The University Of Sheffield.

Different facets of Toll-like receptor 9 activation in trophoblast-endometrium cross-talk

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Abstract

Implantation failure caused by sexually transmitted infections (STI) is one of the major factors involved in pregnancy loss. Successful implantation requires a supportive environment, which is strongly dependent on a healthy endometrium. The presence of any infection at the site of implantation could be sensed by pathogen recognition receptors (PRRs). Toll-like receptors (TLRs) are a major family of PRRs that are wildly expressed in endometrial epithelial cells and react to specific microbial agents. These receptors initiate intracellular signalling leading to the secretion of inflammatory cytokines that might prevent implantation.

The aim of the current investigation was to study TLR9 activation in human endometrial epithelial cells and its effect on trophoblast behaviour. Two and three dimensional *in vitro* cell culture systems of human immortalized cell lines were used to evaluate: (i) the effect of TLR9 ligation on the binding of trophoblast spheroids to endometrial cells, (ii) TLR9 intracellular signalling in human endometrial epithelial cells, and (iii) the effect of TLR9 activation on trophoblast spheroid outgrowth and invasion in a 3D culture system.

The results showed that activation of TLR9 in human endometrial epithelial cells had a detrimental effect on the binding of trophoblasts to endometrial cells in the 2D culture system. The TLR9 intracellular signalling pathway was MyD88 dependent, and there were no alteration in the activation of NFκB and P38 at the time of TLR9 stimulation in endometrial cells. TLR9 activation was able to affect the trophoblast outgrowth and invasion in the 3D culture system. It appears that TLR9 activation can trigger an intracellular signalling pathway in endometrium that is NFκB and P38 independent and could affect the outcomes of human *in vitro* models of implantation.

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"Success is not the key to happiness. Happiness is the key to success. If you love what you are doing, you will be successful."

Albert Schweitzer

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Abbreviation list

ART	Assisted Reproductive Technologies
CpG	Cytidine Phosphate Guanosine repeats
CPP	Cell Penetrating Peptide
ECM	Extracellular Matrix
EDTA	Ethylene Diaminetetra acetatic Acid
ELISA	Enzyme Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
FRT	Female Reproductive Tract
GFP	Green Fluorescent Protein
hCG	human Chorionic Gonadotropin
HCMV	Human Cytomegalovirus
HSV	Herpes Simplex Virus
IFN	Interferon
IKK	IκB Kinase
IL	Interleukin
IRF7	Interferon Regulatory Factor 7
IVF	*in vitro* Fertilization
KD	Knock Down
LIF	Leukaemia Inhibitory Factor
LRR	Leucine Rich Repeats
LSM	Laser Scanning Microscope
MAPK	Mitogen-Activated Protein Kinase
MMP	Matrix Metalloproteinase
MyD88	Myeloid Differentiation Primary gene 88
NK	Natural killer cell
NFκB	Nuclear Factor-κB
NOD	Nucleotide binding Oligomerization Domain
ODN	Oligodeoxynucleotide
PAMP	Pathogen Associated Molecular Pattern
PRR	Pattern Recognition Receptors

PTGS ..... Post-Transcriptional Gene Silencing

qPCR ..... Quantitative Polymerase Chain Reaction

RBGS ..... RNA-Based Gene Silencing

RIG ..... Retinoic acid-Inducible Gene

SEAP ..... Secreted Placental Alkaline Phosphatase

STI ..... Sexually Transmitted Infection

TIR ..... Toll-Interleukin 1 Receptor

TLR ..... Toll-like Receptor

TNF ..... Tumour Necrosis Factor

TRIF ..... TIR Regulated Adaptor Protein Inducing Interferon

VEGF ..... Vascular Endothelial Growth Factor

3DEESCC ..... 3-Dimentional Endometrial Epithelial-Stromal Cell Culture

Chapter 1. Introduction

1.1 Overview

The human body is continuously exposed to a wide variety of organisms and microbial agents. Pathogen recognition and neutralizing of these agents (pathogen removal) are the main roles of the human immune system (Thao Doan 2008). Both innate and adaptive immunity are the two major subdivisions of the human immune system. The innate immune system is our first line of defence against invading organisms with immediate nonspecific responses, whilst the adaptive immune system acts as the second line of defence with specific responses and also provides protection against re-exposure to the same infectious organisms. The innate and adaptive immune systems both protect us against invading organisms in different ways. For example, adaptive immunity requires time to react to an invading organism, whereas the innate immune system is always ready to be mobilized upon detecting infections. In addition, the adaptive immune system is antigen specific; in contrast, the innate immune system reacts equally to a variety of organisms, and finally, in contrast to the innate immune system, adaptive immunity demonstrates immunological memory (Thao Doan 2008).

Toll-like receptors (TLRs), the main group of pathogen recognition receptors (PRRs) in the innate immune system, are found in different tissues and organs in the human body. They act as mediators that connect both the innate and the adaptive immune systems. Recent studies have emphasized the role of TLRs in the female reproductive tract (FRT) and their role in infertility (Aboussahoud et al., 2010b, Montazeri et al., 2015a, Sanchez-Lopez et al., 2014). Implantation failure is one of the major factors involved in pregnancy loss. Successful implantation requires both good embryo quality and endometrial receptivity. In this regard, there is a growing body of evidence that the innate immune system plays a major role in the modulation of endometrial receptivity (Simon et al., 2000). TLRs expression in the endometrial epithelium during the window of implantation probably points to their role in the recognition of infections (such as sexually transmitted infections) at the time of pregnancy and it may suggest a role in the embryo-endometrium interaction.

In this chapter, the writer will try to give the readers an overview of the literature regarding the innate immune system's function and its presence in the female reproductive tract and of Toll-like receptors and their role in implantation and pregnancy. These will be followed by describing the main aim and hypothesis the writer wanted to test and for which she created all the experiments that are explained in detail later in this thesis.

1.2 Innate immune system in human body

Innate immunity is an ancient system, with a similar structure in plants, invertebrates and vertebrates (Sochocka and Blach-Olszewska, 2005). Innate immunity is divided into two main subdivisions regarding its activation: the afferent (sensing) arm, and the efferent (effector) arm. The afferent arm refers to pathogen recognition, and the efferent arm refers to pathogen removal (Beutler, 2004).

After biological barriers like the skin, the innate immune system is the first line of defence against pathogens. Leukocytes, or white blood cells, are recruited by the innate immune system to either prevent an invasion of foreign organisms or to secret soluble molecules to overcome the invaders. Innate immunity is known as a fast responder, since most of its components are genetically active and are able to respond to a wide range of microbial agents. The most important localized response of innate immunity to defend against foreign invaders is called inflammation, which may lead to activation of adaptive immune responses (Thao Doan 2008).

1.3 Pathogen recognition receptors (PRRs):

Innate immunity recognition mechanisms are highly conserved among species, from fruit flies and plants to mammals (Akira et al., 2006, Rosenstiel et al., 2009). Recognition of foreign invaders by the innate immune system in humans normally occurs via germ-line encoded pattern recognition receptors (PRRs). Different classes of PRRs such as Toll-like receptors, nucleotide-binding oligomerization domain receptors (NOD-like receptors), and retinoic acid-inducible gene 1 (RIG-I)-like

receptors, recognize specific microbial components and initiate intracellular signalling pathways, which results in an inflammatory response (Akira et al., 2006). PRRs are responsible for recognition of microorganisms via their pathogen-associated molecular patters (PAMPs). Activation of PRRs by PAMPs would initiate an intracellular signalling pathway leading to innate immune responses (Uematsu and Akira, 2006).

1.4 Toll-like Receptors (TLRs)

Toll-like receptors, as the gate-keepers of innate immunity, are the main family of PRRs. Evidence suggests there are at least 11 members of the TLR family in humans, and 13 in the mouse have been discovered (Yu et al., 2009). TLR 1-9 are conserved between mice and humans, but there is no equivalent discovery of TLR 11 in human that is expressed in the bladder epithelial cells of mice (Aflatoonian and Fazeli, 2008). Each type of TLR has its own signalling pathway that can induce inflammatory responses. Various immune cells, like macrophages, dendritic cells, B cells, T cells and even non-immune cells such as fibroblasts and epithelial cells, express TLRs extra- or intracellularly. TLR 1, 2, 4, 5 and 6 are expressed on the cell surface, while TLR 3, 7, 8 and 9 are found in intracellular compartments like the endoplasmic reticulum (ER) membrane, endosomes and lysosomes (Kawai and Akira, 2010). TLRs are transmembrane proteins with a leucine-rich domain as the recognition site and a TIR (Toll-interleukin 1 receptor) domain in the cytoplasm, which is indispensable for the intracellular signalling cascade (Akira et al., 2006, Kawai and Akira, 2010). They have the capability to recognize different microbial agents as specific ligands (Table 1). The intracellular TLRs are able to recognize nucleic acids (Blasius and Beutler, 2010). In this case, the microbial components should be taken up by cells, which usually happens via phagocytosis, pinocytosis and/or endocytosis. The vesicles then fuse with endosomes or lysosomes, where the stimulation of TLRs occurs (Blasius and Beutler, 2010).

1.4.1 TLRs signalling pathway

Once TLRs are stimulated with their specific ligands, the intracellular signalling pathway is initiated via the main mediators Myeloid differentiation primary response gene 88 (MyD88) or TIR-domain-containing adapter-inducing interferon-β (TRIF),

which then leads to the activation of downstream proteins and results in innate immunity responses such as cytokine secretion. All TLRs except the TLR3 signalling pathways are MyD88-dependent. MyD88 then recruits Interleukin-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) upon ligand stimulation. TRAF6 then activates Transforming growth factor beta-activated kinase 1 (TAK1) complex via ubiquitination, which in turn leads to activation and dimerization of Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and/or Mitogen-activated protein kinases (MAPKs). Activation of MAPKs would consequently result in phosphorylation and the translocation of Activator protein 1 (AP1) into the nucleus. NFkB and AP1 control inflammatory responses by inducing pro-inflammatory cytokines. TRAF6, on the other hand, is able to activate Interferon regulatory factor 3 (IRF3) and/or IRF7. Phosphorylated IRF3 and IRF7 are dimerized and translocated into nuclei to bind to DNA. Among TLRs, TLR3 and TLR4 use TRIF as the main mediator to start their signalling pathways. TRIF interacts with TANK-binding kinase 1 (TBK1). TBK1, together with IκB kinase (IKKi), mediates phosphorylation and activation of IRF3 and/or IRF7, which are then translocated into the nucleus (Blander and Medzhitov, 2004, Kawai and Akira, 2006, Kawai and Akira, 2007, Mitchell et al., 2016, Chen and Yu, 2016). It is well documented that the TLR signalling pathway is highly cell-specific and is also dependent on the type of the stimuli. In this regards, TLR9 ligation with *E. coli* based CpG in human HT-29 cells resulted in activation of p38, a member of MAPKs, while activation of TLR9 with synthetic CpG in human DC cells was TRIF dependent (Akhtar et al., 2003, Volpi et al., 2013). Furthermore, TLR5 ligation with flagella resulted in NFkB activation in the Ishikawa cell line (Caballero et al., 2013). Figure 1.1 (p. 28) deciphers all the possible pathways for both intracellular and extracellular TLRs.

Recent studies have also indicated that there is an interaction between the TLR family and other PRRs like NLR (NOD-like receptor) and RLR (RIG I-like receptor). Hence innate immunity acts not only as a network to respond to invading micro-organisms, but also has an ability to activate adaptive immune responses (Trinchieri and Sher, 2007).

Table -1 Toll-like receptors and their ligands

TLRs:	Ligands:
TLR1	Triacyl lipopeptides
TLR2	LPS
TLR3	Poly (I:C), double stranded RNA
TLR4	LPS, RSV fusion protein
TLR5	Flagellin
TLR6	Diacyl lipopeptides
TLR7	single stranded RNA
TLR8	single stranded RNA
TLR9	CpG DNA
TLR10	No specific ligand has been identified

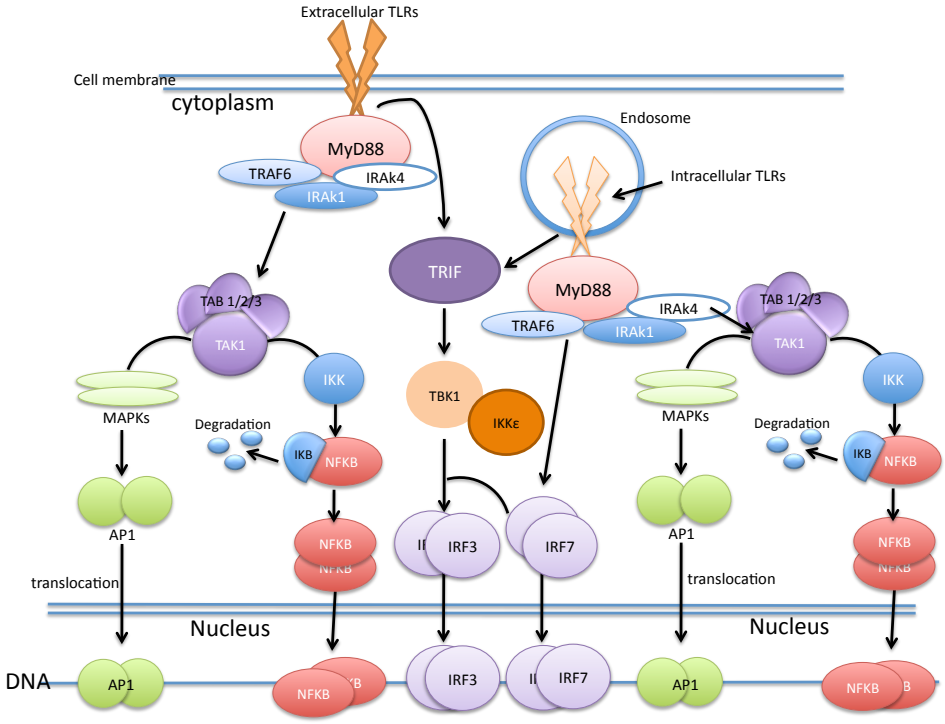


Figure 1.1 Schematic view of all the possible pathways for both intracellular and extracellular TLRs. All the TLRs except TLR3 signalling pathway are MyD88 dependent. MyD88 then recruits IRAK1 and TRAF6 upon ligand stimulation. TRAF6 then activates the TAK1 complex via ubiquitination, which then leads to activation and dimerization of NFκB and/or MAPKs. Activation of MAPKs would consequently cause phosphorylation and translocation of AP1 into the nucleus. NFkB and AP1 control inflammatory responses by inducing pro-inflammatory cytokines. TRAF6, on the other hand, is able to activate IRF3 and/or IRF7. Phosphorylated IRF3 and IRF7 are dimerized and translocated into nucleus to bind to the DNA. Among TLRs are TLR3 and TLR4, which use TRIF as the main mediator to start their signalling pathway. TRIF interacts with TBK1. TBK1, together with IKKi, mediates phosphorylation and activation of IRF3 and/or IRF7.

1.5 Negative regulation of TLRs

Excessive production of cytokines and consistent inflammatory responses usually accelerate the tissue damage process followed by failure of tissue repair. This process is known as chronic inflammation, and is one of the reasons for systemic disorders and autoimmune diseases. Therefore, inflammatory responses, especially TLRs' activation and function, must be precisely controlled at different levels (Foster and Medzhitov, 2009, Wang et al., 2009).

The TLRs signalling pathways lead to not only inflammatory cytokine production but also to production of inflammatory mediators, antimicrobial proteins, and different chemokines. Hence hundreds of genes, with a variety of different functions and definitely different regulatory elements, are involved once a TLR has been activated (Foster and Medzhitov, 2009). In addition to chromatin modification that makes the inflammatory gene become accessible to transcription factors (gene-specific control), degradation, deubiquitination and competition are the main levels of negative regulation of TLRs (Wang et al., 2009, Foster and Medzhitov, 2009). Degradation of proteins and factors involved in TLRs signalling cascade is the most common means of regulating their function. It has been reported that over-expression of the Triad3 protein, which acts as an ubiquitination enhancer, could induce TLR4 and TLR9 degradation and subsequent signal reduction. Deubiquitinating enzymes are involved in negative regulation systems as well. For example, nuclear factor κB (NFκB) activation is inhibited by A20, which is a tumour necrosis factor (TNF)-induced zinc-finger protein. TLRs intracellular signalling pathways depend on adaptor molecules like MyD88 (myeloid differentiation primary response gene 88) and/or TRIF (TIR-domain-containing adapter-inducing interferon-β). Sterile alpha and armadillo motif containing the protein SARM is the negative controller of TRIF. At the time of stimulation, SARM acts in competition with TRIF, and subsequently the signalling pathway is blocked. MyD88 that has a deficiency in its intermediate domain would also act as a negative regulator of the MyD88 dependent signalling pathway. These MyD88 molecules can still form heterodimers, but they are completely dysfunctional

(Wang et al., 2009). These are only a few examples of the variety of proteins and factors that are involved in TLR signalling regulation.

1.6 Innate immunity in the female reproductive tract

Sexually transmitted infections (STIs) and their associated problems, such as infertility, are a major worldwide health issue. The female reproductive tract's (FRT's) innate immune system is equipped to combat STI and other environmental pathogens to keep its milieu safe and to support its cyclic behaviour. FRT is categorized into two distinct regions: the nonsterile, or lower, part, which includes the vagina, cervix and the cervical plug, and the sterile, or upper, part, including the uterus, fallopian tubes and ovaries. Each part of the FRT (upper or lower part) has its own microenvironment, and consequently its own specific innate immune molecules, the expressions of which are controlled by sex hormones. The innate immune system in the FRT is responsible for providing a safe and favourable environment for the embryo to implant, for taking care of the developing foetus, and for maintaining the pregnancy (Horne et al., 2008).

Various anti-microbial peptides such as defensins have been found to prevent infections that pose a threat to the FRT's natural function. Leukocytes and epithelial cells are the main sources of human defensins. Human β-defensins (HBD 1-4) and α-defensins (HD5) are expressed by endometrial epithelial cells. HBD1 and HBD3 are highly expressed during the secretory phase of the menstrual cycle, while HBD2 expression reaches its highest level during menstruation. HBD4 is extensively found during the proliferation phase of the menstrual cycle. While HD5 is present in the vagina, cervix and fallopian tubes, HBD2 is found in cervicovaginal secretions, and HBDs 1-3 are expressed mainly in the pregnant uterus. The presence of anti-microbial peptides at different regions of the FRT suggest that they are involved in the innate immune reactions during pregnancy and that they protect the uterus from infection to present a favourable environment for the developing foetus (Horne et al., 2008). The cyclic behaviour of the FRT is under the control of sex hormones. Different patterns of expression of anti-microbial peptides during the menstrual cycle suggest that sex

hormones may also be involved in the FRT's innate immune regulation (Seavey and Mosmann, 2008). On the other hand, TLRs are also extensively found in the female reproductive tract during all the different stages of the menstrual cycle and in the pregnant uterus (Aflatoonian et al., 2007). Several studies have also probed TLR expression in trophoblast cells, which suggests that the trophoblasts are also able to sense microbial agents and to elevate innate immune responses (Horne et al., 2008). The expression of different anti-microbial peptides and various TLRs in both the FRT and in trophoblasts explains the importance of the innate immune defence in the interaction of trophoblasts and the endometrium (King et al., 2003).

Maternal-foetal communication includes signal exchanges and complex interactions between the mother and the embryo. These communications seem to be crucial for both parties, since failure to communicate may lead to miscarriage or congenital abnormalities in the foetus (Fazeli and Pewsey, 2008). At the time of normal pregnancy, the maternal immune system recognizes the semi-allograft embryo and provokes an immune response to support, rather than suppress, its development. During the first trimester of the pregnancy, different immune cells (Natural killer [NK] cells, macrophages and dendritic cells) assemble around the embryo. The number of activated immune cells at the site of the pregnancy is increased during the second and third trimester. The accumulation of immune cells around the foetus has been found to be supportive of the embryo's development, since the complete depletion of NK cells during pregnancy has a detrimental effect on pregnancy outcome. Moreover, NK cells are also found to be involved in the invasion of trophoblasts and in angiogenesis during the early stages of pregnancy. One must bear in mind that the embryo itself expresses both paternal and maternal antigens, but only the paternal antigens act as foreigners to the mother. Hence there is a maternal immunological tolerance (or immunological ignorance) toward the semi-allograft embryo that prevents its rejection by the mother (Mor, 2008, Seavey and Mosmann, 2008). These data confirm that firstly, the maternal immune system is not indifferent to the embryo, and secondly, by provoking different responses, the maternal immune system supports the developing foetus (Mor, 2008).

1.7 Distribution of TLRs in the female reproductive tract

Several studies have emphasized the presence of TLRs in the FRT. Previous studies performed in our laboratory by Aflatoonian and colleagues have reported the presence of TLRs 1 to 10 in human endometrium (Aflatoonian et al., 2007). These results are in agreement with data published by Young and colleagues regarding the presence of TLRs 1-6 and 9 in endometrial samples (Young et al., 2004), which is similar to the expression of TLR genes previously observed in the human endometrial carcinoma cell line HEC-1B in our laboratory (Aboussahoud et al., 2010a). TLRs 1, 2, 3, 5 and 6 are found in the epithelium of the endocervix, endometrium, uterine tubes, vagina and ectocervix. In contrast, TLR4 is expressed in the endocervix, endometrium and uterine tubes, but could not be found in the vagina or ectocervix (Fazeli et al., 2005). TLR5 has also been recognized in vascular endothelial and muscle cells in the vagina and endocervix (Nasu and Narahara, 2010). Various expressions of TLRs in both the lower and upper regions of the female reproductive tract suggests that they may be involved in immunological tolerance toward the microbial flora in the non-sterile regions of the FRT and may act as immune sensors in the sterile regions of the FRT to support the aseptic milieu (Yu et al., 2009).

