK
Hs.466828	GSK3A	Glycogen synthase kinase 3 alpha	23.07333	26.87	56.52	0.413151	OK
Hs.445733	GSK3B	Glycogen synthase kinase 3 beta	19.80333	21.38333	4.19	0.286821	OK
Hs.714791	JUN	Jun proto-oncogene	22.37667	22.18	-0.45	0.705457	OK

Hs.229335	KREMEN1	Kringle containing transmembrane protein 1	23.31	21.73	-4.22	0.289933	OK
Hs.555947	LEF1	Lymphoid enhancer-binding factor 1	29.08333	26.09667	-8.76	0.080499	OK
Hs.6347	LRP5	Low density lipoprotein receptor-related protein 5	23.35667	27.33667	47.58	0.38509	OK
Hs.584775	LRP6	Low density lipoprotein receptor-related protein 6	24.91	25.52667	1.24	0.871916	OK
Hs.202453	MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	23.64667	24.87667	8.08	0.504485	OK
Hs.592059	NKD1	Naked cuticle homolog 1 (Drosophila)	30.58333	26.64	-42.24	0.307764	OK
Hs.208759	NLK	Nemo-like kinase	23.97333	25.62	3.88	0.136619	OK
Hs.643588	PITX2	Paired-like homeodomain 2	24.62333	30.24	42.33	0.12235	OK
Hs.386453	PORCN	Porcupine homolog (Drosophila)	25.66	27.00667	16.01	0.486248	OK
Hs.483408	PPP2CA	Protein phosphatase 2, catalytic subunit, alpha isozyme	23.25	23.61	0.97	0.984933	OK

Hs.467192	PPP2R1A	Protein phosphatase 2, regulatory subunit A, alpha	19.84667	21.12333	4.16	0.508892	OK
Hs.256587	PYGO1	Pygopus homolog 1 (Drosophila)	27.13667	25.63	-5.17	0.350773	OK
Hs.647774	RHOU	Ras homolog gene family, member U	19.81667	22.13333	5.97	0.105693	OK
Hs.401388	SENP2	SUMO1/sentrin/SMT3 specific peptidase 2	21.48667	23.62667	5.74	0.191076	OK
Hs.713546	SFRP1	Secreted frizzled-related protein 1	22.64333	27.00333	38.60	0.209944	OK
Hs.658169	SFRP4	Secreted frizzled-related protein 4	31.75	23.07333	-316.25	0.206132	OK
Hs.500822	FBXW4	F-box and WD repeat domain containing 4	22.29333	23.41333	2.46	0.111337	OK
Hs.728760	SLC9A3R1	Solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1	22.54333	25.57	19.48	0.310069	OK
Hs.98367	SOX17	SRY (sex determining region Y)-box 17	34.24333	31.02	-8.84	0.085064	OK
Hs.389457	T	T, brachyury homolog (mouse)	37.85	32.34667	-2.62	0.172137	OK

Hs.573153	TCF7	Transcription factor 7 (T-cell specific, HMG-box)	30.73333	29.35333	-12.64	0.504009	OK
Hs.516297	TCF7L1	Transcription factor 7-like 1 (T-cell specific, HMG-box)	27.35667	26.5	-2.49	0.538618	OK
Hs.197320	TLE1	Transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)	21.13333	23.13	4.32	0.004177	OK
Hs.332173	TLE2	Transducin-like enhancer of split 2 (E(sp1) homolog, Drosophila)	22.89667	24.90667	7.79	0.395637	OK
Hs.284122	WIF1	WNT inhibitory factor 1	35	31.86667	-2.42	0.206854	OK
Hs.492974	WISP1	WNT1 inducible signaling pathway protein 1	27.89	24	-186.31	0.415973	OK
Hs.248164	WNT1	Wingless-type MMTV integration site family, member 1	29.99333	26.88	-45.96	0.397336	OK
Hs.121540	WNT10A	Wingless-type MMTV integration site family, member 10A	24.82	31.12	46.12	0.069566	OK
Hs.108219	WNT11	Wingless-type MMTV integration site family, member 11	23.82333	24.43333	1.48	0.847885	OK