1.8 Human implantation and the effect of innate immunity on its success or failure

Human endometrium is a dynamic and multi-layered structure that includes a functional layer laid over a basal layer. The functional layer is shed every month, while the basal layer is attached to the myometrium and remains untouched during menstruation. The main role of a healthy endometrium is to support implantation and pregnancy. Several different cells, alongside of blood vessels, are found in the endometrium, such as luminal and glandular epithelial cells, stromal fibroblastic cells, and cells of the innate immune system. The activity, structure and the function of these cells can change, depending on the situation, such as during pregnancy and the

different stages of the menstrual cycle. A wide variety of anti-microbial peptides can also be produced by immune-competent cells and even by endometrial epithelial cells to protect the uterus from infection (King et al., 2003, Diedrich et al., 2007).

The term *implantation* is used to describe the process of the blastocyst becoming completely embedded inside the uterine endometrium (Koot et al., 2012). In humans, this process is described as interstitially invasive, since the newly hatched blastocyst breaches the epithelium and invades the stroma so deeply that the surface epithelium become restored over it (Johnson, 2014). The first stage of human implantation is known as apposition, during which the blastocyst comes close to the endometrium in the correct orientation. It is believed that trophinin and L-selectin, members of the adhesion molecules, are mediated as weak connections between the blastocyst and endometrium in this stage. The second stage, named attachment, occurs when strong integrins connect the trophoblast and epithelial cells. The last stage is the invasion of the blastocyst into the deeper layers of the endometrium, where it is surrounded with the mother's blood circulation. Subsequent development of the placenta allows maternal support of embryonic and foetal development. The implantation process requires both blastocyst and endometrium interactions during the receptive window (Rosanna Ramhorst, 2015 , Koot et al., 2012, Johnson, 2014).

The receptivity of the endometrium and its capacity to accept the embryo during the implantation depends on characteristic changes in the endometrium, which is strongly dependent on the sex hormones. Progesterone, alongside estrogen, is essential for successful implantation, and, of course, for pregnancy. The appearance of pinopodes, changes in the expression of adhesion molecules such as integrins, loss of the negative charge of the endometrial epithelium, and thinning of the mucin layer over the epithelium are the markers of the receptive endometrium; they are under the control of sex hormones (Reichman and Rosenwaks, 2016, Aagaard-Tillery et al., 2006, Johnson, 2014).

Successful implantation is the result of the perfectly orchestrated cross-talk between an embryo and the receptive endometrium (Imbar et al., 2014). In this complicated process, several key elements, such as different proteins, cytokines and growth factors, play fundamental roles. Leukaemia inhibitory factor (LIF) is known as a

marker for receptive endometrium during a successful implantation. LIF secreted from a receptive endometrium can be recognized by two type of receptors, LIFR and gp130, that are extensively found in trophoblast cells, to induce activation of downstream signalling pathways, including Janus kinase / Signal Transducer and Activator of Transcription (JAK/STAT), MAPKs and Phosphoinositide 3-kinase / Protein kinase B (PI3K/AKT). The result is proliferation, differentiation and development of the embryo (Yue et al., 2015). MicroRNAs are recently found to be involved in the implantation process; however, their exact role in endometrial receptivity and embryo development remains unclear (Yue et al., 2015, Galliano and Pellicer, 2014). During implantation and pregnancy, a perfectly coordinated cytokine-chemokine network is necessary for recruitment of different immune cells to the site of implantation. The cytokine-chemokine network is also involved in switching from a pro-inflammatory environment during implantation to an anti-inflammatory environment during the post-implantation stage to support and maintain the pregnancy (Rosanna Ramhorst, 2015). It is well documented that the presence of any infection at the site of implantation can be recognized by both mother and embryo (Joyee and Yang, 2008, Sanchez-Lopez et al., 2014). Dysregulation of the cytokine-chemokine network as well as other strongly regulated factors (LIF, adhesion molecules, prostaglandins and mucins) at the maternal-foetal interface in response to infection can lead to implantation failure and/or negative pregnancy outcomes (Koot et al., 2012). Trophoblasts are able to express PRRs that act as a kind of sensor to recognize the surrounding environment. They can distinguish the presence of micro-organisms and damaged tissue via PRRs (Evron et al., 2011). Hence, cytokine secretion (in response to stimuli) from trophoblasts at the site of the implantation can stimulate maternal immune cells to provide a safe environment for the growing foetus (Mor, 2008). Therefore, the maternal and embryo immune systems both try to maintain the uterus micro-organism free during implantation and pregnancy. Here in this investigation, I have tried to study the endometrium-trophoblast interaction in the presence of infection to clarify the role of innate immunity during the early stage of pregnancy. Figure 1.2 is a schematic view of different stages of human implantation and the factors involved in its success or failure.

In spite of substantial developments in assisted reproductive technologies (ART), the rate of successful implantation leading to pregnancy is still quite low. Only 30% of

human conceptions lead to successful pregnancies (Diedrich et al., 2007, Johnson, 2014). Many immunological factors are found to be involved in human reproductive failure in different stages of pregnancy, from gamete development to implantation and foetal development and survival. Anti-sperm antibodies and anti-trophoblast antibodies are some examples of these immunological factors. Although the priority of each factor has not yet been clarified, it is believed that any changes in their pattern of expression or any loss of their function may lead to reproductive failure (Choudhury and Knapp, 2001). More investigations are still needed to clarify the immunological status of the FRT during implantation and successful pregnancy.

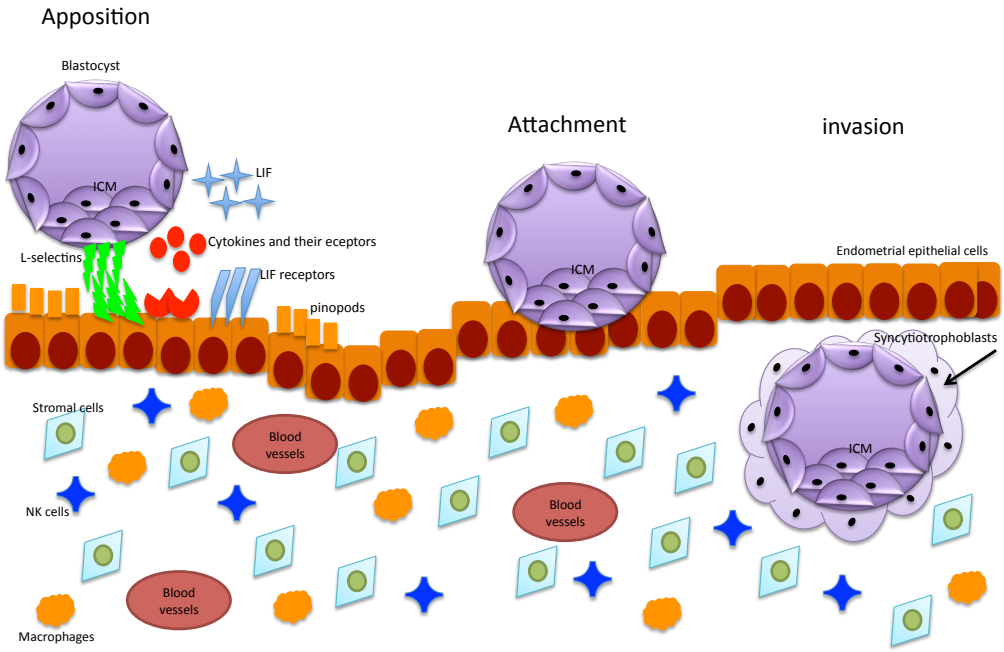


Figure 1.2, schematic view of different stages of human implantation and the factors involved in its success or failure. The term *implantation* is used to describe the process of the blastocyst becoming completely embedded inside the uterine endometrium. In humans, this process is described as interstitially invasive, since the newly hatched blastocyst breaches the epithelium and invades the stroma so deeply that the surface epithelium becomes restored over it. *Apposition* occurs when a weak attachment between blastocyst and endometrium occurs via L-selectins. During *attachment,* a stable and strong contact happens between the blastocyst and the endometrium via adhesion molecules such as integrins. Finally comes *invasion* in which the blastocyst invades into the deeper layer of the endometrium, where it is surrounded by the mother's blood vessels, decidualized stromal cells, and immune cells. Implantation is a complex and multi-factorial process that is highly dependent on several different elements such as cytokines and chemokines, LIF adhesion molecules, etc.

1.9 Foetus-maternal innate immunity interaction

In pregnant mammals, in order to keep the foetus alive, two different immunological approaches are essential. Firstly, the embryo should develop in a sterile, isolated milieu, which needs maternal-foetal innate immune support. Secondly, mutual interactions between the mother and foetus lead to successful growth of the semi-allograft embryo (Aagaard-Tillery et al., 2006). Considering the fact that Toll-like receptors, as the gatekeepers of innate immunity, are widely expressed in various cells of different tissues and organs, such as endometrial epithelial cells, in the FRT and in the trophoblast cells of the developing embryo, they are believed to be involved in the maternal-foetal innate immune interaction (Horne et al., 2008). During pregnancy, distribution of TLRs shows a different pattern in both the FRT and the embryo. For example, TLR6 is not found in the first trimester trophoblast, in contrast to the third trimester, and TLR 2 and 4 are not expressed in the syncytiotrophoblast while they are expressed in villus cytotrophoblast and extravillous trophoblast cells (Mor, 2008).

Successful pregnancy is supported by foetal-maternal immune interaction in three different stages. In the first trimester of pregnancy, pro-inflammatory markers have been up-regulated to support the implanted embryo, leading to successful placentation. Meanwhile, the expression of TLRs have shown a fluctuation in the endometrium and trophoblast (Ma et al., 2007). During the second trimester, to maintain the safety of the pregnancy, humoral immunity is dominant. Finally, in the last trimester, maternal immune responses again shift to make a pro-inflammatory environment in the cervix, amniotic fluid and uterus (Nitsche et al., 2010). During this time, TLR 4 and TLR 2 were found in the placenta, syncytiotrophoblast, and in endothelial cells (Ma et al., 2007).

It had been hypothesized once that an inflammatory milieu is necessary for blastocyst adhesion and invasion. This process is under the control of the maternal innate immunity, which is orchestrated by TLRs. It has been also confirmed that there is a relationship between TLRs polymorphism in the foetus and susceptibility to pre-term parturition (Koga et al., 2009a). There is a growing body of evidence supporting the

role of TLRs in tolerating the normal pregnancy. Further studies are still needed to prove this theory.

1.10 Toll-like receptor 9 (TLR9) and its importance in the female reproductive tract:

TLR9 is an intracellular PRR with a MyD88 dependent signalling pathway that is exclusively found in the endoplasmic reticulum (ER) under normal conditions. After stimulation, TLR9 rapidly transfers to endosomes and lysosomes to be exposed to its stimuli. It has been reported that the UNC93B1 protein in the ER membrane is involved in TLR9 trafficking from ER to endosomes (Kawai and Akira, 2010, Blasius and Beutler, 2010). At the time of stimulation, TLR9 must be cleaved by intracellular proteases. This cleaving is not absolutely necessary for ligand recognition, but rather is for downstream signalling to MyD88 (Kawai and Akira, 2010, Blasius and Beutler, 2010).

TLR9 is able to recognize unmethylated CpG, which is the most abundant motif in viral and bacterial DNA (Huang and Yang, 2010). In response to stimuli, after activation and dimerization of MyD88, IRAK1 and IRAK4 are phosphorylated and dissociated from MyD88, which can lead to activation of TRAF6. TRAF6 in turn activates TAK1. TAK1 then forms a complex with TAB1 (transforming growth factor-beta-activated protein kinase 1), 2, and 3 to maintain its activity. IKK complex (IκB kinase) is activated by TAK1. That can lead to the activation and also the dimerization of NFκB. On the other hand, the TAK1 complex can activate MAPKs (mitogen-activated protein (MAP) kinase), which then in turn activate AP1 (Adaptor protein 1). NFκB and AP1 are translocated into the nucleus to induce the secretion of type 1 IFN and inflammatory cytokines (Figure 1.2) (Akira, 2006, Blasius and Beutler, 2010, Kawai and Akira, 2006). Another possible pathway to induce type I IFN via TLR9 is IRF7 (Interleukine regulatory factor 7). IRF7 is up-regulated after TLR9 stimulation and can be activated by IRAK1 or TRAF6 after the phosphorylation and dimerization; IRF7 is translocated into the nucleus and acts as a promoter activator for IFN secretion (Akira, 2006, Blasius and Beutler, 2010, Kawai and Akira, 2006).

As described above, TLR9 is responsible for the recognition of intracellular bacterial and viral DNA containing unmethylated CpG (Bauer et al., 2001) Herpes simplex viruses (HSV), human cytomegalovirus (HCMV), adenovirus and *Chlamydia trachomatis* are the most important pathogens that can be recognised by TLR9 (Fredlund et al., 2004, Barton, 2007). *Chlamydia trachomatis* and HSV are the most important pathogens affecting women reproductively, since they can be the main causes of infertility-inducing problems such as pelvic inflammatory disease (PID), and fallopian tube and/or endometrium chronic infections (Fredlund et al., 2004, den Hartog et al., 2006). Involvement of different TLRs in the recognition of sexually transmitted diseases and other chronic infections of the FRT is shedding light on the fact that these PRRs, and particularly TLR9, are the right choices for studying the effect of innate immunity on early pregnancy (Joyee and Yang, 2008).

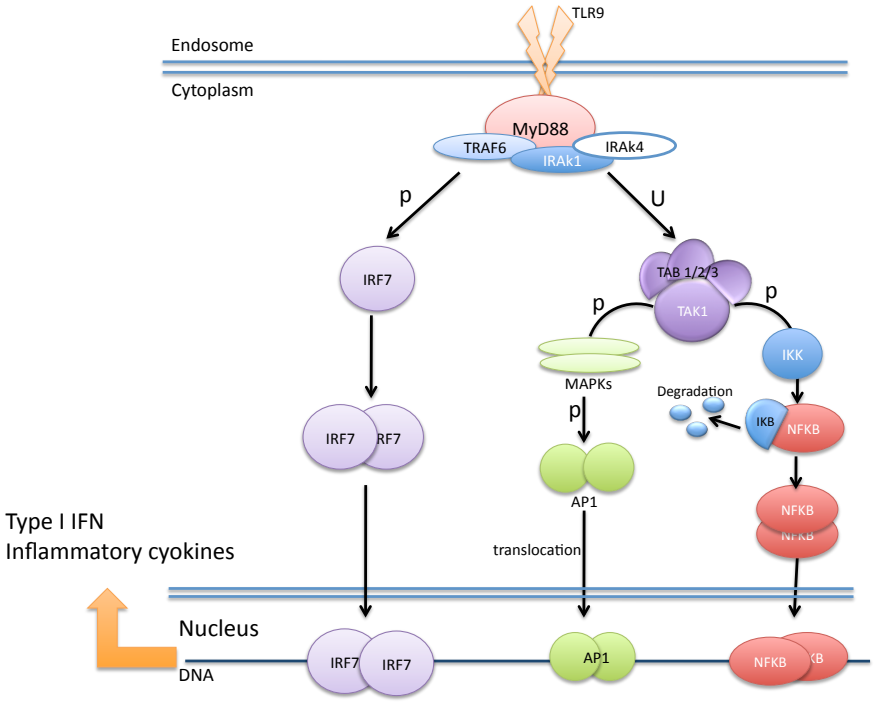


Figure 1.3 TLR9 intracellular signalling pathway. TLR9 is able to recognize unmethylated CpG, which are the most abundant motifs in viral and bacterial DNA. In response to stimuli, after activation and dimerization of MyD88, IRAK1 and IRAK4 are phosphorylated and dissociated from MyD88, which can lead to activation of TRAF6. TRAF6 in turn activates TAK1. TAK1 then forms a complex with TAB1, TAB2, and TAB3 to remain its activity. IKK complex (IκB kinase) is activated by TAK1; that can lead to the activation and also the dimerization of NFκB. On the other hand, the TAK1 complex can activate MAPKs (p38 in the TLR9 signalling pathway), which then in turn activates AP1. NFκB and AP1 are translocated into the nucleus to induce the secretion of type 1 IFN and inflammatory cytokines. Another possible pathway to induce type I IFN via TLR9 is IRF7. IRF7 is up-regulated after TLR9 stimulation and can be activated by IRAK1 or TRAF6 after the phosphorylation and dimerization. IRF7 is translocated into the nucleus and acts as a promoter activator for IFN secretion.

1.11 Studying human implantation *in vitro*

Studying human implantation is challenging, since *in vivo* studies are unethical and also impractical. Animal studies are not always reliable due to the vast physiological differences from humans (Weimar et al., 2013). To overcome these limitations, *in vitro* studies have been shown to be promising to provide consistent results. Different *in vitro* models have been designed to mimic certain aspects of *in vivo* human implantation. *In vitro* models of human implantation are generally divided into two categories. (i) The first category is 2-dimentional culture systems, which is simply a co-culture of endometrial epithelial or endometrial stromal cells with human embryos or human embryo surrogates. Both human immortalized cell lines and primary cells can be used to prepare such models. *In vitro* co-culture systems of the early stage of human implantation are primarily focused on the interaction of the endometrial epithelium with the embryo or the trophoblast cells. Hence they are used to study the apposition and attachment stages of the implantation (Weimar et al., 2013, Hannan et al., 2010, Evron et al., 2011, Montazeri et al., 2015b). (ii) The second category is 3-dimentional culture systems, which is more complicated compared to former models due to the presence of multilayers of the epithelial and stromal cells and their paracrine activity. Both human immortalized cell lines and primary cells can be used to prepare such models. 3D culture systems allow the study of both early and later stages (invasion) of embryo implantation (Wang et al., 2013, Wang et al., 2012, Buck et al., 2015, Weimar et al., 2013, Hannan et al., 2010).

In the current investigation, both 2D and 3D *in vitro* culture systems, with the help of human immortalized cell lines, were used to study early and late stages of implantation. Using immortalized cell lines has some advantages. They are easy to handle, economical, there are unlimited supplies of material for them, and most importantly, they bypass the ethical concerns. Bearing in mind that immortalized cell lines are powerful tools to study *in vitro* implantation, there are yet still some limitations such as genotypic or phenotypic changes in immortal cell lines and mycoplasma contamination of the culture (Weimar et al., 2013). Therefore, this researcher has decided to use the most common immortalized cell lines suggested in

the literature to study implantation to prepare both 2D and 3D culture systems (Hannan et al., 2010, Weimar et al., 2013).

1.12 Hypothesis and Aims:

A close interaction between mother and embryo is needed to establish a favourable environment for a successful implantation. Different factors, such as microbial infections, can affect the maternal-embryo communication and result in implantation failure. The innate immune system in the epithelium of the female reproductive tract is the first line of defence against invading pathogens. This system recognizes microorganisms via Toll-like receptors (TLRs) as pattern recognition receptors. TLRs are expressed in the endometrial epithelium during the window of implantation. They have the ability to recognize infection, suggesting that they may have an effect on embryo attachment to the endometrium.

The aim of the current investigation was to identify whether activation of TLR9 via its specific ligand (CpG) can affect the trophoblast-endometrium interaction. We hypothesized that the activation of TLR9 in endometrial epithelial cells negatively affects trophoblast attachment to the endometrium.

To validate our main hypothesis, a 2D endometrial culture system was developed and the attachment of trophoblast spheres (as the first stage of human implantation) was quantified at the time of TLR9 ligation. There were several questions, such as where does the reduction of attachment of the trophoblasts to the 2D endometrial culture system come from? How are both trophoblasts and endometrial cells involved in the reduction of attachment? To answer these questions, TLR9 receptor functionality in both endometrial epithelial cells and trophoblast spheres was eliminated, and the consequences were investigated. In addition, the TLR9 intracellular signalling pathway involved in the trophoblasts-endometrium interaction was extensively investigated. Finally, to study the human implantation in more detail *in vitro,* a 3D endometrium culture system was created in which endometrial epithelial, endometrial stromal and trophoblast cells were in close interaction. This 3D culture system,

resembling the *in vivo* environment, allowed us to closely investigate and examine the trophoblasts' outgrowth and invasion in response to TLR9 ligation.

Taken together, these observations indicated that the TLR9/CpG complex acts as a double-edged sword that can negatively and/or positively affect the interaction and behaviour of trophoblasts and endometrial cells. All in all, we have tried to extend the knowledge in this field one step closer to the discovery of a novel therapeutic method that might help increase the rate of successful implantation.

Chapter 2. TLR9 activation alters the attachment of trophoblast spheroids to endometrial epithelial cells in a 2-dimentional in-vitro cell culture system.

2.1 Introduction:

Immediately after fertilization, the zygote starts to divide whilst moving out of the fallopian tube to reach the uterus, where implantation takes place 4 to 5 days after fertilization (Johnson, 2014, Johnson, 2000b). Human implantation is defined as a successful event when all three stages of the implantation process i.e., apposition, attachment, and invasion, are fully completed. Hence, a blastocyst must be in the correct orientation toward the endometrium to guarantee that adhesion can take place. After firm attachment, the blastocyst invades the stroma to grow further and penetrate the maternal blood circulation. Simultaneously, the endometrium should reach its highest level of receptivity (the "window of implantation") to allow embryonic implantation to take place. Both embryo quality and a receptive endometrium are necessary for successful implantation. Approximately 70% of unsuccessful implantations are due to inappropriate endometrial receptivity, while the rest are due to low embryo quality (Ledee-Bataille et al., 2002). All in all, less than 30% of human conceptions lead to successful pregnancies. Therefore, in humans, failure to conceive is a frequent event (Choudhury and Knapp, 2001). Investigating the human implantation process in more detail will help increase potential new methods to increase the rate of successful pregnancies, whether by natural mating or assisted reproduction processes.

The innate immune system, as the first line of defence against invading organisms, is believed to have a vital role in providing a safe and aseptic environment for the embryo, allowing it to survive and successfully implant into the uterus wall without being recognized as a foreign invader and being rejected (Koga et al., 2009a). In this regard, the innate immune system can detect the presence of microorganisms responsible for sexually transmitted or any other infections at the site of implantation, triggering an immune response that provides a safe milieu for successful implantation (King et al., 2003, Horne et al., 2008). The innate immune system recognizes microorganisms via pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs), and NOD-like receptors (NLRs) (Akira et al., 2006).

TLRs are extensively found in different cell types, especially in immune and epithelial cells. There are ten different members in humans. They can be divided into two main groups based on their location. TLR 1, 2, 5, 6 and 10 are extracellular, whilst TLR 3, 7, 8 and 9 are intracellular (Akira et al., 2006). The structure of TLRs is composed of a leucine-rich extra-membranous domain as a specific pathogen recognition region and a cytoplasmic toll/interleukine-1 receptor (TIR) domain that is responsible for the initiation of the downstream intracellular signalling cascade. They have the ability to recognize specific components from different microbial agents. Once TLRs are stimulated by their specific ligands, an intracellular signalling pathway is initiated that leads to various inflammatory responses at the site of infection (Kawai and Akira, 2006).

TLR9, as an intracellular PPR, is largely found in endosomes or in the endoplasmic reticulum (ER) of cells. TLR9 is able to recognize short unmethylated DNA sequences containing cytosine-guanine nucleotides (CpG), which are highly abundant in both bacterial and viral genomic sequences (Akira et al., 2006, Aflatoonian and Fazeli, 2008). Although it is well documented that the TLR9 intracellular signalling pathway is cell specific and is dependent on the dose of the ligand used for its activation (Volpi et al., 2013), generally, once stimulated, TLR9 signalling leads to activation of nuclear factor-kappa B (NF-κB), activating protein 1 (AP1), and interleukin regulatory factor 7 (IRF7) through the MyD88-dependent signalling pathway (Kawai and Akira, 2006). Translocation of these transcription factors into the nucleus induces the production of a set of inflammatory cytokines (Kawai and Akira, 2006, Kawai and Akira, 2007).

Sexually transmitted infections (STI) are nowadays one of the most important worldwide health problems that can affect the natural function of the female reproductive tract and increase the risk of infertility (Joyee & Yang, 2008). Scientists are trying to clarify the role of the innate immune system, especially TLR-induced responses, in the recognition of such diseases (Fredlund et al., 2004, Barton, 2007). There is a growing body of evidence focusing on this subject. For example, it has been reported that TLR9, TLR2 and even TLR3 are involved in Herpes simplex virus type 2 (HSV-2) recognition and immunomodulation by the secretion of type I interferons that can suppress the virus' replication (Triantafilou K, *et al*. 2013., Gill,

N. *et al*. 2006). In addition, it has been found that stimulation of TLR9 and TLR3 with their specific ligands could also control HSV-1 infection by suppressing its replication in an interferon β (IFN β) dependent and independent manner (Giel R.Gaajetaan, *et al.* 2011). *Chlamydia trachomatis* infection is the most common sexually transmitted disease among women. TLR9 is reported to act as a modulator in *Chlamydia* infection, since TLR9 knockout mice showed significant protection against Chlamydia re-infection, which has not been observed in wild type mice (Ouburg et al., 2009). The mechanism of the immune response against *Chlamydia* usually starts with the recognition of the pathogen by the TLR receptors and continues with activation of transcription factors such as interferon regulatory factor 3 (IRF3), mitogen-activated protein kinases (MAPK), and nuclear factor kappa B (NF-κB), and results in the production of type I interferons (IFN), especially IFNβ, which can either pacify or suppress the viral replication (Prantner D *et al*. 2010). What is particularly interesting about the role of TLRs in STIs is that TLR stimulation can be used as a therapeutic application to produce type I interferons to suppress viral replication and soothe the infection (Gill, N. *et al*. 2006, Giel R. Gaajetaan, *et al*. 2011, Triantafilou K, *et al*. 2013). Since *Chlamydia trachomatis* infection is known to be one of the main causes of female infertility (Fredlund et al., 2004), and TLR2/6, TLR3 activation were reported to have a negative effect on the trophoblast-endometrium interaction (Montazeri et al., 2015b, Sanchez-Lopez et al., 2014), therefore, studying the role of TLR9's function in the female reproductive tract and clarifying the therapeutic application of its specific ligand (CpG) is a worthwhile endeavour.

In this chapter, the effect of TLR9 stimulation on the early stages of human implantation is investigated. For this purpose, an *in vitro* model of human implantation that had been previously developed in our lab was used. This model employed three different cell lines: a breast cancer epithelial cell line (MCF-7) simulating the receptive endometrium, an epithelial endometrial cell line called RL95-2 (Way et al., 1983) that also simulating the receptive endometrium, and a choriocarcinoma cell line (JAr cells) simulating an implanting embryo. All cell lines have been previously validated for use in implantation assays and mimic the endometrium and the trophoblast respectively (Aboussahoud et al., 2010b). The experiments reported in this chapter demonstrated that stimulation of TLR9 with CpG

decreased the percentage of JAr spheroids attaching to the endometrial cells. The suppressing effect of CpG on JAr spheroid attachment was specifically through TLR9. At least four hours was needed for CpG to induce its suppressing effect on JAr spheroid attachment to the endometrial cells. Finally, both trophobalst and epithelial cells were involved in the process of reducing trophoblast attachment to the endometrial epithelial cells in the presence of CpG.

2.2 Material and Methods:

2.2.1 Cell lines and Culture

The Michigan Cancer Foundation (MCF)-7 cells, human endometrial adenosquamous carcinoma cell line (RL95-2), and human choriocarcinoma cell line (JAr) derived from first trimester trophoblast cells were purchased from American Type Culture Collection (ATCC, Teddington, UK). RL95-2 cells were used to represent a receptive endometrium, while JArs were used to simulate the human embryo trophoblast, and finally, MCF-7 cells were used to represent epithelial cells.

RL95-2 cells were cultured at 37°C in Dulbecco-modified Eagle medium (DMEM-F12) (Sigma, Dorset, UK), supplemented with 10% Fetal Bovine Serum (FBS) (Sigma), 1% L-glutamine (Sigma), 0.005 mg/ml Insulin (human recombinant insulin, Gibco, Life technologies, Paisley, UK) and 1% Penicillin/Streptomycin (Sigma) in a 5% CO2 atmosphere until confluent. MCF-7 cells were cultured at 37°C in DMEM-F12 (Sigma), supplemented with 10% foetal bovine serum (Sigma), 1% L-glutamine (Sigma), 160 ng/ml insulin (Gibco), and 1% penicillin/streptomycin (Sigma) in a 5% CO2 atmosphere until confluent. The JAr cells were grown in RPMI (Roswell Park Memorial Institute) 1640 (Sigma) supplemented with 10% FBS (Sigma), 1% L-glutamine (Sigma), and 1% penicillin/streptomycin (Sigma) in T75 flasks in a 5% CO2 atmosphere until confluent.

At confluency, the cells were washed with Ca^{+2} and Mg^{+2} free Dulbecco's phosphate-buffered saline (PBS) (Sigma), harvested using trypsin-EDTA (Sigma), and pelleted by centrifugation at 300 g for 4 min.

2.2.2 Ligands and inhibitors

Type B CpG oligonucleotide-human TLR9 ligand (5'-tcgtcgtttgtcgttttgtcgtt-3') was used to stimulate the TLR9 receptor (Invivogen, Toulouse, France). Oligodeoxynucleotide (ODN) TTAGGG, TLR9 antagonist-inhibitory oligonucleotide (5'-ttt agg gtt agg gtt agg gtt agg g-3') was used to suppress the TLR9 receptor (Invivogen).

2.2.3. Viability test

The Live/Dead Viability/Cytotoxicity Kit for Mammalian Cells (Invitrogen, Loughborough, UK) was used to check the viability of both the RL95-2 and JAr cells in the presence of the TLR9 ligand.

2.2.4 *In vitro* human implantation assay

2.2.4.1 Formation of JAr spheroids

To generate multicellular spheroids (200-250 μm in diameter) from the JAr cell monolayer, after harvesting the cells and re-suspension of the pellets in 3 ml RPMI media (Sigma), 1 x 10^6 cells were counted with a haemocytometer and cultured in 60 x 15 mm Petri dishes (Greiner bio-one, Stonehouse, UK) with 5 ml of RPMI media (Sigma) in a humid atmosphere containing 5% CO2 at 37°C on a gyratory shaker (IKA MTS 2/4, Staufen, Germany) set at 300 rpm. The spheroids were formed after 24 hours. Approximately 3000 spheroids were obtained per Petri dish.

2.2.4.2 Formation of endometrial monolayer

RL95-2 and MCF-7 cells were both cultured in T75 flasks (Greiner Bio-One) with supplemented DMEM-F12 (Sigma), as described above, until confluent. Cells were then harvested using trypsin-EDTA (Sigma), and 2.5 x 10^5 cells were transferred to each well of a 12 well plate (Greiner Bio-One) in triplicate and incubated at 37°C for 2-3 days until confluent.

2.2.4.3 Co-incubation of JAr spheroids with endometrial monolayer

Fifty JAr spheroids were gently transferred to the upper surface of the confluent monolayer in each well of 12 well culture plates (Greiner Bio-One). They were then

co-incubated in supplemented DMEM-F12 (Sigma) at 37°C and 5% CO2. The incubation time was dependent on the experimental design.

2.2.4.4 Removal of unattached JAr spheroids and counting the attached spheroids

An automatic horizontal shaker (Labman Automation LTD) was used to detach loosely bound or unbound spheroids from the endometrial monolayer. In brief, 12-well plates (Greiner Bio-One) were placed on the horizontal shaker, which was set at 200 rpm for four minutes. The media was then discarded by inverting the plate. Each well was then filled with 1 ml of warmed PBS with Ca^{+2} and Mg^{+2} (Sigma), and the plate was set to shake again for four minutes. The plate was then inverted to discard the PBS. The final number of tightly attached spheroids was counted under the stereoscopic microscope (LEICA MZ125, Newcastle upon Tyne, UK), and the total percentage of remaining spheroids was calculated.

2.2.5 EXPERIMENTAL DESIGN;

2.2.5.1 Determination of the effect of different concentration of CpG on viability of RL95-2 and JAr cells

To determine the effect of TLR9 ligation on the viability of the cells, the RL95-2 monolayer and JAr spheroids were treated with 0 (Control), 0.001, 0.01, 0.1 and 1 μM of CpG in serum free media for 24 hours. Thereafter, 10 μM of both Calcein AM and Ethidium homodimer-1 were added to the media of the cells following the protocol provided by the kit. The viability of the cells was checked under the fluorescent microscope (Olympus CKX41, Southend-on-Sea, UK) and photos were taken with the help of a camera (Nikon DS-Fi1, Surrey, UK) connected to the microscope.

2.2.5.2 Determination of the effect of different concentrations of CpG on the JAr spheroids attachment to the endometrial epithelial cells monolayer

To determine the effect of TLR9 activation with different concentrations of CpG on trophoblast attachment to the endometrium, both RL95-2 and MCF-7 cells were grown in 12-well plates (Greiner bio-one) to 75% confluence. The media was then replaced with serum-free media containing CpG at different concentrations (0, 0.01, 0.1 and 1 μM) and incubated for 24 hours. Thereafter, the JAr spheroids were delivered to each well and co-incubated with the monolayer for 1 hour. The spheroids

were then washed and counted as described elsewhere. The experiment was repeated three times on different days.

2.2.5.3 Determination of the effect of different JAr-MCF-7 and JAr-RL95-2 cell co-incubation times on trophoblast spheroids attachment to epithelial cells in the presence of CpG

To find out if the detrimental effect of the TLR9 activation on the ability of the JAr spheroids to attach to the epithelial cells was dependent on the kinetics of the interaction between both the JAr spheroids and the MCF-7 and/or RL95-2 cells, we developed the following experiment. MCF-7 and RL95-2 cells were grown in triplicate in 12-well plates (Greiner Bio-One) as described, until there was 75% confluence. The media was replaced with serum-free media with and without CpG (0 and 0.1 μM). After 24 hours of incubation, the JAr spheroids were delivered to each well and co-incubated for 30 min, 1, 2 and 4 hours. The spheroids were then washed and counted as described earlier. The experiment was repeated three times on different days.

2.2.5.4 Determination of the effect of pre-incubation of endometrial epithelial cells with CpG on JAr spheroids attachment

To investigate the effect of the pre-treatment of endometrial epithelial cells with CpG on JAr spheroids attachment to the endometrium, both RL95-2 and MCF-7 cells were cultured as described earlier. They were then stimulated with CpG (0.01 μM for MCF-7 and 0.1μM for RL95-2 cells) or without CpG (controls) in serum free media for 0, 2, 4, 8, 24 and 48 hours. After stimulation, JAr spheroids were added to each well and co-incubated for one hour. The spheroids were then washed and counted as described earlier. The experiment was repeated three times on different days.

2.2.5.5 Determination of the effect of blocking TLR9 on JAr spheroids attachment to the endometrial epithelial cells in the presence of CpG

To find out if the observed effect of the treatment of the endometrial epithelial cells with CpG was specifically mediated through TLR9, both MCF-7 and RL95-2 cells were treated with ODN TTAGGG (Invivogen), a specific inhibitor of TLR9. The endometrial epithelial monolayers were incubated in serum-free media with either (i) 0.01μM CpG, (ii) 0.01μM CpG + 0.1 μM ODN TTAGGG or (iii) 0.1 μM ODN TTAGGG for 24 hours. Negative controls were done by omitting both ODN

TTAAGGG and CpG in the culture. JAr spheroids were added to each well and co-incubated for 30 minutes. The JAr spheroids were then washed and counted as described earlier. The experiment was repeated three times on different days.

2.2.5.6 Determination of whether CpG stimulated JAr cells are also involved in the process of the reduction of attached spheroids to the epithelial cells

This experiment was designed to find out whether the CpG interference was due to the effects on the JAr spheroids, or the epithelial cells, or both. For this purpose, MCF-7 monolayers and/or JAr spheroids were pre-treated with CpG (0.01 μM) for 24 hours and 1 hour respectively, alone or simultaneously together. Controls were done by omitting CpG and adding the same volume of water. The CpG had been washed away from both cells before one hour of co-incubation by rinsing the cells twice with fresh media. The JAr spheroids were then washed and counted as described earlier. The experiment was repeated three times on different days.

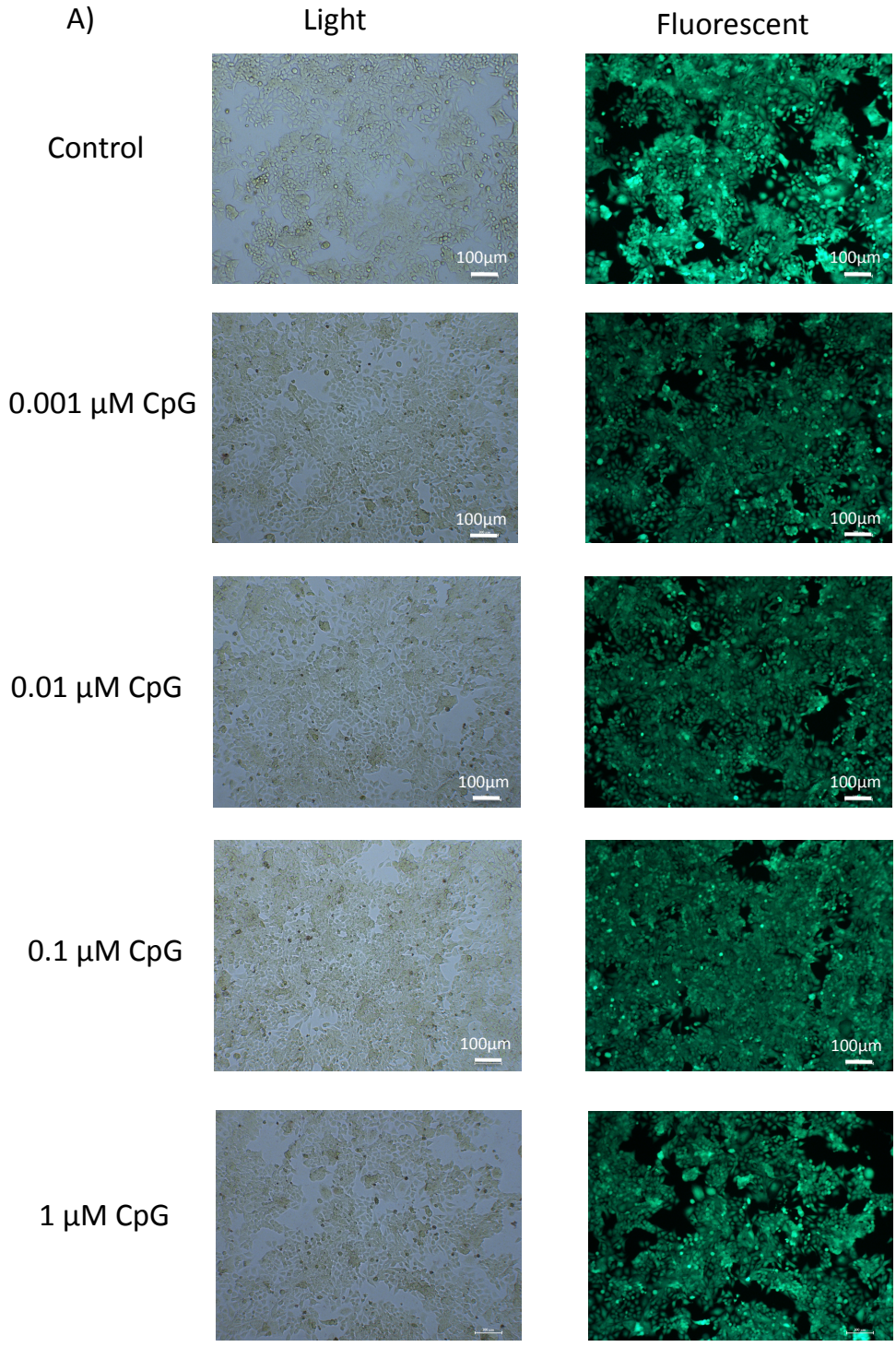
2.2.6 Statistical analysis

The results were expressed as mean ± S.E.M. of the percentage of JAr spheres bound to the endometrial epithelial monolayer. A statistical analysis was performed using ANOVA with Fischer's multiple comparison test. $P<0.05$ was considered to be significant.

2.3 Results:

2.3.1 The presence of CpG did not affect the viability of RL95-2 and JAr spheroids

Treatment of both the RL95-2 and the JAr spheroids with the different concentration of CpG did not affect the viability of the cells (Figure 2.1, A and B).


A)
Light
Fluorescent
Control
0.001 µM CpG
0.01 µM CpG
0.1 µM CpG
1 µM CpG
100µm

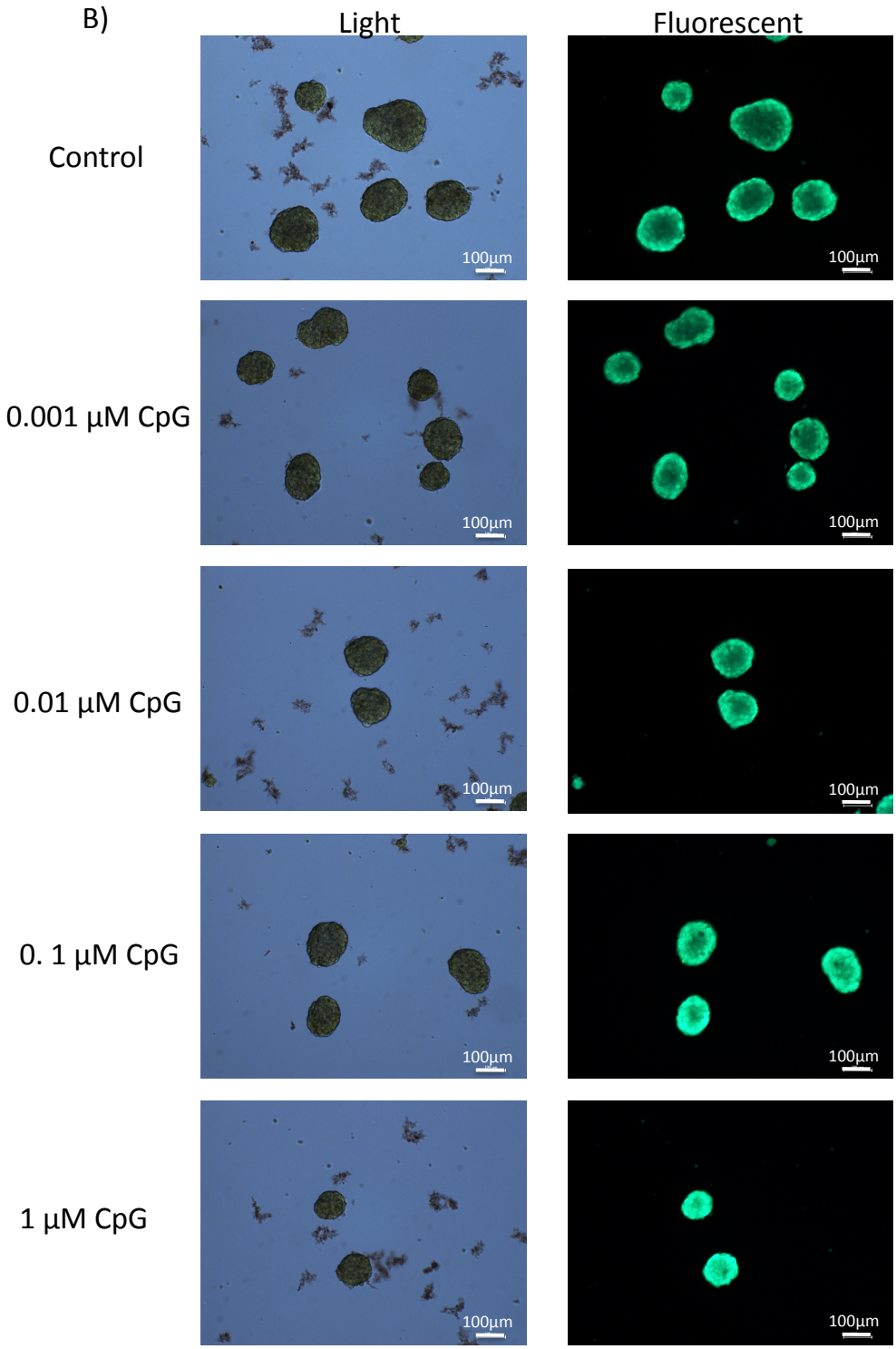


Figure 2.1 Determination of the effect of different concentrations of CpG on the viability of RL95-2 and JAr cells. To determine the effect of TLR9 ligation on the viability of the cells, the RL95-2 monolayer (A) and JAr spheroids (B) were treated with 0 (Control), 0.001, 0.01, 0.1 and 1 µM of CpG in serum free media for 24 hours. Thereafter, 10 µM of both Calcein AM and Ethidium homodimer-1 were added to the media of the cells. Viability of the cells was checked under the fluorescent microscope. Treatment of both RL95-2 and JAr spheroids with the different concentrations of CpG did not affect the viability of the cells.