Hs.272375	WNT16	Wingless-type MMTV integration site family, member 16	30.14667	28.22	-10.00	0.265834	OK
Hs.567356	WNT2	Wingless-type MMTV integration site family member 2	25.69333	24.44333	-4.00	0.397021	OK
Hs.258575	WNT2B	Wingless-type MMTV integration site family, member 2B	27.85667	27.51667	-0.55	0.274719	OK
Hs.445884	WNT3	Wingless-type MMTV integration site family, member 3	27.59667	26.44333	-2.35	0.289086	OK
Hs.336930	WNT3A	Wingless-type MMTV integration site family, member 3A					No expression
Hs.25766	WNT4	Wingless-type MMTV integration site family, member 4	25.14333	25.89667	6.97	0.580069	OK
Hs.696364	WNT5A	Wingless-type MMTV integration site family, member 5A	22.78333	20.46	-9.90	0.235773	OK
Hs.306051	WNT5B	Wingless-type MMTV integration site family, member 5B	23.94333	21.66667	-7.01	0.185741	OK
Hs.29764	WNT6	Wingless-type MMTV integration site family, member 6	23.87333	27.29667	55.38	0.417513	OK

Hs.72290	WNT7A	Wingless-type MMTV integration site family, member 7A	23.52	28.14667	47.46	0.336543	OK
Hs.512714	WNT7B	Wingless-type MMTV integration site family, member 7B	29.3	30.64	1.54	0.771741	OK
Hs.591274	WNT8A	Wingless-type MMTV integration site family, member 8A					No expression
Hs.149504	WNT9A	Wingless-type MMTV integration site family, member 9A	26.59	28.04333	3.97	0.663707	OK

Appendix 3: Complete comparative gene expression levels of trophoblast and decidua

The following data presents the Comparative gene expression levels of trophoblast and decidua of the complete set of results from PCR array analysis. The average 2^-ΔCt values from both trophoblast and decidua cells and the p-values (Paired t Student's-tests) of all 84 Wnt related genes are presented in the table.

Table III: Complete Comparative gene expression levels of trophoblast and decidua. Genes that were undetected are coloured red. Genes with significant P values (≤0.05) are indicated in bold text.

UniGene	Symbol	Description	Trophoblast 2^-ΔCt	Decidua 2^-ΔCt	P value
Hs.515053	AES	Amino-terminal enhancer of split	4.3E-02	4.3E-02	0.4937
Hs.158932	APC	Adenomatous polyposis coli	8.9E-04	1.0E-03	0.4013
Hs.592082	AXIN1	Axin 1	2.2E-03	2.2E-03	0.4835
Hs.415209	BCL9	B-cell CLL/lymphoma 9	2.1E-04	1.8E-04	0.3389
Hs.643802	BTRC	Beta-transducin repeat containing	3.2E-03	3.0E-03	0.4438
Hs.17631	FZD5	Frizzled family receptor 5	6.1E-04	1.8E-03	0.1704

Hs.523852	CCND1	Cyclin D1	4.3E-02	4.3E-02	0.4997
Hs.376071	CCND2	Cyclin D2	1.9E-02	2.1E-02	0.2933
Hs.534307	CCND3	Cyclin D3	3.2E-02	2.3E-02	0.2716
Hs.529862	CSNK1A1	Casein kinase 1, alpha 1	2.5E-02	2.4E-02	0.4803
Hs.631725	CSNK1D	Casein kinase 1, delta	4.5E-02	5.8E-02	0.2106
Hs.646508	CSNK1G1	Casein kinase 1, gamma 1	1.0E-04	3.2E-04	0.1704
Hs.644056	CSNK2A1	Casein kinase 2, alpha 1 polypeptide	4.6E-02	4.3E-02	0.437
Hs.208597	CTBP1	C-terminal binding protein 1	2.2E-02	2.0E-02	0.4113
Hs.501345	CTBP2	C-terminal binding protein 2	3.3E-02	5.9E-02	0.1021
Hs.476018	CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa	5.6E-02	4.8E-02	0.367
Hs.463759	CTNNBIP1	Catenin, beta interacting protein 1	2.4E-04	2.8E-04	0.379
Hs.12248	CXXC4	CXXC finger protein 4	1.2E-04	1.4E-04	0.2433
Hs.654934	**DAAM1**	**Dishevelled associated activator of morphogenesis 1**	**1.2E-02**	**1.7E-02**	**0.022**
Hs.655626	DIXDC1	DIX domain containing 1	9.6E-03	8.4E-03	0.3245