2.3.2 The presence of CpG decreased JAr spheroid attachment to the endometrial cells in a dose-dependent manner in the 2D culture system

Treatment of both the RL95-2 and MCF-7 cells with different concentrations of CpG significantly decreased the number of JAr spheroids attached to the endometrial epithelial cells ($p<0.05$). The inhibitory effect of CpG on the JAr spheroids binding to the endometrial monolayer was dose-dependent; with higher concentrations of CpG, a lower percentage of attaching spheroids was seen (Figure 2.2A and 2.2B).

2.3.3 Attachment of JAr spheroids to epithelial cells was dependent on the JAr-MCF-7/RL95-2 co-incubation time in the presence of CpG

Different co-incubation times for the JAr-MCF-7 and JAr-RL95-2 cell lines used in our experiments had a significant effect on the number of spheres binding to the epithelial cells ($p<0.003$). The addition of 0.1 μM of CpG decreased the percentage of attached spheroids at 0.5, 1 and 4 hours co-incubation times for the JAr-MCF-7 cells, but surprisingly, this effect was not observed at two hours of co-incubation (Figure 2.3 A). Furthermore, the addition of 0.1 μM of CpG significantly decreased the percentage of attached spheroids at 0.5, 1, 2 and 4 hours co-incubation times of the JAR-RL95-2 cell lines (Figure 2.3 B).

2.3.4 Pre-treatment of endometrial epithelial cells with CpG longer than 2 hours was necessary to decrease the percentage of JAr spheroids binding to endometrial cells

A minimum of four hours of pre-treatment of both MCF-7 and RL95-2 cells with 0.01μM and 0.1μM of CpG respectively was necessary to produce a significant effect on the capacity of the JAr spheroids to bind to the endometrial epithelial cells (Fig 2.4 A and 2.4 B) ($p<0.05$). No significant alterations were observed in the inhibitory effect of CpG when the monolayers were pre-incubated up to 48 hours with ligand.

2.3.5 Blocking the TLR9 function restored the attachment of JAr spheroids to endometrial epithelial monolayer

Pre-incubation of both MCF-7 and RL95-2 cells with a functional TLR9 inhibitor restored the binding ability of the JAr spheroids to the endometrial epithelial cells in the presence of CpG. Furthermore, endometrial epithelial monolayers solely pre-treated with TLR9 inhibitor showed no significant alteration in the number of attached JAr spheroids compared to the untreated controls (Figures 2.5 A and 2.5 B).

2.3.6 Both JAr and epithelial cells were involved in the process of the reduction of attachment of the spheroids to the epithelial cells

Pre-treatment of the MCF-7 cells and JAr spheroids with CpG alone or simultaneously together had a significant effect on the number of spheroids binding to the endometrial cells ($p<0.0001$). Both the JAr and MCF-7 cells seemed to be involved in the reduction of spheroid attachment to the epithelial cells in the presence of CpG (Fig 2.6).

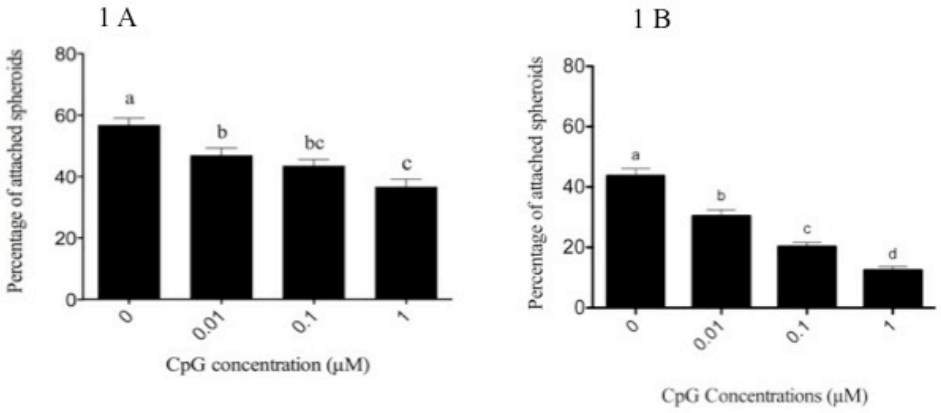


Figure 2.2 The effect of different CpG concentrations on the percentage of attached spheroids to endometrial epithelial cells. MCF-7 cells (A) and RL95-2 cells (B) pre-treated with CpG for 24 hours reduced the percentage of JAr spheroids attached to the both endometrial and epithelial cells significantly. The data are the average of three independent experiments (n=9). The results are presented as the mean ± S.E.M. ANOVA was used to compare the percentage of the attached spheroids at each CpG concentration. Different letters indicate significant statistical differences. $p<0.05$ was considered to be significant.

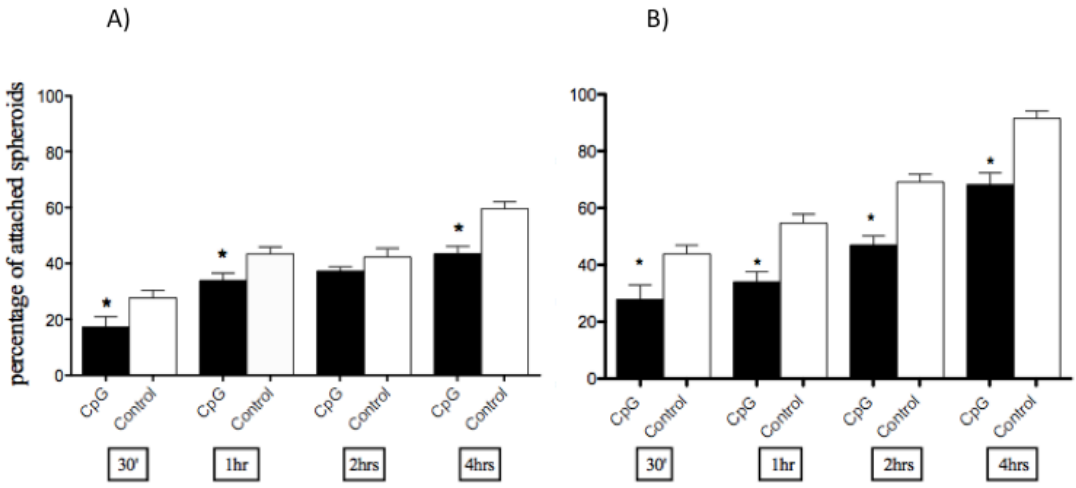


Figure 2.3 Kinetics of JAr spheroids adhesion to the epithelial cells. A) MCF-7 cells and B) RL95-2 cells were pre-treated or not (control group) with 0.1μM CpG for 24 hours. JAr spheres were added to the epithelial endometrial monolayers and co-incubated for 4 different time points (30 min, 1 hr, 2 hrs and 4 hrs). The data are the average of three independent experiments (n=9). The results are presented as the mean ± S.E.M. ANOVA was used to analyse the results. Control= no CpG added. * indicates the significant difference compare to control. $p<0.05$ was considered to be significant.

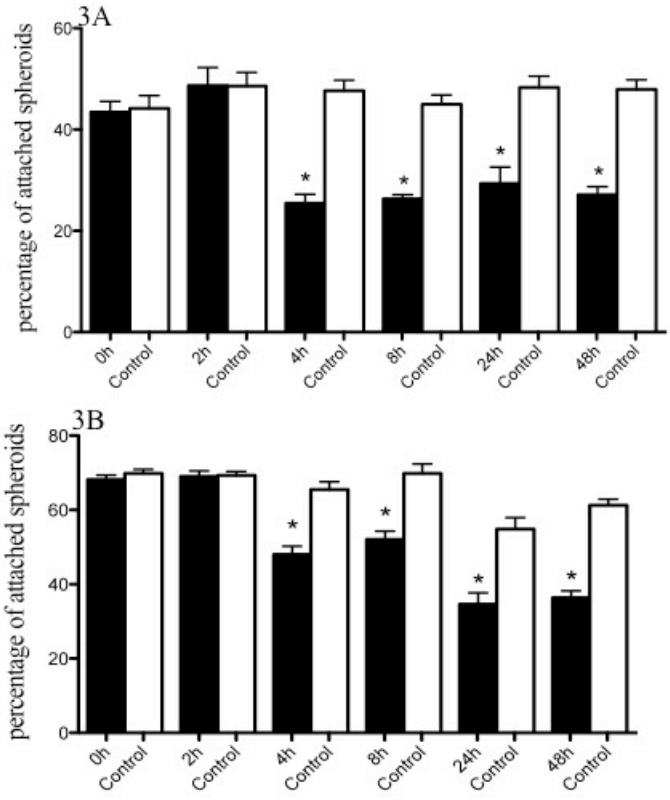


Figure 2.4 The effect of different pre-incubation times of endometrial epithelial cells with CpG on JAr spheroid attachment. (A) The MCF-7s were pre-treated with 0.01μM of CpG for 0 hr, 2 hrs, 4 hrs, 8 hrs, 24 hrs and 48 hrs. (B) The RL95-2 cells were pre-treated with 0.1μM of CpG for 0 hr, 2 hrs, 4 hrs, 8 hrs, 24 hrs and 48 hrs. JAr spheroids were then added to the endometrial cells and co-incubated for 1 hour. There was no significant reduction in the percentage of attached spheres after pre-incubation of endometrial cells with CpG for 0 and 2 hours compared to the control group. The data of each graph are the average of three independent experiments (n=9). The results are presented as the mean ± S.E.M. ANOVA was used to analyse the results. Control= no CpG added. 0h= CpG and JAr spheroids were added to endometrial cells simultaneously. * indicates the significant difference compare to control. $p<0.05$ was considered to be significant.

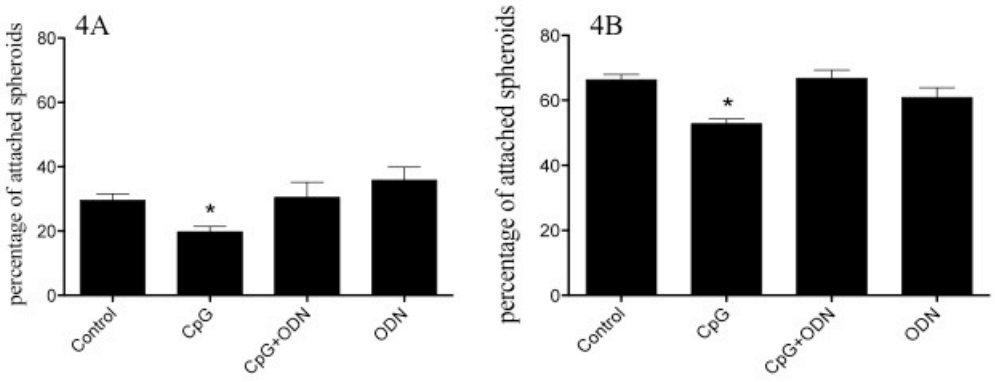


Figure 2.5 The effect of blocking TLR9 in MCF-7s and RL95-2 cells on the percentage of JAr spheroids binding to endometrial cells. (A) MCF-7s and (B) RL95-2 cells were pre-treated with either 0.01μM CpG, 0.01μM CpG + 0.1μM ODN TTAAGGG, or 0.1μM ODN TTAAGGG alone. No CpG was added to the control group. The addition of ODN TTAAGG significantly restored the ability of JAr spheres to bind to the endometrial cells. The data are the average of three independent experiments (n=9). The results are presented as the mean ± S.E.M. ANOVA was used to analyse the results. Control= untreated cells. * indicates significant differences of the CpG treated group compared to other treatments (CpG+ODN and ODN) and to control. $P<0.05$ was considered to be significant.

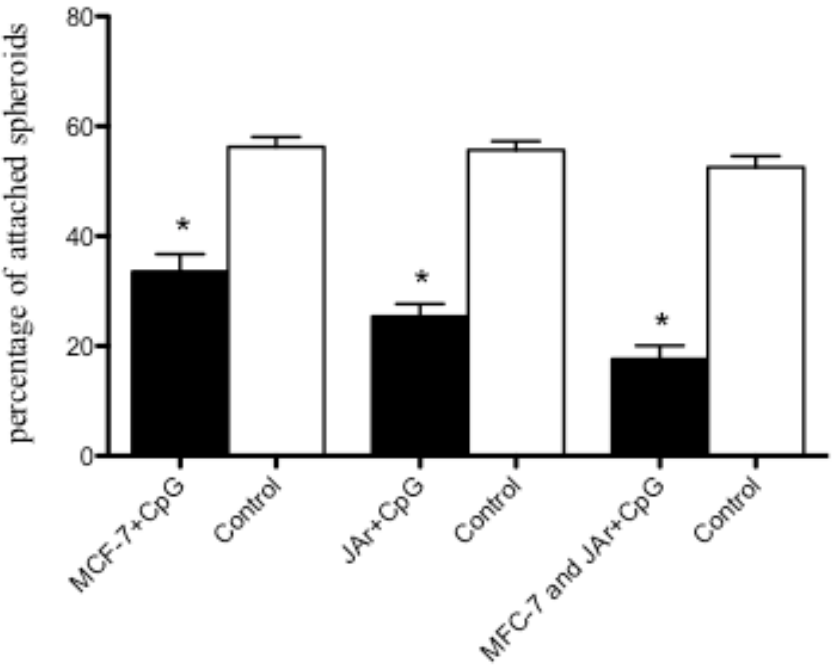


Figure 2.6 Both JArs and MCF-7s are involved in the process of reduction of attachment of spheroids to endometrial cells. Pre-treatment of MCF-7 cells and JAr spheroids with 0.01μM of CpG for 24 hours and 1 hour respectively, alone and together, significantly decreased the percentage of attached spheroids compared to the control (untreated) group. The results indicate that both cell lines are involved in the process of reducing the attachment of the spheroids to the epithelial cells. Data are the average of three independent experiments (n=9). Results are presented as the mean ± S.E.M. ANOVA was used to analyse the results. Control= Untreated cells. * indicates significant differences compared to its control. $P<0.05$ was considered to be significant.

2.4 Discussion

Investigations of the molecular interactions between mother and embryo at the site of implantation cannot be performed *in vivo* in humans due to ethical issues. Therefore, there is always a need for suitable *in vitro* models to allow researchers to study human implantation. Such limitations has been covered by the use of different endometrial and trophoblast cell lines (Hannan et al., 2010). In our study, MCF-7s, a breast cancer epithelial cell line, RL95-2, a human endometrial carcinoma cell line, and cellular spheroids made from a choriocarcinoma trophoblast cell line (JAr), were selected to evaluate the effect of TLR9 activation on the early stage of human implantation *in vitro*. These cell lines have previously been tested as suitable models to study human implantation (Hombach-Klonisch et al., 2005, Aboussahoud et al., 2010b, Hannan et al., 2010, Way et al., 1983).

The results demonstrated that stimulation of TLR9 in both the MCF-7 and RL95-2 cells by CpG significantly decreased the percentage of JAr spheroids attaching to the endometrial cells in a dose-dependent manner. This is a strong indication that TLR9 activation during the periconception period can lead to implantation failure. It is also well documented that stimulation of other TLRs, such as TLR5, TLR3 and TLR2/6, in human endometrial epithelial cells leads to failure of attachment of JAr spheroids to the endometrial culture (Aboussahoud et al., 2010b, Montazeri et al., 2015a, Sanchez-Lopez et al., 2014). TLR2/6 and TLR3 ligation has also been reported to reduce the implantation and uterine vascular remodelling *in vivo* respectively (Zhang J, 2007, Aboussahoud et al., 2010b)Sanchez-Lopez *et al.* 2014). Furthermore, it has been reported that stimulation of TLRs not only in endometrial cells but also in first trimester trophoblast cells can induce different immune responses such as inflammation and apoptosis (Koga and Mor, 2008). In our study, as the CpG remained in the co-culture of endometrial monolayers with JAr spheroids for one hour, it is probable that the inhibitory effect on binding originated from either the trophoblast, the endometrial cells, or a combination of both cell types.

The percentage of JAr spheres adhering to the epithelial cells was dependent on the trophoblast-epithelial co-incubation time. These findings were very similar to those

observed in previous studies (Aboussahoud et al., 2010b, John et al., 1993). There is a time dependent increase in the number of attached spheres, reaching from 30% to nearly 60%, after 30 minutes and after 4 hours of co-incubation, respectively. John *et al.* showed that attachment of JAr spheroids to different endometrial cell lines is time-dependent, reaching nearly 100% adhesion after 24 hours (John et al., 1993). The results are also in agreement with those observed by Montazeri *et al.* and Sanchez-Lopez *et al.,* where the adhesion of the JAr spheroids to the RL95-2 cells increased in a time-dependent manner, allowing approximately 70% of the spheres to attach to epithelial cells after one hour of co-incubation (Sanchez-Lopez et al., 2014, Aboussahoud et al., 2010b, Montazeri et al., 2015b). In the current experiments, one hour co-incubation of the cells was also enough for 50% of the JAr spheroids to attach to the MCF-7 cells and approximately 65-70% of the Jar spheroids in the 2D culture containing RL95-2 cells.

Stimulation of TLR9 in both MCF-7 and RL95-2 cells decreased the percentage of spheroids attaching as soon as four hours after the addition of CpG to the epithelial monolayer. Increasing the pre-treatment times of the epithelial endometrial cells with CpG up to 48 hours did not produce any significant increase in the inhibitory effect of TLR9 stimulation over trophoblast attachment. It has been reported that CpG needs only ten minutes to associate with TLR9; approximately another two hours is probably needed for endosomes containing TLR9/CpG to increase in size and recruit downstream mediators to initiate the required signalling pathway (Takeshita et al., 2004). Moreover, when mice spleen cells where stimulated with CpG, there was a time-dependent increase in the production of IL-6 and IL-12 already after four hours, reaching peak production after 12 hours of CpG treatment (Klinman et al., 1996). These observations indicated that CpG could stimulate TLR9 in a relatively quick manner, triggering downstream signalling that results in the transcription of a selected set of genes and protein translation in less than four hours. In our experiments, we have also seen that at least four hours was needed for CpG to activate TLR9 and its downstream signalling pathway that negatively affected the JAr spheroid attachment to the endometrial cells. Whether MCF-7 or/and RL95-2 cells present similar quick kinetics for cytokine production when encountered with trophoblast cells is still a matter for future investigations.

It is clear that any alterations in molecules related to implantation, such as cytokines, hormones, or adhesion molecules, may have a negative effect on implantation (Simon et al., 1997, Makrigiannakis et al., 2006, Valles and Dominguez, 2006, Singh and Aplin, 2009). Furthermore, a finely regulated endometrial cytokine balance is required for implantation (Valles and Dominguez, 2006). Hence any local or systemic dysregulation of the cytokine presence at the site of implantation may lead to inadequate uterine receptivity (Kwak-Kim et al., 2010). We speculate that activation of TLR9 may lead to an early inflammatory response that could affect the balance of cytokines and chemokines required to provide proper endometrial receptivity (Abrahams et al., 2005). Broadly speaking, cytokines and chemokines that are released at the site of implantation might have a direct or indirect impact on the implantation process. Triggering the intracellular signalling in a very short time after TLR9 activation may lead to the alteration of any of these molecules, which may result to a delay or inhibition of trophoblast cells binding to endometrial cells in our 2D *in vitro* cell culture system. Obviously, further studies are needed to prove the role of the different cytokines and chemokines secretion during the implantation process and their involvement in the TLR-mediated reduction of trophoblast cells binding to endometrial cells.

Blocking TLR9 by the pre-treatment of epithelial cells with the ODN TTAGGG was sufficient to restore the number of JAr spheroids attaching to the endometrial cells to the levels when CpG was not present in the culture system. This confirmed the role of CpGs in suppressing JAr attachment specifically through TLR9 signalling. TLR9 detects the CpG ODN (unmethylated CpG oligodeoxynucleotides) as a specific ligand, which are excessively found in bacterial and viral genomic DNA but are rarely found in mammalian DNA (Krieg, 2007). Since the original DNA with a phosphodiester sugar backbone is sensitive to nucleases, to date researchers have been using synthetic CpGs with phosphorothionate sugar, which makes them resistant to nucleases (Hemmi et al., 2003, Krieg, 2007). It has also been established that gouanine (G)-rich ODN with a phosphorothionate sugar backbone is able to inhibit CpG ODN activity. Although it is believed that G-rich ODN competes with CpG in uptake and reaches the TLR9 receptor, experiments have shown that G-rich ODN were still able to halt CpG activity when it was added to the system four hours later than the CpG (Heeg et al., 2008). These findings confirm that G-rich ODN is a strong

antagonist for the TLR9 receptor. In our experiments, TLR9 antagonist was added to the endometrial cells 30 minutes before CpG, which was enough to block the CpG/TLR9 activity and restore the number of attached JAr spheroids to the endometrial monolayer.

During the past few years, TLRs agonists have been under development as possible therapeutic vaccine adjuvants to provoke optimum inflammatory responses with less toxicity to target the innate immune system in cases of infectious diseases and cancer. As an example, different pharmaceutical companies are developing TLR9 agonist-based drugs for the treatment of hepatitis C and metastatic colorectal cancer (Kanzler et al., 2007). Pregnancy outcomes can be affected by intrauterine infection and inflammation. TLR9 agonist (CpG) in low doses rapidly induced foetal resorption and preterm birth in IL-10 knockout mice, while CpG in higher doses induced malformation in C57BL/6 offspring (Thaxton et al., 2009, Prater et al., 2006). CpG has also been used as an adjuvant to modulate intrauterine inflammation induced by *Lysteria Monocytogenes* in pregnant mice in a positive manner (Ito et al., 2004). These findings are evidence indicating that, depending on different conditions, CpG is capable of initiating a TLR9-induced immune response that may positively or negatively affect pregnancy outcomes. But can this knowledge be translated to the treatment of infertility cases and embryonic implantation failure in humans and livestock? More investigation is needed to clarify the details about the role of TLRs and their intracellular signalling pathways in human implantation before it can direct us to a cure for implantation failure.

Pre-treatment of the JAr spheroids with CpG for one hour resulted in a significant reduction in the percentage of the attached spheroids to the endometrial epithelial monolayer. These results confirmed that trophoblast cells as well as endometrial cells are involved in the reduction of the attachment of trophoblast cells to epithelial cells via the TLR9 pathway stimulation in these cells. It has been reported that all TLRs and their associated molecules are found in the JAr cell line. Hence JAr cells, in principal, should be able to dynamically respond to different types of infections similar to those of the human placenta. Stimulation of the JAr cells with CpG leads to activation of a downstream signalling pathway through MAPKs (p38 phosphorylation) (Klaffenbach et al., 2005). We bear that in mind, since in all of our

experiments, remnants of CpG may have been present in the culture system at the time of co-incubation. Hence trophoblast cells may have also responded to the stimuli, resulting in failure to attach to the endometrium. The detrimental effect of CpG on the attachment of trophoblast cells to the endometrial cells seems to originate from both of the cell lines involved in the *in vitro* culture system.

To sum up, we have demonstrated that TLR9 stimulation by CpG leads to decreased JAr spheroid attachment to the endometrial epithelial cells *in vitro*. This detrimental effect of CpG on trophoblast attachment was completely TLR9-specific, since TLR9 antagonist (G-rich ODN) was able to suppress the negative effect of CpG. This data corroborates previous studies, which highlighted the importance of the innate immunity, and specifically of TLRs, in the mediation of implantation failure.

Chapter 3. Both endometrial epithelial and trophoblast cells respond to TLR9 ligation in a two-dimensional culture system

3.1 Introduction

Assisted reproductive technologies (ART) are used to help infertile couples conceive healthy offspring. *In vitro* fertilization (IVF) is the most common technology used by experts to help patients who want to enjoy the experience of parenthood. IVF is now used as a treatment for various female fertility problems, such as endometriosis and fallopian tube damage, and also for male factor reproductive problems or when the couple's infertility is unexplained (Min JK, 2006).

Gene modification (via gene-targeting technologies) is a technique used to study biological systems at the molecular level. Recent studies have focused on gene-targeting techniques to investigate the *in vivo* functions and phenotype characteristics of each individual gene involved in fertility. For example, by using these technologies, 83 different genes were discovered to be involved in female mice fertility (Naz and Rajesh, 2005, Santiago et al., 2008). Although not all of this data is comparable to human studies due to physiological differences between species, these findings indicate the importance of every single gene involved in fertility, and may help us to understand the process of pregnancy in better detail. Both molecular techniques and ART are the strategies used to broaden our knowledge of reproductive mechanisms, to find an answer to unexplained infertility, and to increase the chances of pregnancy.

Genome modification with the purpose of complete gene deletion, known as "gene knockout", is a common method to study the possible phenotype and other changes that the absence of a particular gene could cause. In recent years, this technique has been widely used in generating null (knockout) mice that are genetically designed to lack a particular single gene (Galli-Taliadoros et al., 1995, Sun et al., 2008). Rather than the complete deletion of a single gene, such as that which takes place in the knockout method, another method, called the gene-knockdown technique, is used to reduce the expression of one or more genes at the same time. Hence the gene of interest is still available, yet the level of its expression has been decreased due to degradation of the messenger RNA (mRNA) (Turner and Morris, 2010). Currently,

gene knockdown using RNA interference is one of the most reliable methods of gene-targeting technologies used by researchers (Kiger et al., 2003).

The concept of RNA interference (RNAi) was first disclosed in detail as a gene silencing strategy by Fire *et al.* at Johns Hopkins University, followed by Baulcombe's research group in the UK (reviewed in (Bagasra and Prilliman, 2004). Among eukaryotes, RNA-based gene silencing (RBGS) is a kind of molecular defence mechanism against retroviruses, transposons, retrotransposons and RNA-viruses (Bagasra and Prilliman, 2004). RBGS technology has become very common in cell culture and other *in vivo* studies for discovering the function of particular genes or proteins, and numerous investigations based on this method have been carried out. In this approach, double stranded RNA (dsRNA), siRNA, or short hairpin RNA (shRNA) is introduced into the cells by micro-injection or electroporation as a physical delivery method, or by lipid- or peptide-mediated gene delivery as a chemical method (Schellander et al., 2007, Stanislawska and Olszewski, 2005). RBGS can either be defined as post-transcriptional gene silencing (PTGS) or as transcriptional gene silencing (TGS). TGS directly affects the level of DNA and lasts for longer periods, similar to non-coding RNAs that regulate the genes involved in chromatin modifications, such as histone methylation and/or DNA methylation (Turner and Morris, 2010). In contrast to TGS, the PTGS mechanism transiently affects the mRNA of the gene and depends on the presence of the siRNA molecule in the system (Turner and Morris, 2010).

dsRNAs and/or shRNAs, which are homologous to the sequence of the gene of interest, can either be intracellularly synthesized or exogenously induced into the cells. They are first recognized and then cleaved by a dimeric enzyme from the Rnase III ribonuclease family called Dicer to form siRNAs. Thereafter, RNA-induced silencing complex (RISC), an ATP dependent RNA helicase with an Argonaute protein core, uses the antisense strand of siRNA to find and destroy the mRNA intended to be knocked-down. This cycle of cleavage can be continued as long as the siRNA molecule is induced into the cell, and as a result, the gene of interest would be constantly disrupted (Stanislawska and Olszewski, 2005, Kobayashi and Tomari, 2015, Lam et al., 2015). Figure 3.1 depicts the PTGS mechanism in mammals.

The RNAi technique for gene-silencing purposes is a powerful tool in *in vitro* studies. It has been reported that nearly 100 genes have been knocked-down in cell culture systems using dsRNA transfection. Applying this method has some outstanding beneficiary outcomes (Kiger et al., 2003); RNAi technology has a potential role in fighting against different diseases by destroying the mRNA of the defective proteins causing the illness (Wittrup and Lieberman, 2015). Hence RNAi may be utilized as a therapeutic approach in cancers and different diseases such as Haemophilia A and B and Hepatitis C virus (HCV) infection (Stanislawska and Olszewski, 2005, Wittrup and Lieberman, 2015, Turner and Morris, 2010).

In the last chapter, it was demonstrated that CpG stimulation of endometrial epithelial cells in our 2D culture system led to a decrease of JAr spheroid attachment to endometrial epithelial cells *in vitro*. Blocking TLR9 in endometrial epithelial cells suppressed the negative effect of CpG on JAr spheroid attachment, which confirmed that the CpG effect on Jar-endometrial interaction is via TLR9. In the current investigation, we hypothesized that both trophoblast spheroids and endometrial epithelial cells are involved in reducing JAr attachment to endometrial cells when CpG is present in the *in vitro* culture system. The siRNA technique was used to stably suppress the TLR9 function in endometrial epithelial cells. The results demonstrated a significant reduction of JAr spheroid attachment to the TLR9 knocked-down endometrial epithelial cells in the presence of CPG in the i*n vitro* culture system. It seems JAr spheroids also respond to CpG, which negatively affect their ability to bind to endometrial epithelial cells.

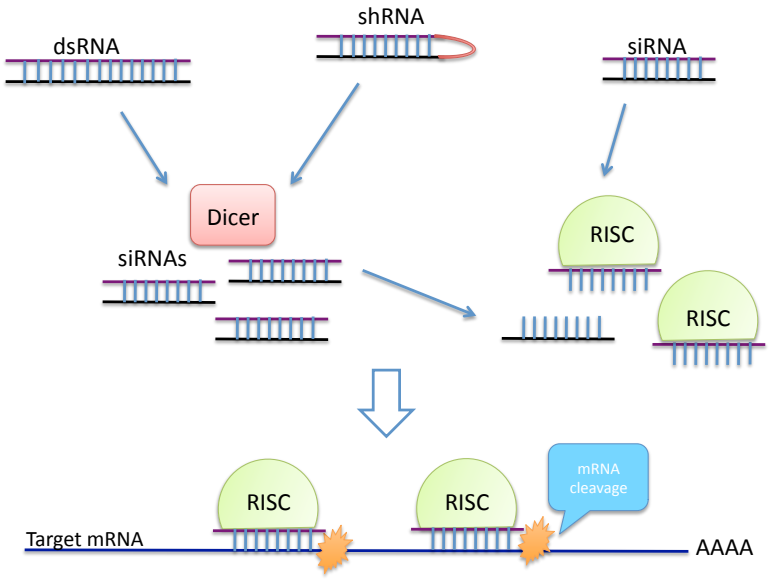


Figure 3.1 RNAi mechanism in mammals. dsRNA: double-stranded RNA, shRNA: short hairpin RNA, siRNA: small interfering RNA, RISC: RNA induced silencing complex, Dicer: endoribonuclease in RNase III. dsRNAs and/or shRNAs, which are homologous to the sequence of the gene of interest, can either be intracellularly synthesized or exogenously induced into the cells. They are first recognized and then cleaved by a dimeric enzyme from the Rnase III ribonuclease family called Dicer to form siRNAs. RNA-induced silencing complex (RISC), an ATP dependent RNA helicase with an Argonaute protein core, uses the antisense strand of siRNA to find and destroy the mRNA intended to be knocked-down. The cycle of cleavage can be continued as long as the siRNA molecule is induced into the cell, and as a result, the gene of interest would be constantly disrupted.

3.2 Material and Methods

3.2.1 Cell lines and Culture

The human endometrial adenosquamous carcinoma cell line RL95-2 and the human choriocarcinoma cell line JAr derived from first trimester trophoblast cells were purchased from American Type Culture Collection (ATCC, Teddington, UK). RL95-2 cells were used to represent a receptive endometrium, while JArs were used to simulate the human embryo trophoblast.

The process of culturing and maintaining the cells has been explained in detail in chapter 2 (2.2.1).

3.2.2 Ligand and inhibitor

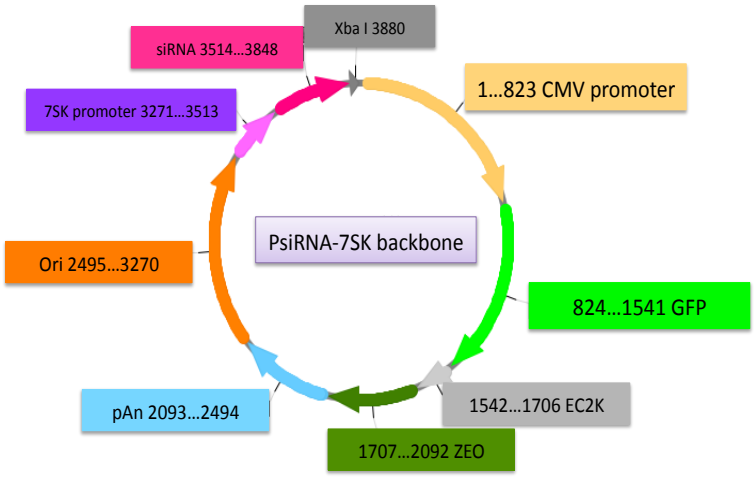
The type B CpG oligonucleotide-human TLR9 ligand (5'-tcgtcgtttgtcgttttgtcgtt-3') was used to stimulate the TLR9 receptor (Invivogen, Toulouse, France). The oligodeoxynuvleotide (ODN) TTAGGG TLR9 antagonist-inhibitory oligonucleotide (5'-ttt agg gtt agg gtt agg gtt agg g-3') was used to suppress the TLR9 receptor (Invivogen).

3.2.3 Creation of stable TLR9 knocked-down RL95 endometrial epithelial cells:

The psiRNA-hTLR9 and psiRNA-lucGL3 (control) plasmids (Figure 3.2) (InvivoGen, Toulouse, France) were resuspended in 20 μl of sterile water. Plasmids were transformed into the E. coli GT116 strain provided with the PsiRNA kit following the protocol of the manufacturer (PsiRNA KIT, InvivoGen). Briefly, the GT116 container was placed in ice for five minutes, a volume of 1ml of cold reconstitutive solution was added to the bacteria, and the mix was then incubated in ice for another five minutes. The bacterial solution was gently homogenized to allow the bacteria to rehydrate for 25 minutes in ice. 1 μg of each plasmid was separately added to the 100 μl of the rehydrated bacteria, mixed, and placed in ice for 30 minutes. The bacterial solution was incubated in a 42°C water bath for 30 seconds and replaced in ice for another 2 minutes. The bacterial solution were then added to the 900 μl of LB (Lyzogeny broth) medium incubated at 37°C for two hours, with shaking at 250 rpm. The transformation reaction was then spread onto an agar plate

prepared with the fast-media Zeo provided with the kit. On the next day, one colony of each transformed bacteria containing siTLR9 or the control plasmid were selected for further actions. In order to increase the quantity of plasmid DNA, transformed bacteria from the colony were cultured in 100 ml Fast-Media Zeo for 24 hours on a gyratory surface (≈300 rpm) at 37°C. Plasmids were then extracted using a plasmid DNA purification kit (Plasmid plus midi kit, QIAGEN, Manchester, UK) based on the manufacturer's protocol. The final concentration of each plasmid was measured using a nanophotometer (GENFLOW, Lichfield, UK). Both the control and siTLR9 plasmids were digested by XbaI restriction enzyme (Biolabs New England, Ipswich, MA, USA). This allowed linearization and random insertion of vectors into the genome of the cells for the purpose of creating stable transfected cell lines. RL95-2 cells were cultured in 12 well plate culture dishes (Greiner bio-one) till 70% confluency. Cells were then transfected by 500 ng/ml of linearized control or by the siRNA-hTLR9 plasmid in separate wells. 48 hours post transfection, confluent RL95-2 cells were trypsinized and transferred into 100x20 mm tissue culture dishes (Greiner bio-one) with 8ml DMED-F12 media (Sigma) containing 500μg/ml of Zeocin antibiotic (InvivoGen) as a selective marker to obtain RL95-2 cell colonies that carried the linearized plasmids. Colony selection was performed using cloning cylinders (Sigma). Briefly, colonies of transfected cells were placed in the centre of cylinders, trypsinized, harvested, and transferred into T25 cell culture flasks (Greiner bio-one) to grow. Transfected RL95-2 cells were also visible under the fluorescent microscope, since both the psiRNA-hTLR9 and the control plasmids contained the green fluorescent protein (GFP) coding sequence. Stable Toll-like receptor 9 knocked down RL95-2 cells (TLR9KD-RL95-2) and control plasmid transfected RL95-2 cells (CP-RL95-2) were kept in liquid nitrogen or in the culture for further experiments.

A)



B)

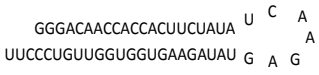


Figure 3.2 siRNA Plasmid and siTLR9 structure. A) Schematic view of the siRNA plasmid backbone used to create the stably knocked-down TLR9 endometrial epithelial cell line. CMV promoter: a promoter obtained from cytomegalovirus for efficient expression of GFP. GFP: Green Fluorescent Protein, which exhibits bright green fluorescence when exposed to blue light. EC2K: efficient promoter for Zeo. Zeo: Zoecin antibiotic resistance sequence. pAn: Human Beta-globin 3' UTR, efficient for transgene transcription. Ori: Origin of plasmid replication. 7SK: a promoter for efficient expression of siRNA. siRNA: contains the sequence of the siRNA to silence the gene of interest. XbaI: restriction site of the XbaI restriction enzyme. **B)** siTLR9 hairpin structure used in the plasmid.

3.2.4 RNA isolation, cDNA synthesis

Total RNA from TLR9KD-RL95-2, CP-RL95-2, and normal RL95-2 cells were extracted using TRI-reagent (Sigma) following the exact protocol supplied by the manufacturer. Briefly, cells were homogenized in 1 ml of TRI-reagent. A total value of 200 µl chloroform (Sigma) were added to the cell homogenate, mixed vigorously, and incubated at room temperature for at least 10 minutes. The mix was then centrifuged at 12,000x g for 10 minutes at 4°C. The aqueous layer was transferred to a clean tube with 500 µl isopropanol (Sigma), mixed well, and incubated at room temperature for 10 minutes. The sample was again centrifuged at 12,000x g for 10 minutes at 4°C. After that, the supernatant was discarded and the pellet was washed once with 1ml cold 75% ethanol (Sigma) and centrifuged at 7500x g for five minutes at 4°C. Finally the pellet was suspended in 50 µl of RNase free water (Sigma). The RNA was then treated twice with Dnase I (DNA-freeTM, Ambion, Austin, TX, USA) to remove the genomic DNA from the samples. cDNA synthesis was performed using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Paisley, UK) with 2µg of sample RNA. The RNA was mixed with 10 µl of the RT buffer and 1µl of the enzyme provided with the kit (no enzyme was used for the negative controls). RNase free water were added to the mix to make the total volume reach 20 µl. The mix was placed into the thermocycler (Eppendorf, Stevenage, UK) for just one cycle of 37°C for 60 minutes, followed by 95°C for five minutes. The cDNA was stored at -20 °C for further action.

3.2.5 TLR9 primer optimization and validation of qPCR

qPCR was carried out with prepared the cDNAs according to the MIQE guidelines. The TLR9 primer sequences were, forward, 5'-CGTCTTGAAGGCCTGGTGTTGA-3', and reverse, 5'-CTGGAAGGCCTTGGTTTTAGTGA-3' (Fathallah et al., 2010). The results were normalized using two housekeeping genes as references, β-actin (B.act), forward, 5'-CAAGATCATTGCTCCTCCTG-3' and reverse, 5'-ATCCACATCTGCTGGAAGG-3', B2M (Beta-2 microglobulin); forward, 5'-TATGCCTGCCGTGTGAACCA-3', and reverse, 5'-GCGGCATCTTCAAACCTCCA-3'. A standard curve was created using serial

dilutions of the cDNA samples (1:5, 1:15, 1:45, 1:135 and 1:405) and plotted using the logarithm of the cDNA dilution versus the average threshold cycle number (C_t). The efficiency of the TLR9 primers was calculated using this formula:

$$E=10^{\{-1/slope\}}$$

The percentage of the efficiency was estimated as follows:

$$\%E=\{-1+(10^{\{-1/slope\}})\}*100$$

An efficiency of 80-120% for the primers was considered suitable for analysing the gene expression.

3.2.6 Quantitative real-time PCR

SYBR Green Jump start *taq ready* mix (Sigma) was used to prepare the qPCR master mix (10 µl SYBR Green, 7 µl water, 1 µl of each forward and reverse primer, and 1 µl of cDNA in each well of the PCR plate). Amplification was carried out under the following conditions: 45 cycles of 95° for 30 seconds, 62° for 1 minute, and 72° for 30 seconds. qPCR was performed with the help of Mx3005P QPCR (Stratagene, Agilent technologies, Stckport, UK) and MxPro QPCR, version 4.01 software (Stratagene).

3.2.7 IL-8 ELISA

IL-8 ELISA was conducted using a commercial enzyme-linked immunosorbent assay kit (R&D Systems, Abingdon, UK). Briefly, Capture antibody was diluted to the working concentration suggested in the protocol in PBS and was immediately added to a 96-well microplate (R&D Systems) with 100 µl per well. The plate was sealed and incubated overnight at room temperature. On the next day, the microplate was washed 3 times with wash buffer (R&D Systems). A total volume of 300 µl of blocking buffer (R&D Systems) was added to each well of the microplate and incubated at room temperature for one hour. The microplate was washed three times. A volume of 100 µl of Samples and/or IL-8 standards (recombinant human IL-8 in serial dilutions) was added to the wells and incubated two hours at room temperature.

Meanwhile, the detection antibody was diluted to the working concentration suggested in the protocol in reagent diluent (R&D Systems). After washing the microplate three times, 100 µl of detection antibody was added to each well and incubated two hours at room temperature. The microplate was than washed three times, and 100 µl of the working dilution of streptavidin-HRP was added to each well and incubated at room temperature for 20 minutes. The washing procedure was repeated three times again, and 100 µl of substrate solution (R&D Systems) were added to each well. After 20 minutes, 50 µl of stop solution (R&D Systems) was added to each well, and the microplate was placed into the microplate reader, set to 450nm (Molecular Devices, Sunnyvale, CA, USA), to determine the optical density of the samples.

3.2.8 *In vitro* human implantation assay;

3.2.8.1 Formation of JAr spheroids

The process of JAr spheroid formation has been explained in detail in Chapter 2 (2.2.4.1).

3.2.8.2 Formation of the endometrial monolayer

TLR9KD-RL95-2, CP-RL95-2 cells were cultured in T75 flasks (Greiner Bio-One) with supplemented DMEM-F12 (Sigma), as described above, until confluent. Cells were then harvested using trypsin-EDTA (Sigma), and 2.5 x 10^5 cells were transferred to each well of a 12 well plate (Greiner Bio-One) in triplicate and incubated at 37°C for 2-3 days until confluent.