Hs.40499	DKK1	Dickkopf homolog 1 (Xenopus laevis)	1.1E-01	8.6E-02	0.3305
Hs.74375	DVL1	Dishevelled, dsh homolog 1 (Drosophila)	3.8E-02	4.4E-02	0.3447
Hs.118640	DVL2	Dishevelled, dsh homolog 2 (Drosophila)	2.5E-03	2.6E-03	0.4581
Hs.517517	EP300	E1A binding protein p300	1.1E-02	1.0E-02	0.4367
Hs.484138	FBXW11	F-box and WD repeat domain containing 11	1.2E-02	1.2E-02	0.4874
Hs.494985	FBXW2	F-box and WD repeat domain containing 2	1.2E-03	1.5E-03	0.1075
Hs.1755	FGF4	Fibroblast growth factor 4	No expression		
Hs.283565	FOSL1	FOS-like antigen 1	1.9E-01	1.4E-01	0.3475
Hs.663679	FOXN1	Forkhead box N1	8.4E-06	2.2E-05	0.2
Hs.126057	FRAT1	Frequently rearranged in advanced T-cell lymphomas	5.5E-04	5.1E-04	0.4044

Hs.128453	FRZB	Frizzled-related protein	2.6E-04	4.8E-04	0.1334
Hs.36975	FSHB	Follicle stimulating hormone, beta polypeptide	No expression		
Hs.94234	FZD1	Frizzled family receptor 1	4.5E-03	4.2E-03	0.4425
Hs.142912	FZD2	Frizzled family receptor 2	1.8E-03	1.6E-03	0.2627
Hs.40735	FZD3	Frizzled family receptor 3	1.5E-03	1.3E-03	0.3943
Hs.19545	FZD4	Frizzled family receptor 4	8.3E-03	8.7E-03	0.394
Hs.591863	FZD6	Frizzled family receptor 6	4.5E-03	5.1E-03	0.3634
Hs.173859	FZD7	Frizzled family receptor 7	5.2E-04	5.4E-04	0.4673
Hs.302634	FZD8	Frizzled family receptor 8	1.6E-02	2.1E-02	0.1254
Hs.466828	GSK3A	Glycogen synthase kinase 3 alpha	8.4E-03	7.5E-03	0.3336
Hs.445733	GSK3B	Glycogen synthase kinase 3 beta	3.8E-02	5.7E-02	0.0646
Hs.714791	JUN	Jun proto-oncogene	8.4E-02	6.8E-02	0.3298

Hs.229335	KREMEN1	Kringle containing transmembrane protein 1	7.7E-02	8.0E-02	0.4701
Hs.555947	**LEF1**	**Lymphoid enhancer-binding factor 1**	**1.3E-03**	**1.9E-03**	**0.0358**
Hs.6347	LRP5	Low density lipoprotein receptor-related protein 5	2.9E-03	2.7E-03	0.4007
Hs.584775	LRP6	Low density lipoprotein receptor-related protein 6	3.9E-03	3.6E-03	0.433
Hs.202453	MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	1.3E-02	1.4E-02	0.3781
Hs.592059	NKD1	Naked cuticle homolog 1 (Drosophila)	8.2E-04	1.5E-03	0.06
Hs.208759	NLK	Nemo-like kinase	3.6E-03	3.3E-03	0.413
Hs.643588	PITX2	Paired-like homeodomain 2	4.9E-04	3.6E-04	0.1357
Hs.386453	PORCN	Porcupine homolog (Drosophila)	2.3E-03	2.7E-03	0.3754
Hs.483408	PPP2CA	Protein phosphatase 2, catalytic subunit, alpha isozyme	5.8E-03	1.1E-02	0.0921