3.2.8.3 Co-incubation of the JAr spheroids with the endometrial monolayer

The process of co-incubation of JAr spheroids with endometrial epithelial monolayer has been described in detail in Chapter 2 (2.2.4.3).

3.2.8.4 Removal of unattached JAr spheroids and counting the attached spheroids

The process of removing the unattached JAr spheroids and counting the attached spheroids has been described in detail in Chapter 2 (2.2.4.4).

3.2.9 Experimental design

3.2.9.1 Determination of the TLR9 gene expression in TLR9KD-RL95-2 and CP-RL95-2 cells

To determine whether the TLR9 gene was successfully knocked-down in the TLR9KD-RL95-2 cells, total RNA was extracted from TLR9KD-RL95-2, CP-RL95-2 and normal RL95-2 cells, cDNA was synthesized, and the qPCR was performed as described before, to check the level of TLR9 gene expression.

3.2.9.2 Determination of the TLR9kD-RL95-2 cell line functionality in response to CpG

To determine the functionality of the TLR9KD-RL95-2 cell line in response to CpG, TLR9KD-RL95-2, CP-RL95-2 and normal RL95-2 cells were cultured in 24 well plates till 90% confluence. The media were then replaced with fresh serum free media with and without 1μM CpG in a total volume of 200μl of serum free DMEM-F12 media for each well for 24 hours. The supernatants were then collected and used for further analysis. The IL-8 level in each sample was then measured as described earlier.

3.2.9.3 Determination of the effect of different concentrations of CpG on the JAr spheroid attachment to the TLR9KD-RL95-2 and CP-RL95-2 monolayer

To determine the effect of TLR9 activation with different concentrations of CpG on trophoblast attachment to the endometrial epithelial cells, TLR9KD-RL95-2 and CP-RL95-2 cells were grown in 12-well plates to 75% confluence. The media was then replaced with serum-free media containing CpG as a TLR9 ligand at different concentrations (0, 0.01, 0.1 and 1 μM) and incubated for 24 hours. The epithelial monolayers were then rinsed once with PBS or not to omit the excess CpG. Thereafter, 50 JAr spheroids were delivered to each well and co-incubated for 1 hour. The spheroids were then washed and counted as described earlier.

3.2.9.4 Determination of the effect of different CpG concentrations on JAr spheroid attachment to the transfected endometrial cells when the TLR9 antagonist inhibitory oligonucleotide was added to the JAr spheroids before co-incubation

To determine whether the CpG effect on trophoblast attachment was specifically originated from the endometrial epithelial cells, TLR9KD-RL95-2 and CP-RL95-2 cells were grown in 12-well plates to 75% confluence. The media was then replaced with serum-free media containing CpG at different concentrations (0, 0.1 and 1 μM) and incubated for 24 hours. JAr spheroid media was also replaced with serum-free media containing 0.1 μM TLR9 antagonist inhibitory oligonucleotide (ODN TTAGGG). After six hours, the JAr spheroids were washed once with BPS, and 50 of them were delivered to each well and co-incubated with the endometrial epithelial monolayer for one hour. The spheroids were then washed and counted as described earlier.

3.2.10 Statistical analysis

The qPCR results were analysed with the $\Delta\Delta C_t$ method. The C_t of the gene of interest was normalized to the standard sample (a pool of cDNA of all the samples) and with the C_t of the reference genes:

$$\Delta\Delta C_t = \Delta C_t \text{ sample} - \Delta C_t \text{ reference}$$

The relative mRNA expression was then calculated by: $2^{-\Delta\Delta Ct}$.

The results were expressed as mean ± S.E.M. Statistical analysis was performed by using ANOVA with Bonferroni's post hoc test. $p<0.05$ was considered to be significant. Three replicates were performed in each experiment.

3.3 Results

3.3.1 Creation of stable TLR9 knocked-down RL95 endometrial epithelial cells

3.3.1.1 PsiRNA-hTLR9 and PsiRNA-LucGL3 were amplified and extracted from E. coli bacteria

In order to enhance the yield of the plasmids, both siRNA-hTLR9 and siRNA-LucGL3 (control) plasmids were amplified to a large scale and extracted from the *E.*

coli bacteria, as described earlier. To check the molecular weight, each plasmid was run on the agarose gel. Bands were shown at 3.6 kb size, which was the exact weight of the plasmids (Figure 3.3).

3.3.1.2 PsiRNA-hTLR9 and PsiRNA-LucGL3 were linearized with XbaI restriction enzyme

For the purpose of random insertion of the plasmids into the genome of the RL95-2 cells, both siRNA-hTLR9 and siRNA-LucGL3 were cut with the XbaI restriction enzyme. The linear plasmids were then run on the agarose gel to check their weight (Figure 3.4).

3.3.1.3 TLR9KD-RL95-2 and CP-RL95-2 cell lines were grown in the culture

siTLR9 and siLuc transfected RL95-2 colonies (TLR9KD-RL95-2 and CP-RL95-2 cells) were grown in the culture with the maintenance conditions described earlier for the further experiments. Since both siRNA-hTLR9 and siRNA-LucGL3 plasmids have a GFP coding sequence, transfected RL95-2 cells were easily traceable with a fluorescent microscope. Figure 3.5 shows the stably transfected RL95-2 cells under the microscope.

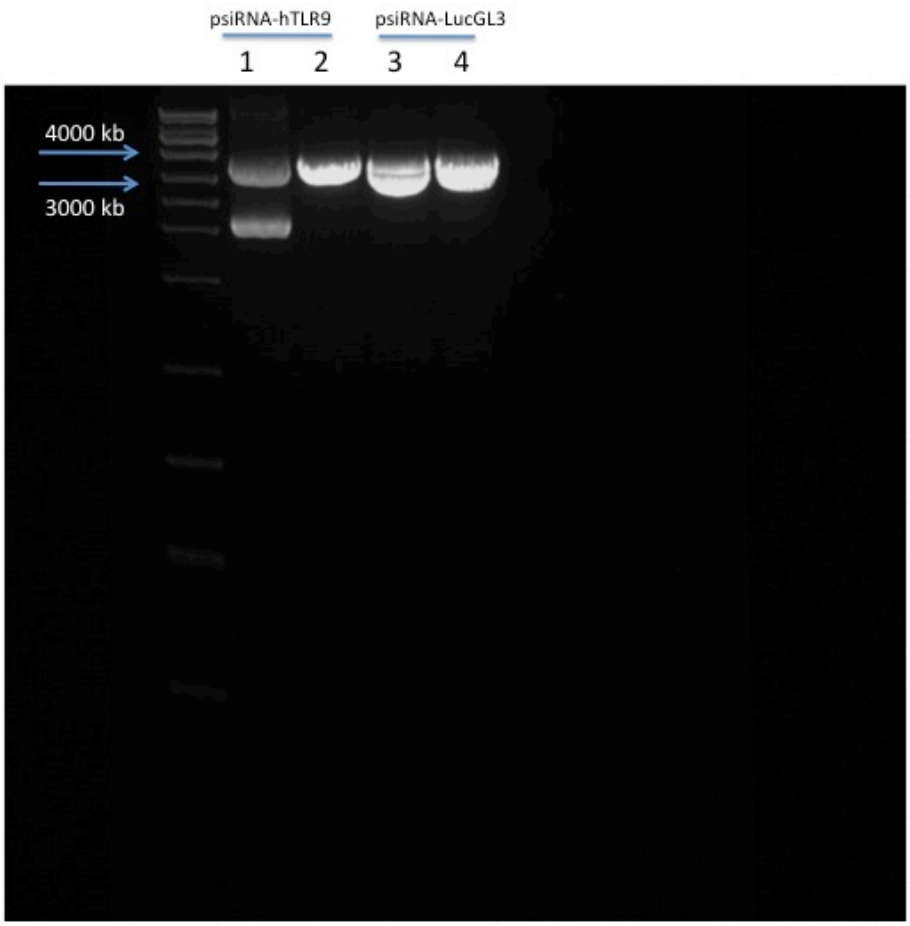


Figure 3.3 psiRNA-hTLR9 and psiRNA-LucGL3 on agarose gel. psiRNA-hTLR9 and psiRNA-LucGL3 (control plasmid) were extracted from the bacteria in large scale. Despite reading their final concentration, their weight has also been checked by running them in the agarose gel. Bands 1 and 2 show two different samples of psiRNA-hTLR9 (3.6 kb). Bands 3 and 4 show two different samples of the control plasmid (3.6 kb).

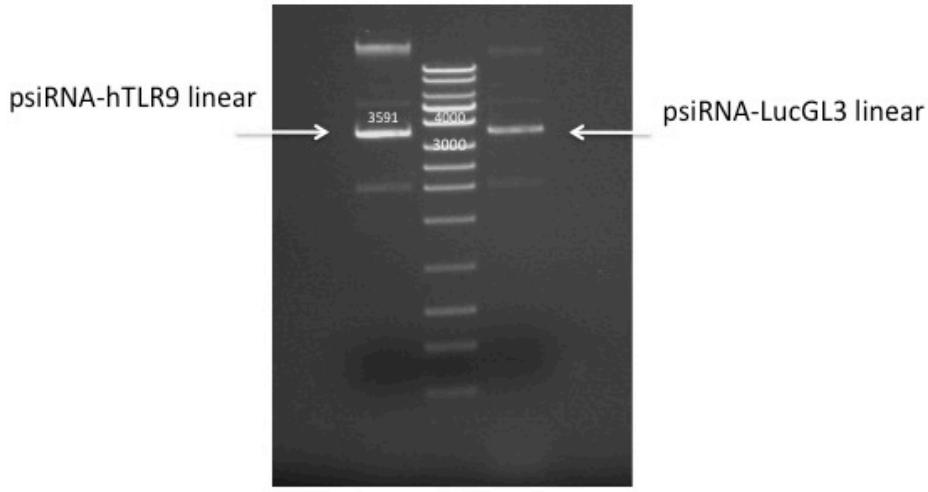


Figure 3.4 linear psiRNA-hTLR9 and psiRNA-LucGL3 on agarose gel. Both psiRNA-hTLR9 and control plasmids (3591 bp) should be linear to randomly insert into the chromosomes of the cells. XbaI enzyme was used to cut the plasmid with sticky ends. The bands shown by arrows are the linear plasmids.

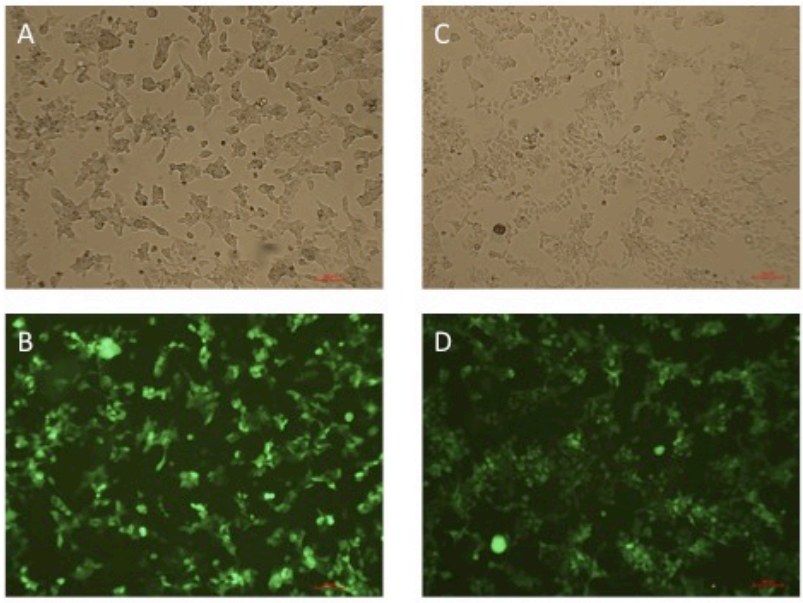


Figure 3.5 TLR9KD-RL95-2 cells. TLR9KD and Control plasmid transfected RL95-2 cells were cultured as a monolayer, and the amount of transfection (based on GFP expression) has been checked under the microscope. A) TLR9 stably knocked down RL95-2 cells, as shown by viewing under light microscopy. B) TLR9 stably knocked down RL95-2 cells cells, as shown by viewing under fluorescent microscopy (it shows 100% transfection, as all the cells are producing GFP). C) Stably Control plasmid transfected RL-95-2 cells viewed under light microscopy. D) Stably control plasmid transfected RL95-2 cells viewed under fluorescent microscopy.

3.3.2 TLR9 primer optimization

The efficiency of the TLR9 primers was confirmed by standard and dissociation curves. The primers for the reference genes had been optimized before in our lab with efficiencies of 96% and 94% for B.Act and B2M respectively. The TLR9 primers showed an efficiency of 95.8%, with only one amplification peak (Figure 3.6).

3.3.3 TLR9 gene expression was significantly decreased in TLR9KD-RL95-2 cells

Transfecting RL95-2 cells with the linear control and siRNA-hTLR9 plasmids and selecting the colonies from media supplemented with Zeocin, as descried earlier, resulted in the creation of two types of RL95-2 cell lines: A) RL95-2 cells, which were transfected with the control plasmid with normal TLR9 function (CP-RL95-2 cells), and B) RL95-2 cells, which were transfected with the siRNA-hTLR9 plasmid with the TLR9 knocked-down function (TLR9KD-RL95-2 cells). qPCR results indicated that TLR9 gene expression did not significantly change in the CP-RL95-2 cells compared to the normal RL95-2 cell line, but it was significantly decreased in the TLR9KD-RL95-2 cells (Figure 3.7).

3.3.4 TLR9KD-RL95-2 cells did not produce IL-8 in response to CpG

After treatment of TLR9KD-RL95-2 cells with 1μM CpG for 24 hours, no significant alterations in IL-8 production ($p>0.05$) was observed, while normal RL95-2 and CP-RL95-2 cells showed significant production of IL-8 in response to CpG compared to the controls (untreated cells) ($p<0.05$) (Figure 3.8).

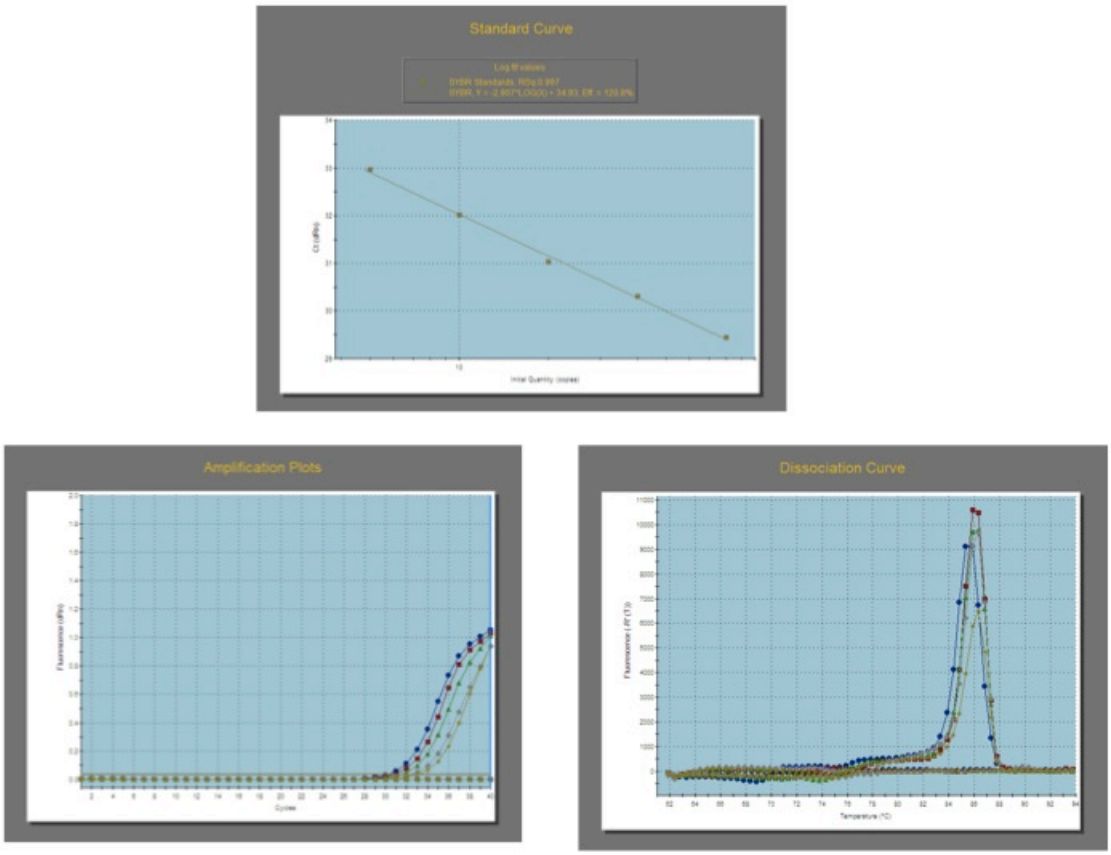


Figure 3.6 TLR9 primer optimization. Amplification was carried out under the following conditions: 45 cycles of 95° for 30 s, 62° for 1 min, and 72° for 30 s. The upper photo shows the standard curve created using serial dilutions of the cDNA samples (1:5, 1:15, 1:45, 1:135 and 1:405) and plotted using the logarithm of the cDNA dilution versus the average threshold cycle number (C_t). The bottom-right photo represents the dissociation curve, with only one peak specific to TLR9. The bottom-left photo shows the amplification plot of the different serial dilutions of the cDNA samples (1:5, 1:15, 1:45, 1:135 and 1:405).

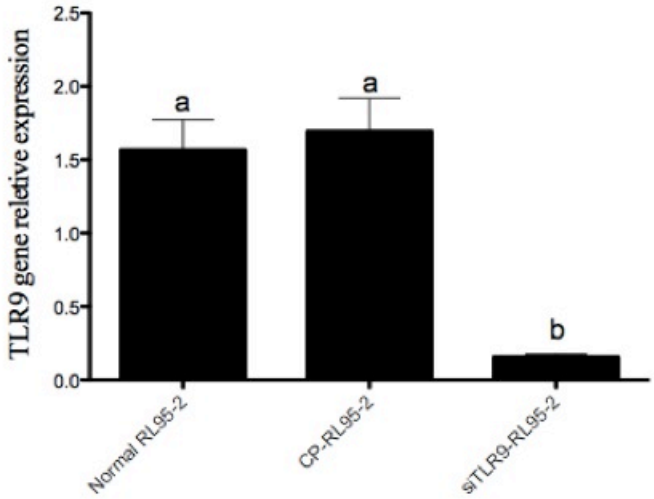


Figure 3.7 TLR9 gene expression analysis. TLR9 gene expression did not change significantly in CP-RL95-2 cells compare to the normal RL95-2 cell line, but it has been significantly decreased in the TLR9KD-RL95-2 cells (siTLR9-RL95-2) compared to the normal RL95-2 cell line. qPCR was performed based on the MIQE guidelines. Data are the average of three independent experiments and three technical replicates on each day (n=9). Results are presented as the mean ± S.E.M. ANOVA was used to analyse the results. Different letters indicate significant statistical differences. $P<0.05$ was considered to be significant.

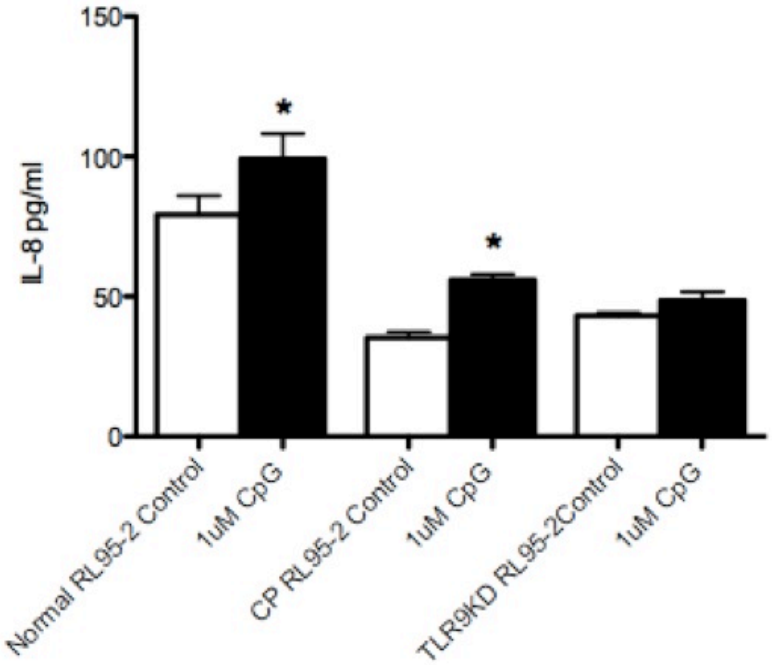


Figure 3.8 IL-8 production analysis. To determine the functionality of TLR9KD-RL95-2 cell line in response to CpG, TLR9KD, CP and normal RL95-2 cells were stimulated with 1μM CpG for 24 hours and the level of IL-8 was measured by ELISA. Treatment of TLR9KD-RL95-2 cells with 1μM CpG for 24 hours did not show any significant production of IL-8 ($p>0.05$), while the Normal and CP RL95-2 cells showed significant production of IL-8 in response to CpG compared to the negative control (untreated cells) ($p<0.05$). The data are the average of three independent experiments and 3 technical replicates in each day (n=9). The results are presented as the mean ± S.E.M. ANOVA was used to compare the amount of IL-8 production. Control= untreated cells. * indicates significant statistical differences. $p<0.05$ was considered to be significant.

3.3.5 Effect of different concentrations of CpG on the JAr spheroid attachment to the TLR9KD-RL95-2 and CP-RL95-2 monolayer

Pre-treatment of both the TLR9KD and the CP RL95-2 cells with different concentrations of CpG significantly decreased the percentage of JAr spheroids attaching to the endometrial cells ($p<0.05$) when the excess CpG had not been washed away from the endometrial monolayers before transferring the JAr spheroids (Figure 3.9).