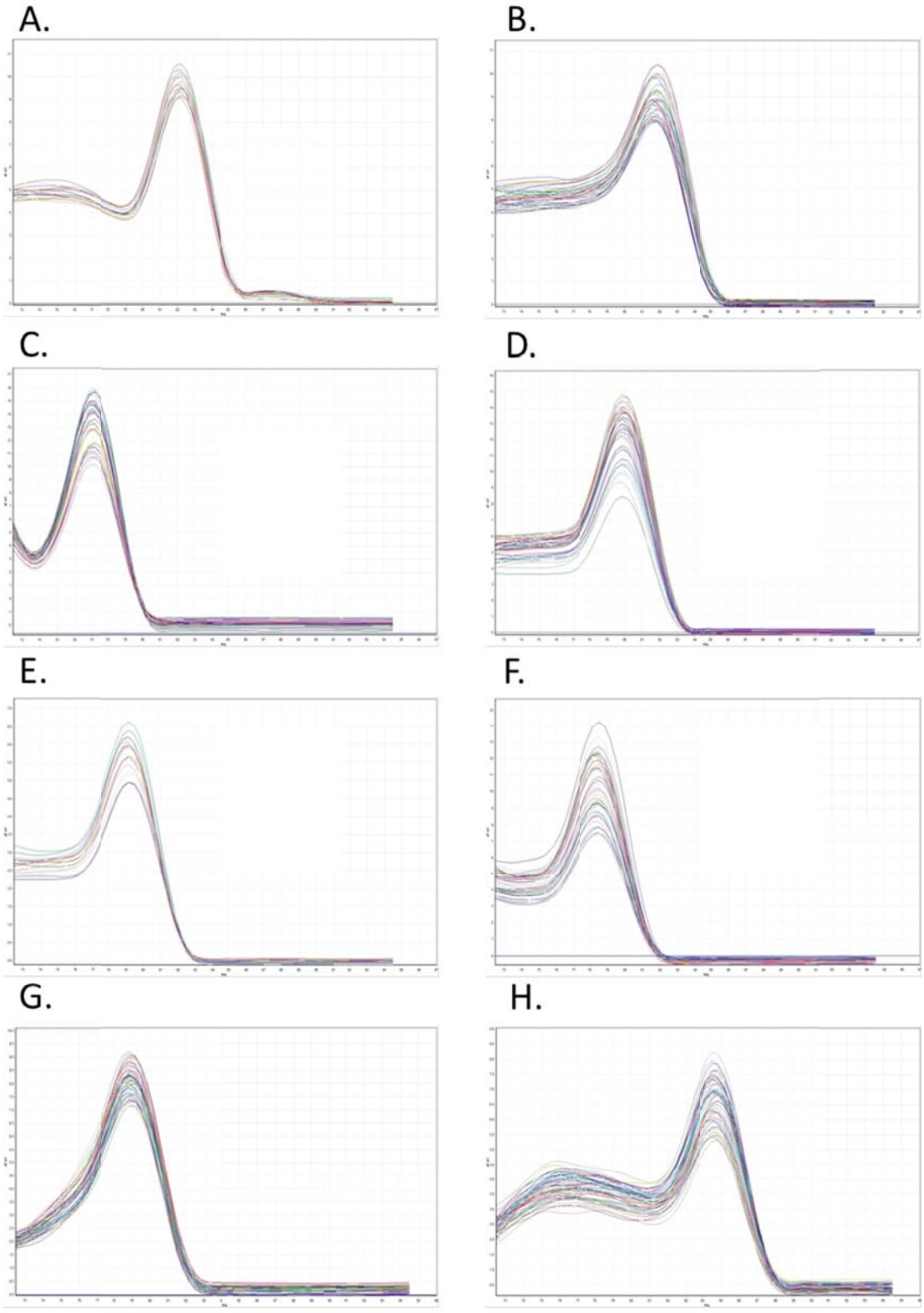
Hs.467192	PPP2R1A	Protein phosphatase 2, regulatory subunit A, alpha	1.3E-01	1.0E-01	0.2991
Hs.256587	PYGO1	Pygopus homolog 1 (Drosophila)	3.5E-03	3.5E-03	0.4962
Hs.647774	RHOU	Ras homolog gene family, member U	2.5E-02	3.2E-02	0.1995
Hs.401388	SENP2	SUMO1/sentrin/SMT3 specific peptidase 2	1.2E-02	1.3E-02	0.4611
Hs.713546	SFRP1	Secreted frizzled-related protein 1	2.0E-03	1.8E-03	0.3836
Hs.658169	**SFRP4**	**Secreted frizzled-related protein 4**	**5.2E-03**	**1.7E-02**	**0.0429**
Hs.500822	FBXW4	F-box and WD repeat domain containing 4	6.6E-03	1.3E-02	0.0924
Hs.728760	SLC9A3R1	Solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1	8.5E-03	7.8E-03	0.4103
Hs.98367	SOX17	SRY (sex determining region Y)-box 17	1.0E-04	1.1E-04	0.1355
Hs.389457	T	T, brachyury homolog (mouse)	2.2E-05	4.1E-05	0.0835