Pre-treatment of TLR9KD-RL95-2 cells with 0, 0.01 and 0.1μM concentrations of CpG did not significantly decrease the percentage of JAr spheroids attaching to the endometrial cells ($p>0.05$) when the excess CpG had been washed away from the endometrial monolayers just before transferring the JAr spheroids. Interestingly, significant reduction of the spheroid attachment were only seen when the highest dose of CpG (1μM) was used (Figure 3.10).

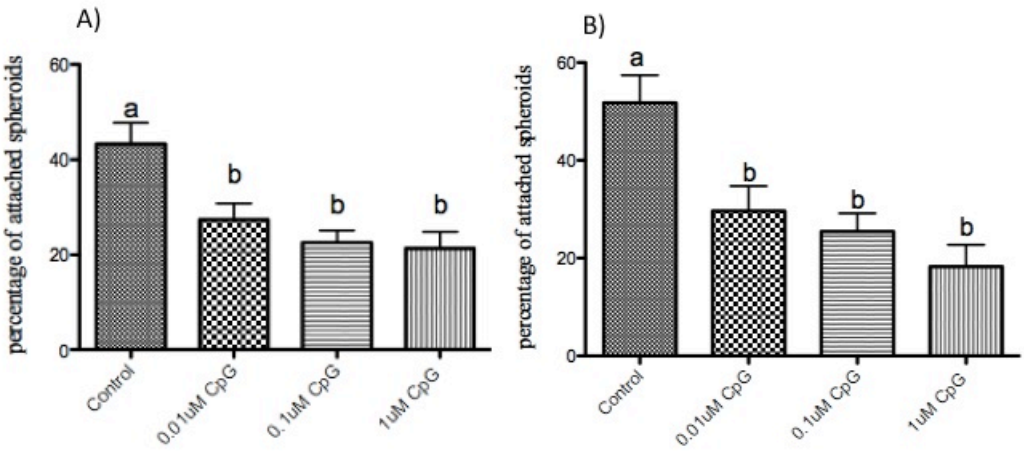


Figure 3.9 The effect of CpG on JAr spheroid attachment to TLR9KD-RL95-2 cells when CpG remained in the system. Pre-treatment of CP-RL95-2 (A) and TLR9KD-RL95-2 (B) cells with different concentrations of CpG had a detrimental effect on JAr spheroid attachment to the endometrial cells compare to the control when the excess CpG has not been removed from the endometrial monolayers just before transferring the JAr spheroids. The data are the average of three independent experiments and 3 technical replicates in each day (n=9). The results are presented as the mean ± S.E.M. ANOVA was used to compare the percentage of the attachment of JAr spheroids. Control= untreated cells. Different letters indicate significant statistical differences. $p<0.05$ was considered to be significant.

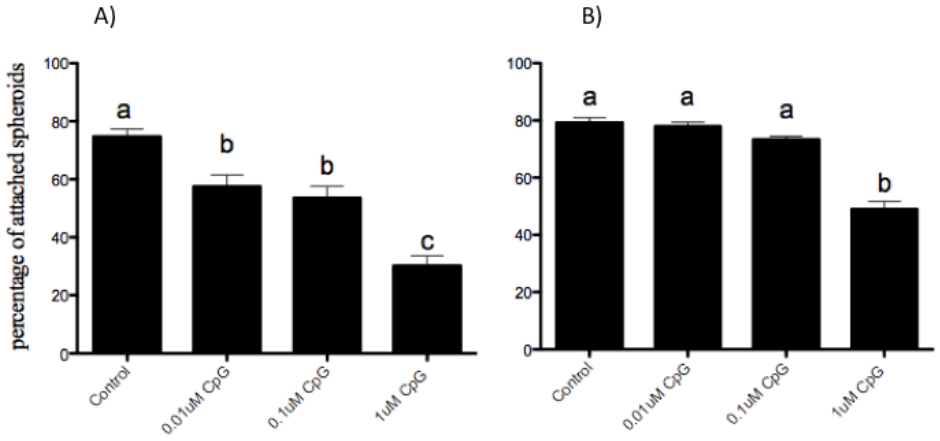


Figure 3.10 The effect of CpG on JAr spheroid attachment to TLR9KD-RL95-2 cells when CpG was washed from the system. A) pre-treatment of CP-RL95-2 cells with different concentrations of CpG had a detrimental effect on JAr spheroid attachment to endometrial cells compared to the control when the excess CpG was removed from the endometrial monolayers just before transferring the JAr spheroids. B) Pre-treatment of TLR9KD-RL95-2 cells with 0, 0.01 and 0.1μM concentrations of CpG did not significantly decrease the percentage of JAr spheroids attaching to the endometrial cells when the excess CpG was washed away from the endometrial monolayers just before transferring the JAr spheroids. Interestingly, significant reduction in spheroid attachment was only seen when the highest dose of CpG (1μM) was used. The data are the average of three independent experiments and three technical replicates in each day (n=9). The results are presented as the mean ± S.E.M. ANOVA was used to compare the percentage of the attachment of the JAr spheroids. Control=untreated cells. Different letters indicate significant statistical differences. $p<0.05$ was considered to be significant.

3.3.6 Pre-treatment of TLR9KD-RL95-2 cells with CpG did not show a detrimental effect on JAr spheroid attachment to endometrial epithelial cells when the CpG remained in the in-vitro culture system but the JAr spheroids were treated with TLR9 antagonist inhibitory oligonucleotide

Pre-treatment of TLR9KD-RL95-2 cells with 0, 0.01 and 0.1μM concentrations of CpG did not significantly decrease the percentage of JAr spheroids attaching to the endometrial cells ($p>0.05$) when the excess CpG remained in the endometrial culture and while the JAr spheroids TLR9 receptors was blocked using the TLR9 antagonist inhibitory oligonucleotide before being transferred onto the endometrial culture. Interestingly, a significant reduction of spheroid attachment was only seen when the highest dose of CpG (1μM) was used. The negative effect of CpG on JAr spheroid attachment was present when the experiment was repeated with CP-RL95-2 endometrial cells (Figure 3.11).

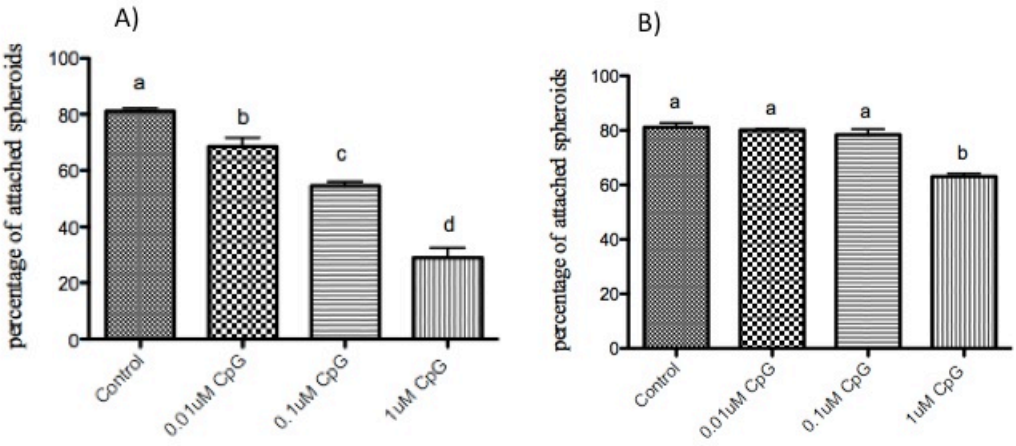


Figure 3.11 The effect of CpG on JAr spheroid attachment to TLR9KD-RL95-2 cells when TLR9 was blocked in the JAr spheroids. A) pre-treatment of CP-RL95-2 cells with different concentrations of CpG had a detrimental effect on JAr spheroid attachment to the endometrial cells compare to the control when the excess CpG was not removed from the endometrial monolayers but the TLR9 receptors in the JAr spheroids were blocked using an inhibitory oligonucleotide. B) Pre-treatment of TLR9KD-RL95-2 cells with 0, 0.01 and 0.1μM concentrations of CpG did not significantly decreased the percentage of JAr spheroids attaching to the endometrial cells when the excess CpG remained in the endometrial culture and while the JAr spheroid TLR9 receptors were blocked before being transferred onto the endometrial culture. Interestingly, significant reduction of the spheroid attachment was again only seen when the highest dose of CpG (1μM) had been used. The data are the average of three independent experiments and three technical replicates in each day (n=9). The results are presented as the mean ± S.E.M. ANOVA was used to compare the percentage of the attachment of the JAr spheroids. Control = untreated cells. Different letters indicate significant statistical differences. $p<0.05$ was considered to be significant.

3.4 Discussion

RNAi was found to be a potential molecular defence mechanism against intracellular invaders in plants and other living organisms such as Drosophila and *Caenorhabditis elegans*. Currently, RNAi application is a popular method of gene silencing (Paddison et al., 2002a, Sliva and Schnierle, 2010). Using RNAi gene-silencing technologies are usually problematic in mammalian somatic cells mostly because of their robust antiviral system. That is why shorter sequences of siRNAs (21-25 nucleotides) are more popular among scientists to avoid provoking unwanted responses. Hence siRNA has become a very powerful genetic tool in molecular engineering to apply both transient and stable gene-silencing in mammalian cells (Sindhu et al., 2012).

For the purpose of creating stable knocked-down cell lines for *in vitro* studies with a particular gene being knocked down, researchers have been trying to design different types of expression vectors containing an efficient shRNA sequence to overcome the limitations that transient transfection would cause (van de Wetering et al., 2003, Paddison et al., 2002b, Gomez-Martinez et al., 2013). Expression vectors beside a specific siRNA insertion site usually contain a selection marker, like an antibiotic resistance gene and/or a traceable marker such as a fluorescent protein coding sequence. After transfecting the cells with the expression vector, by constant replication of the plasmids, the expression of the gene of interest would be interrupted (van de Wetering et al., 2003, Triantafilou et al., 2013, Wu and Kuo, 2012). Here in this study, to create a stable TLR9 knocked-down endometrial epithelial cell line, RL95-2 cells were transfected with a plasmid containing a TLR9 specific shRNA with Zeocin resistance and a green fluorescent protein (GFP) coding sequence. By making the plasmids linear, we increased the chance of random insertion of the vector into the chromosomes of the cells. This helped to have constant expression of the TLR9 siRNA over long periods of time in the culture, even in the cells with high passage numbers.

TLR9 gene expression significantly decreased in the TLR9KD-RL95-2 cells compared to normal RL95-2 and CP-RL95-2 cells. This indicated that the siRNA

used to disrupt our target gene was TLR9 sequence-specific and efficient enough to decrease the gene expression. It is well established that introducing the shRNA into the cells initiates the RNA based gene silencing (RBGS) process, in which shRNA transforms to siRNA that associates with RISC to find its homologous sequence on the target RNA and then degrade the mRNA of the target gene (Elbashir et al., 2002, Bagasra and Prilliman, 2004). RBGS function is highly dependent on the quantity of siRNA that should be constantly introduced into or endogenously replicated in the cells. The existence of a silencing memory (a memory of the RNA sequence that has been silenced once) has been reported in both *C. elegans* and *Neurospora* that epigenetically is inherited through several generations. It seems that the memory signals are also exchanged between the cells via cellular communication and interactions (reviewed by (Bagasra and Prilliman, 2004). Silencing memory has not yet been found in humans; hence siRNA should be constantly transferred into the cells for the purpose of transient RBGS. In our experiment to overcome such limitations, we have tried to randomly insert the siRNA sequence into the genome of the cells to produce a sufficient amount of siRNA in future generations. Significant reduction in the TLR9 gene expression indicated successful stable gene silencing.

TLR9KD-RL95-2 cells were not able to produce IL-8 in response to CpG. These results confirmed that TLR9 gene in the knockdown cells was no longer functional in the presence of its specific ligand. It is well documented that IL-8 is one of the most common cytokines produced in response to TLR9 ligation in both humans and mice, while type I interferons win second place (Ghadimi et al., 2010, Parilla et al., 2006, Andersen et al., 2006, Sathe and Reddy, 2014, Volpi et al., 2013, Steinhagen et al., 2012, Pedersen et al., 2005, Greene et al., 2005, Platz et al., 2004, Xu et al., 2012, Yu et al., 2011, Wan et al., 2011, Xu et al., 2009, Li et al., 2007, Osawa et al., 2006, Hemmi et al., 2003, Erridge et al., 2008). Furthermore, in contrast to high doses of CpG that result in the production of significant amount of cytokines over a short period of time, low doses of CpG need more time to induce cytokine production from stimulated cells (Akhtar et al., 2003, Volpi et al., 2013, Platz et al., 2004). Therefore, TLR9KD-RL95-2 cells were pre-treated with a high dose of CpG (1 μM) to check whether they would be able to produce significant amounts of IL-8 in response to TLR9 ligation. These findings confirmed our results obtained from the TLR9 gene

expression analysis of these cells. Hence it seems that the TLR9 gene was disrupted at both the gene and functional levels in the RL95-2 cells in our experiments.

Pre-treatment of TLR9KD-RL95-2 cells (as well as CP-RL95-2 cells) with CpG had a detrimental effect on JAr spheroid attachment to endometrial epithelial cells when the excess CpG was not removed from the *in vitro* culture system during the experiments. These results confirmed that our control cell line (CP-RL95-2 cells) was responsive to CpG, the same as normal RL-95-2 cells, when encountering the same ligand. In theory, a detrimental effect of CpG should not be seen, since TLR9KD-RL95-2 cells were used to prepare the endometrial monolayer and they did not respond to TLR9 ligation unless the remaining CpG in the culture during the co-incubation time affected the JAr spheroids. TLRs are found extensively in JAr cells and are able to respond to pathogens in a quick manner (it took nearly ten minutes for the TLR9 receptor to sense CpG in the endosomes) (Takeshita et al., 2004). Hence, it was not outside of our expectations that the JAr spheroids also sensed the CpG in our *in vitro* system and tried to respond to the ligand while they were co-cultured with endometrial epithelial cells. That is why we believe the JAr cells as well as the endometrial epithelial cells were involved in the process of reducing JAr spheroid attachment to the endometrium in our *in vitro* model.

Pre-treatment of the TLR9KD-RL95 cells with CpG (0.01 and 0.1 μM) did not have a negative effect on JAr spheroid attachment to the endometrial epithelial cells when the excess CpG was removed from the monolayer before adding the JAr spheroids. The same results were observed when the excess CpG was not removed from the co-culture but the JAr spheroids were pre-treated with TLR9 inhibitory oligodeoxynucleotide (ODN). Interestingly, only high doses of CpG (1 μM) induced its detrimental effect on JAr spheroid attachment when TLR9KD-RL95-2 cells and ODN pre-treated JAr spheroids were used in the culture system. One possible explanation for this inhibitory effect using a 1 μM CpG concentration is that CpG simply overloaded the system. Hence washing the extra CpG from the endometrial monolayer before adding the JAr spheroids was not enough to entirely clean the system from CpG. Another explanation could be that even ODN was not able to block all the CpG responsive sites. On the other hand, CpG uptake is not hundred percent

dependent on classic endocytosis (Heeg et al., 2008). Several surface receptors are now found in various human and mice cells involved in CpG uptake and trafficking. One of these surface receptors, called human killer immunoglobulin (Ig)-like receptor (KIR3DL2), is highly expressed in NK cells. Engagement of KIR3DL2 with CpG on the surface of the cell leads to cytokine release (Sivori et al., 2010). Mannose receptor 1 is also known to be another surface receptor involved in the uptake and endosomal delivery of CpG in mice macrophages (Moseman et al., 2013). β-defensins (antimicrobial peptides HBD2 and 3) and DEC-205 (multi-lectin receptor) are two other surface receptors widely found in human epithelial cells that can facilitate and enhance immune responses against bacterial and viral DNAs by uptaking both self and non-self nucleic acids and transferring them to endosomes to encounter TLR9 and/or TLR7 (Tewary et al., 2013, Lahoud et al., 2012). Although it is still not clear if different cells used different receptors specifically to uptake nucleic acids, it is believed that they are mostly sequence, structure, length and concentration (of CpG) dependent (Sivori et al., 2010). Hence, one can speculate that CpG in high doses is rapidly being taken up by different surface receptors as well as by endocytosis, triggering a fast immune response independent of TLR9 that may lead to spheroid attachment failure. More research is still needed to clarify the TLR9 independent intracellular signalling pathway downstream of the CpG surface receptors.

To sum up, both JAr trophoblasts and endometrial epithelial cells seems to respond to CpG, which negatively affected the binding of trophoblast spheroids to endometrial epithelial cells in our *in vitro* system.

Chapter 4: Understanding the TLR9 intracellular signalling pathway in human endometrial epithelial cells

4.1 Introduction

Implantation failure caused by infection is a major factor involved in pregnancy loss (Barton, 2007, Fredlund et al., 2004). Successful implantation requires a supportive environment, which is strongly dependent on a healthy endometrium (Ledee-Bataille et al., 2002). According to a survey conducted by Public Health England between the years 2000 to 2010, the number of people who suffered from different sexually transmitted infections (STIs), *Chlamydia* in particular, has sharply increased (Volume 8 Number 24 Advanced Access report published on: 17 June 2014). Pathogen recognition receptors (PRRs) detect the presence of infection at the site of implantation. Toll-like receptors (TLRs) are a major family of PRRs and are widely expressed in endometrial epithelial cells. TLRs are able to react to specific microbial agents, initiating an intracellular signalling pathway and the secretion of inflammatory cytokines that may result in implantation failure.

Among the TLRs, TLR9 is believed to be involved in the recognition of various STIs such as Herpes simplex viruses (HSV) and *Chlamydia trachomatis* (Fredlund et al., 2004, Barton, 2007). Furthermore, we have previously shown that the activation of TLR9 in human endometrial epithelial cells has a detrimental effect on the binding of trophoblast cells to RL95-2 cells in our 2D *in vitro* model of human implantation (Chapter 2). Hence clarifying the TLR9 intracellular signalling pathway in human endometrial epithelial cells and understanding the signalling pathway that is being activated by TLR9 ligation and that results in a reduction of trophoblast cells binding to the endometrium would be helpful for further discovering the role of innate immune reactions during pregnancy.

TLRs consists of two main regions that are common to all of their family members: a pathogen binding ectodomain (15 to 19 Leucine rich repeats [LRRs]) and a trans-membrane cytoplasmic domain called Toll IL-1 receptor (TIR) (Heeg et al., 2008). X-ray crystallography used to investigate the three-dimensional structure of TLR3 has revealed the horse-shoe structure of LRRs and its possible ligand binding sites (Bell et al., 2005). Nowadays scientists have deduced that other intracellular TLRs, such as

TLR9, also contain this structure due to their conservative protein sequences (Heeg et al., 2008).

TLR9 is responsible for the recognition of CpG, a short sequence of un-methylated DNA containing CG dinucleotides linked together with strong phosphodiester bonds. They are widely found in prokaryotic organisms such as bacteria and viruses rather than in eukaryotes such as mammalian cells (Akira and Hemmi, 2003). The first generation of synthesized CpG oligodeoxynucleatides (CpG ODNs) was not quite satisfactory, mainly because of their phosphodiester deoxyribose (PO) backbone that made them degradable by intracellular nucleases (Lipford et al., 1997), but this problem was soon overcome by using a thionate deoxyribose backbone (PTO) instead. However, CpG with a PTO backbone may also be able to provoke unwanted immune responses completely independent of TLR9 (Heeg et al., 2008, Lipford et al., 2000). Many different sequences and structures of CpG have therefore been developed to minimize defects and increase their efficiency. Three different classes of synthetic CpG ODNs with PTO backbones are now available for research purposes: Class A usually contains a central CpG palindromic motif and a 3' poly G tail. This structure strongly induces interferon-α (IFNα) production from plasmacytoid dendritic cells, but a very weak nuclear factor κB (NFκB) signal. Class B contains more than one CpG motif in its structure and effectively activates B cells. Finally, Class C has the characteristics of both classes A and B (Krieg et al., 1995, Bauer et al., 2001, Krug et al., 2001). According to the literature, Class B CpGs are more commonly used among scientists to induce TLR9 signalling in both *in vivo* and *in vitro* based investigations (Ghadimi et al., 2010, Parilla et al., 2006, Andersen et al., 2006, Guiducci et al., 2008, Sato et al., 2010, Volpi et al., 2013, Greene et al., 2005, Steinhagen et al., 2013, Xu et al., 2012, Lou et al., 2010, Osawa et al., 2006). Hence CpG B was selected for the present study.

Once CpG has stimulated TLR9, an intracellular signalling pathway is initiated from the myeloid differentiation primary response gene 88 (MyD88) mediator. The signalling pathway continues downstream via three different possible pathways: either through NFκB, mitogen-activated protein kinases (MAPKs), or interferon regulatory factor-7 (IRF7) (explained in more detail in Chapter 1) (Akira, 2006, Blasius and

Beutler, 2010, Kawai and Akira, 2006). One must bear in mind that the TLR9 signalling pathway is reported to be cell-specific and to differ from one cell type to another (Ghadimi et al., 2010, Parilla et al., 2006, Volpi et al., 2013, Sathe and Reddy, 2014, Fitzner et al., 2011, Douagi et al., 2009, Sorrentino et al., 2011). Here we aimed to clarify which TLR9 intracellular signalling pathway is involved in the inhibition of trophoblast spheroid binding to endometrial epithelial cells in a 2D *in vitro* model of the early stages of human pregnancy.

Cell penetrating peptides (CPPs) are positively charged short peptides (not exceeding 30 aminoacid residues) that are able to cross the cellular membrane with no toxicity and without any specific receptors. CPPs can transport a variety of cargos (nanoparticles, peptides, inhibitors, siRNA, dsRNA, etc.) into living cells. Hence they are popular among researchers as vectors to transport various macromolecules *in vitro* and *in vivo* for targeted cellular therapies (Bechara and Sagan, 2013). Here in this investigation, CPP-inhibitors were used to study the TLR9 intracellular signalling pathway.