Hs.573153	TCF7	Transcription factor 7 (T-cell specific, HMG-box)	8.0E-04	6.8E-04	0.3934
Hs.516297	TCF7L1	Transcription factor 7-like 1 (T-cell specific, HMG-box)	3.8E-03	3.1E-03	0.367
Hs.197320	TLE1	Transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)	9.5E-03	1.5E-02	0.0642
Hs.332173	TLE2	Transducin-like enhancer of split 2 (E(sp1) homolog, Drosophila)	5.5E-03	7.2E-03	0.2705
Hs.284122	WIF1	WNT inhibitory factor 1	7.1E-06	3.9E-05	0.2788
Hs.492974	WISP1	WNT1 inducible signaling pathway protein 1	2.8E-02	1.7E-02	0.1895
Hs.248164	WNT1	Wingless-type MMTV integration site family, member 1	1.2E-03	1.3E-03	0.4156
Hs.121540	WNT10A	Wingless-type MMTV integration site family, member 10A	1.7E-04	1.4E-04	0.3271
Hs.108219	WNT11	Wingless-type MMTV integration site family, member 11	3.9E-03	6.5E-03	0.1004

Hs.272375	WNT16	Wingless-type MMTV integration site family, member 16	2.2E-03	1.5E-03	0.0453
Hs.567356	WNT2	Wingless-type MMTV integration site family member 2	3.2E-03	6.8E-03	0.0884
Hs.258575	**WNT2B**	**Wingless-type MMTV integration site family, member 2B**	**2.6E-04**	**7.2E-04**	**0.0044**
Hs.445884	WNT3	Wingless-type MMTV integration site family, member 3	2.9E-03	2.8E-03	0.4472
Hs.336930	WNT3A	Wingless-type MMTV integration site family, member 3A	No expression		
Hs.25766	WNT4	Wingless-type MMTV integration site family, member 4	7.3E-03	6.7E-03	0.2097
Hs.696364	WNT5A	Wingless-type MMTV integration site family, member 5A	2.9E-01	2.2E-01	0.282
Hs.306051	WNT5B	Wingless-type MMTV integration site family, member 5B	7.4E-02	5.9E-02	0.3449
Hs.29764	WNT6	Wingless-type MMTV integration site family, member 6	9.1E-03	8.2E-03	0.0091

Hs.72290	WNT7A	Wingless-type MMTV integration site family, member 7A	1.4E-03	1.2E-03	0.3042
Hs.512714	WNT7B	Wingless-type MMTV integration site family, member 7B	2.3E-04	1.6E-04	0.2924
Hs.591274	WNT8A	Wingless-type MMTV integration site family, member 8A	No expression		
Hs.149504	WNT9A	Wingless-type MMTV integration site family, member 9A	2.9E-03	2.4E-03	0.2625

Appendix 4: Melt curve analysis for genes studied.



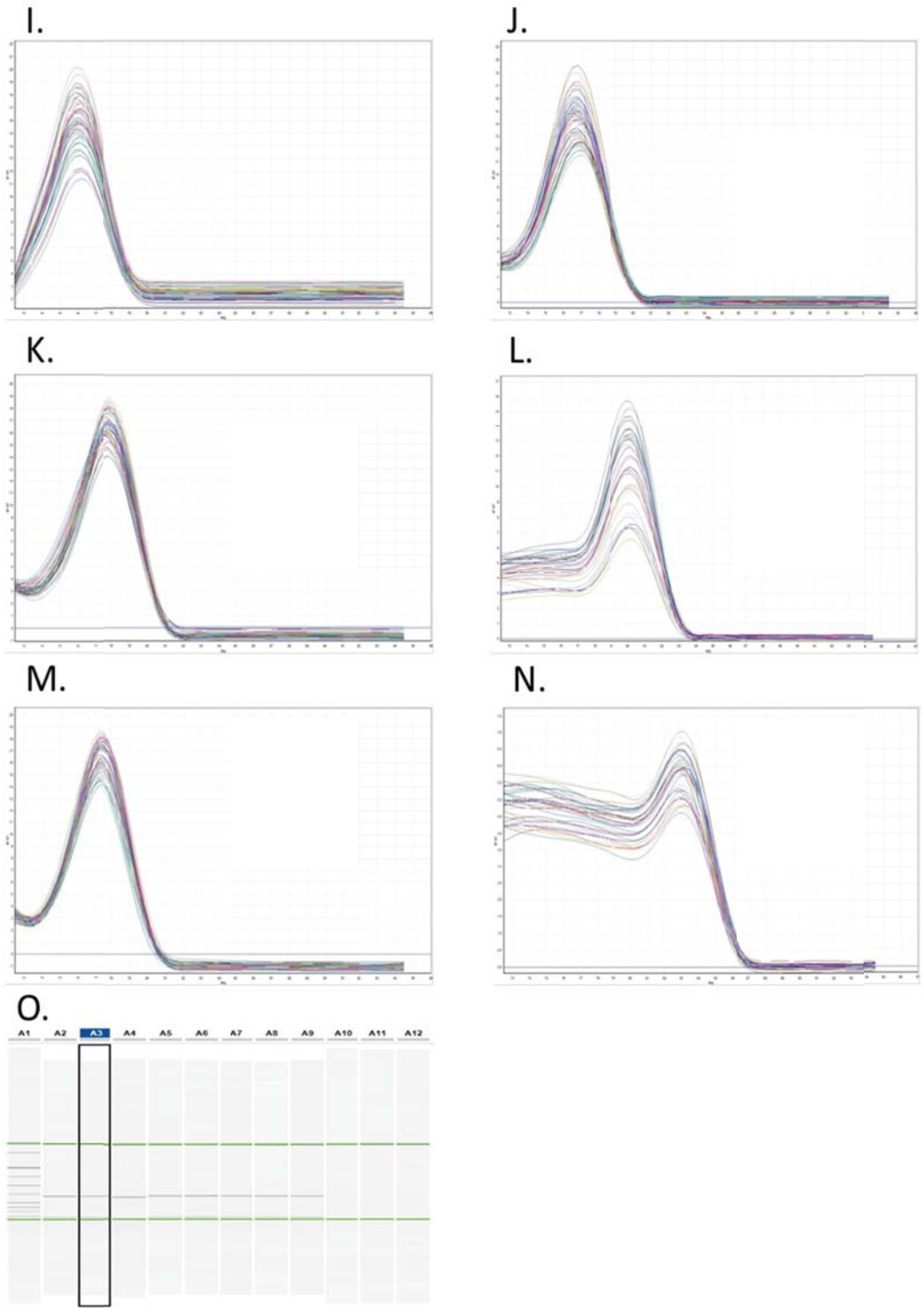


Figure I: Melt curve analysis for genes studied. Denaturation of a single DNA product is represented by a single peak in the derivation of fluorescence plotted against temperature. Graphs display reaction products of primers designed against. **(A) WNT5A, (B) FZD4 (C) FZD5, (D) DKK1, (E) HOXA10, (F) sFRP4, (G) MSX1, (H) MSX2, (I) PTGS1, (J) PTGS2, (K) PTGES, (L) WIF1, (M) SDHA, (N) sFRP1 and (O) gel analysis of sFRP1 product.** Gel shows single product of 135bp size.