4.2 Material and Methods

4.2.1 Cell lines and Culture

The Michigan Cancer Foundation (MCF)-7 cells, the human endometrial adenosquamous carcinoma cell line (RL95-2), and the human choriocarcinoma cell line (JAr) derived from first trimester trophoblast cells, were purchased from American Type Culture Collection (ATCC, Teddington, UK). RL95-2 cells were used to represent a receptive endometrium, while JArs were used to simulate the human embryo trophoblast, and finally, MCF-7 cells were used to represent epithelial cells.
The process of culturing the cells has been described in detail in Chapter 2 (2.2.4.1).

4.2.2 Ligand and inhibitors

Type B CpG oligonucleotide-human TLR9 ligand (5'-tcgtcgtttgtcgttttgtcgtt-3') was used to stimulate TLR9 (Invivogen, Toulouse, France). Oligodeoxynucleotide (ODN) TTAGGG, TLR9 antagonist-inhibitory oligonucleotide (5'-ttt agg gtt agg gtt agg gtt agg g-3') was used to suppress the TLR9 (Invivogen). Pepinh-MyD: a 26 amino acid

peptide that blocks MyD88 signalling by inhibiting its homodimerisation through binding. Pepinh-MyD contains a sequence homolog to the MyD88 TIR homodimerisation domain followed by a protein transduction sequence, which enables the peptide to translocate through the cell membrane. Pepinh-control: a nonfunctional control peptide structurally similar to Pepinh-MyD (used as a negative control). BAY11-7082: (E)-3-(4-Methylphenylsulfonyl)-2-propenenitrile ($C_{10}H_9NO_2S$) irreversible IKB kinase (IKK) inhibitor that inhibits IKB-α phosphorylation resulting in the inactivation of NFκB. SB203580: a Pyridinyl imidazole ($C_6H_{16}FN_3OS$) that suppresses the MAPK signalling by inhibiting p38 phosphorylation.

4.2.3 Plasmids and reagents

PNiFty2-SEAP, a NFκB-inducible reporter plasmid (Invivogen), and pNiFty3-SEAEP, an AP1-inducible reporter plasmid (Invivogen), were used to transfect the endometrial epithelial cells as a tool for measuring the amount of NFκB and AP1 activation in response to TLR9 ligation. Both PNiFty2-SEAP and PNiFty3-SEAP reporter plasmids contain a specific promoter for SEAP (Secreted Embryonic Alkaline Phosphatase) called Endothelial Leukocyte Adhesion Molecule (ELAM). ELAM promoter contains five NFκB and/or AP1 repeated transcription factor binding sites. Once NFκB and/or AP1 are attached to the ELAM, the promoter will be activated, which leads to the secretion of embryonic alkaline phosphatase from the transfected cells (Figure 4.1)

X-tremeGENE HP DNA transfection reagent (Roche Life Science, Burgess Hill, UK) was used to transfect the endometrial epithelial cells with plasmids. Briefly, RL95-2 and/or MCF-7 cells were cultured in 12 well plates (Greiner Bio-One, Stonehouse, UK) till 65% confluence, the media of each well was replaced with fresh serum free media, and the mix was prepared using 1:3 ratio of plasmid: transfection reagent in 50μl of serum free media. The mix was then added to each well for 24 hours. The Phospha-light System (a chemiluminescent reporter gene assay system for detection of alkaline phosphatase) (Applied Biosystems, Paisely, UK) was used to detect the amount of alkaline phosphatase in response to CpG in the supernatant of each sample of endometrial epithelial cells, as suggested in the manufacturer's protocol.

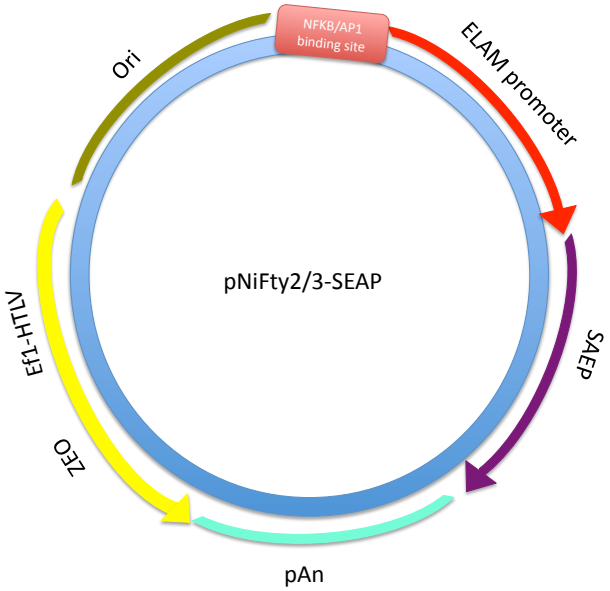


Figure 4.1 Schematic view of pNiFty 2 and/or pNiFty 3- SEAP reporter plasmid. ELAM promoter: Endothelial Leukocyte adhesion molecule with NFKB and/or AP1 binding sites. SEAP: reporter gene. Secreted alkaline phosphatase (SEAP) is a reporter widely used to study promoter activity or gene expression. SEAP expression can be rapidly and readily measured in supernatants of transfected cells. pAn: Human beta-globin 3' UTR, efficient for transgene transcription. Ef1-HTLV-ZEO: Zeocin antibiotic resistance gene with its promoter. Ori: Origin of plasmid replication.

4.2.4 RNA isolation, cDNA synthesis

Total RNA from the RL95-2 cells was extracted using TRI reagent (Sigma) following the exact protocol supplied by the manufacturer. The RNA was then treated twice with Dnase I (DNA-freeTM, Ambion, Austin, TX, USA) to remove the genomic DNA from the samples. cDNA synthesis was performed using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Paisley, UK) with 2μg of sample RNA following the manufacturer's protocol.

4.2.5 IRF7 primer optimization and validation of qPCR

qPCR was carried out with prepared cDNAs according to the MIQE guidelines. The IRF7 primer sequences were, forward, 5'-TGGTCCTGGTGAAGCTGGAA-3', and reverse, 5'-GATGTCGTCATAGAGGCTGTTGG-3' (Izaguirre et al., 2003). The results were normalized using two housekeeping genes as reference, β-actin (B.act), forward, 5'-CAAGATCATTGCTCCTCCTG-3', and reverse, 5'-ATCCACATCTGCTGGAAGG-3', B2M (Beta-2 microglobulin); forward, 5'-TATGCCTGCCGTGTGAACCA-3', and reverse, 5'-GCGGCATCTTCAAACCTCCA-3'. A standard curve was created using serial dilutions of the cDNA samples (1:5, 1:15, 1:45, 1:135 and 1:405) and plotted using the logarithm of the cDNA dilution versus the average threshold cycle number (C_t). The efficiency of the TLR9 primers was calculated using this formula:

$$E=10^{\{-1/slope\}}$$

The percentage of the efficiency was estimated as follows:

$$\%E=\{-1+(10^{\{-1/slope\}})\}*100$$

An efficiency of 80-120% for primers was considered suitable for analysing the gene expression.

4.2.6 Quantitative real-time PCR

SYBR Green Jump start *taq ready* mix (Sigma) was used to prepare a qPCR master mix (10 μl SYBR Green, 7 μl water, 1 μl of each forward and reverse primer, and 1 μl of cDNA in each well of the PCR plate). Amplification was carried out under the

following conditions: 40 cycles of 95° for 30 s, 62° for 1 minute, and 72° for 30 seconds. qPCR was performed with the help of Mx3005P QPCR (Stratagene, Agilent Technologies, Stckport, UK) and MxPro QPCR, version 4.01 software (Stratagene).

4.2.7 IL-8 ELISA

IL-8 ELISA was conducted using a commercial enzyme-linked immunosorbent assay kit (R&D systems, Abingdon, UK) as recommended by the manufacturer with 100µl of cell-free supernatant. This was explained in more detail in Chapter 3 (3.2.7).

4.2.8 *In vitro* human implantation assay

4.2.8.1 Formation of JAr spheroids

The process of the formation of JAr spheroids has been described in detail in Chapter 2 (2.2.4.1).

4.2.8.2 Formation of endometrial monolayer

MCF-7 and RL95-2 cells were cultured in T75 flasks (Greiner Bio-One) with supplemented DMEM-F12 (Sigma), as described in Chapter 2 (2.2.4.2).

4.2.8.3 Co-incubation of JAr spheroids with endometrial monolayer

The process of co-incubation of the JAr spheroids with endometrial epithelial cells has been described in detail in Chapter 2 (2.2.4.3).

4.2.8.4 Removal of unattached JAr spheroids and counting the attached spheroids

The process of the removal of unattached JAr spheroids and counting the attached spheroids has been explained in detail in Chapter 2 (2.2.4.4).

4.2.9 Experimental design

4.2.9.1 Determination of the effect of blocking the MyD88 mediator on the JAr spheroid attachment to the endometrial cells in the presence of CpG

To determine whether blocking the MyD88 mediator would affect the attachment of JAr spheroids to the endometrial epithelial cells, both RL95-2 and MCF-7 cells were grown in 12 well plates till 75% confluence. The media was then replaced with fresh serum free media containing 5μM Pepinh-MYD and/or Pepinh-control for 6 hours. Thereafter 0.01μM of CpG was added to the cells and incubated for 24 hours. JAr spheroids were then delivered to the endometrial epithelial monolayer and co-incubated for 1 hour. The spheroids were washed and counted as described elsewhere. The experiment was repeated three times on different days.

4.2.9.2 Determination of the effect of blocking the NFκB transcription factor on the JAr spheroid attachment to the endometrial cells in the presence of CpG

To determine whether blocking the NFκB transcription factor would affect the attachment of JAr spheroids to the endometrial epithelial cells, both RL95-2 and MCF-7 cells were grown in 12 well plates till 75% confluence. The media was then replaced with fresh serum free media containing 10μM BAY-11 for 30 minutes. Then 0.01μM of CpG was added to the cells and incubated for 24 hours. Thereafter, JAr spheroids were delivered to the endometrial epithelial monolayer and co-incubated for one hour. The spheroids were washed and counted as described elsewhere. The experiment was repeated three times on different days.

4.2.9.3 Determination of the amount of NFκB activation in endometrial epithelial cells in response to CpG

To determine whether NFκB would be activated in the epithelial endometrial cells in response to CpG, both RL95-2 and MCF-7 cells were grown in 12 well plates till 65% confluence. The cells were then transfected by 1μg/ml pNifty2 plasmid. On the next day, the media of the cells was replaced with fresh serum free media containing 0.01μM CpG or 100 ng/ml Flagellin (TLR5 ligand, as a positive control) and

incubated for another 24 hours. The supernatants were then collected and the amount of the secreted embryonic alkaline phosphatase (SEAP) production was detected by Phospha-light System, as described before. The final concentration of SEAP in the supernatant of each sample was measured using a luminometer (Berthold Detection Systems). Data were reported as the fold induction of SEAP activity over the non-stimulated control for MCF-7 cells and as row data for the RL95-2 cells. The experiment was repeated three times on different days.

4.2.9.4 Determination of the effect of blocking the p38 MAPK on the JAr spheroid attachment to the endometrial cells in the presence of CpG

To determine whether blocking the p38 MAPK would affect the attachment of JAr spheroids to the endometrial epithelial cells, both RL95-2 and MCF-7 cells were grown in 12 well plates till 75% confluence. The media was then replaced with fresh serum free media containing 10μM SB203580 for one hour. Thereafter, 0.01μM of CpG was added to the cells and incubated for 24 hours. JAr spheroids were delivered to the endometrial epithelial monolayer and co-incubated for one hour. The spheroids were then washed and counted as described elsewhere. The experiment was repeated three times on different days.

4.2.9.5 Determination of the amount of AP1 activation in endometrial epithelial cells in response to CpG

To determine whether AP1 would be activated in epithelial endometrial cells in response to CpG, both RL95-2 and MCF-7 cells were grown in 12 well plates till 65% confluence. The cells were then transfected by 1μg/ml pNifty3 plasmid. On the next day, the media was replaced with fresh serum free media containing 0.01μM CpG or 10 μg/ml Poly I:C (TLR3 ligand, as a positive control) and incubated for another 24 hours. The supernatants were then collected, and the amount of the secreted embryonic alkaline phosphatase (SEAP) production was detected by the Phospha-light System, as describe before. The final concentration of SEAP in the supernatant of each sample was measured using a luminometer (Berthold Detection Systems). Data were reported as raw data for both the MCF-7 and RL95-2 cells. The experiment was repeated three times on different days.

4.2.9.6 Determination of changes in IRF7 gene expression in endometrial epithelial cells in response to CpG

To determine whether IRF7 gene expression would change in endometrial epithelial cells in response to CpG, RL95-2 cells were grown till 75% confluence. The media were then replaced with fresh serum free media containing 0.01μM CpG and incubated for two and/or four hours. The total RNA was then extracted from the cells, cDNA was synthesized, and qPCR was performed, as described earlier.

4.2.9.7 Determination of the TLR9 signalling pathway in RL95-2 cells that leads to IL-8 production

In chapter 3 we showed that RL95-2 cells were only able to produce IL-8 when they were treated with a 1μM CpG concentration for 24 hours. To clarify the TLR9 downstream signalling pathway resulting in IL-8 secretion, the following experiments were designed.

RL95-2 cells were grown till 75% confluence. The media was then replaced with fresh serum free media containing 10μM SB203580 and /or BAY-11 and/or 5μM Pepinh-MYD peptides. Thereafter, 1μM CpG was added to the cells and incubated for 24 hours. The supernatants were then collected, and the amount of IL-8 production was measured by ELISA.

To determine any changes in IRF7 gene expression level in response to a higher dose of CpG, RL95-2 cells were grown till 75% confluence. The media was replaced with fresh serum free media containing 1μM CpG and incubated for 24 hours. The total RNA was then extracted from the cells, cDNA was synthesized, and qPCR was performed as described earlier.

4.2.10 Statistic analysis

The qPCR results were analysed with the $\Delta\Delta C_t$ method. The C_t of the gene of interest was normalized to a standard sample (consisting of a pool of cDNA of all the samples) and with the C_t of the reference genes:

$$\Delta\Delta C_t = \Delta C_t \text{ sample} - \Delta C_t \text{ reference}$$

The relative mRNA expression was then calculated by: $2^{-\Delta\Delta Ct}$.

The results were expressed as mean ± S.E.M. Statistical analysis was performed using ANOVA with Bonferroni's post hoc test or student t-test (for IRF7 expression only). $p<0.05$ was considered to be significant. Three replicates were performed in each experiment.

4.3 Results

4.3.1 IRF7 primer optimization

The efficiency of the IRF7 primers was confirmed via production of a standard curve and a dissociation curve. The primers for the reference genes had been optimized before in our lab with efficiencies of 96% and 94% for B.act and B2M respectively. The IRF7 primers showed an efficiency of 94.8% with a single amplification peak (Figure 4.2).

4.3.2 Blocking the MyD88 mediator restored the JAr spheroid attachment to the endometrial epithelial cells

Pre-treatment of both the MCF-7 and RL95-2 cells with MyD88 inhibitor restored the JAr spheroid attachment to the endometrial epithelial cells in the presence of CpG, while using the MyD88 control peptide could not suppress the CpG negative effect on JAr spheroid attachment (Figure 4.3).

4.3.3 Blocking the NFκB transcription factor could not restore the JAr spheroids attachment to the endometrial epithelial cells

Pre-treatment of both the MCF-7 and the RL95-2 cells with BAY-11 could not restore the JAr spheroid attachment to endometrial epithelial cells in the presence of CpG. Furthermore, endometrial epithelial monolayers pre-treated with NFκB inhibitor

solely showed no significant alteration in the percentage of attached JAr spheroids compared to the untreated controls (Figure 4.4).

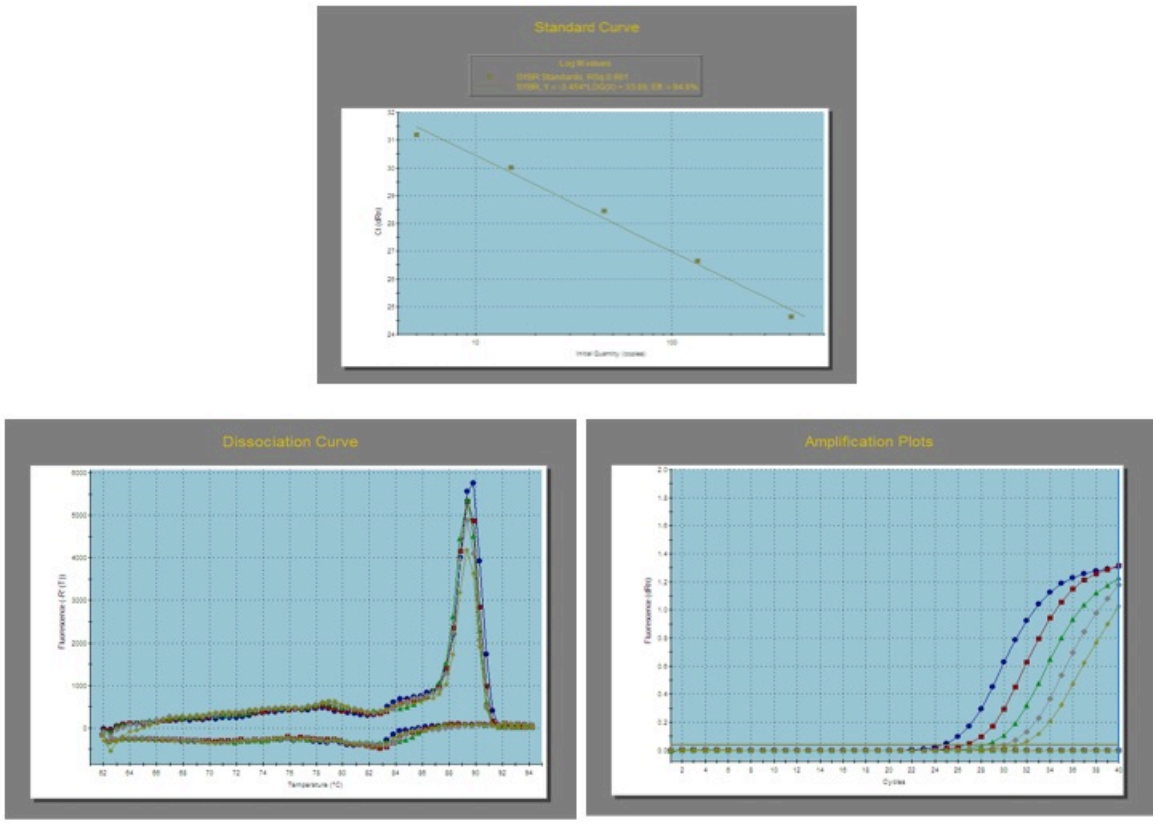


Figure 4.2 IRF7 Primer optimization. Amplification was carried out under the following conditions: 40 cycles of 95° for 30 s, 62° for 1 min, and 72° for 30 s. The upper photo shows the standard curve created using serial dilutions of the cDNA samples (1:5, 1:15, 1:45, 1:135 and 1:405) and plotted using the logarithm of the cDNA dilution versus the average threshold cycle number (C_t). The bottom-left photo represents the dissociation curve with only one peak specific to IRF7. The bottom-right photo shows the amplification plot of the different serial dilutions of the cDNA samples (1:5, 1:15, 1:45, 1:135 and 1:405).

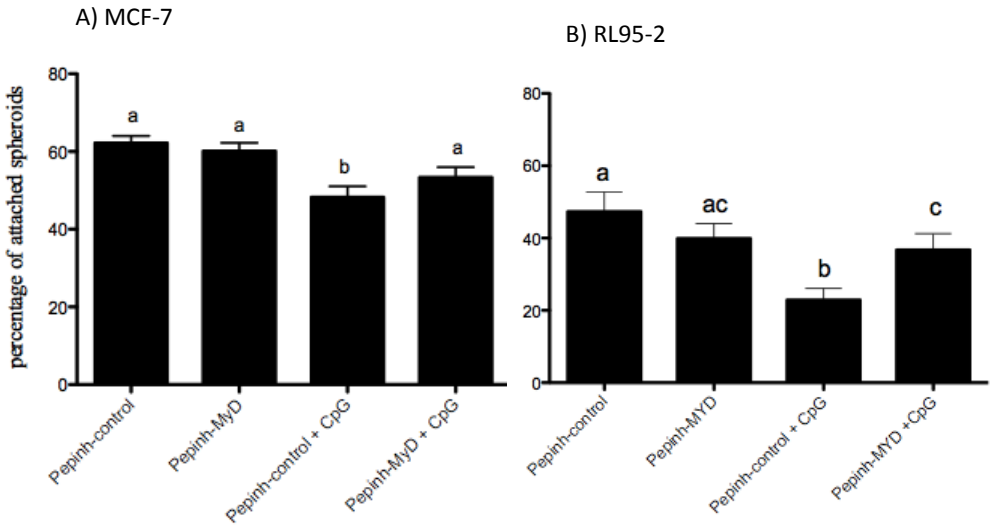


Figure 4.3 Blocking the MyD88 mediator restored the JAr spheroid attachment to the endometrial epithelial cells. Pre-treatment of both MCF-7 (A) and RL95-2 (B) cells with MyD88 inhibitor (Pepinh-MyD) restored the JAr spheroid attachment to the endometrial epithelial cells in the presence of CpG, while using the MyD88 control peptide (Pepinh-MyD) could not suppress the negative effect of CpG on JAr spheroid attachment. The data are the average of three independent experiments (n=9). The results are presented as the mean ± S.E.M. ANOVA was used to compare the percentage of the attached spheroids at each condition. Different letters indicate significant statistical differences. $p<0.05$ was considered to be significant.

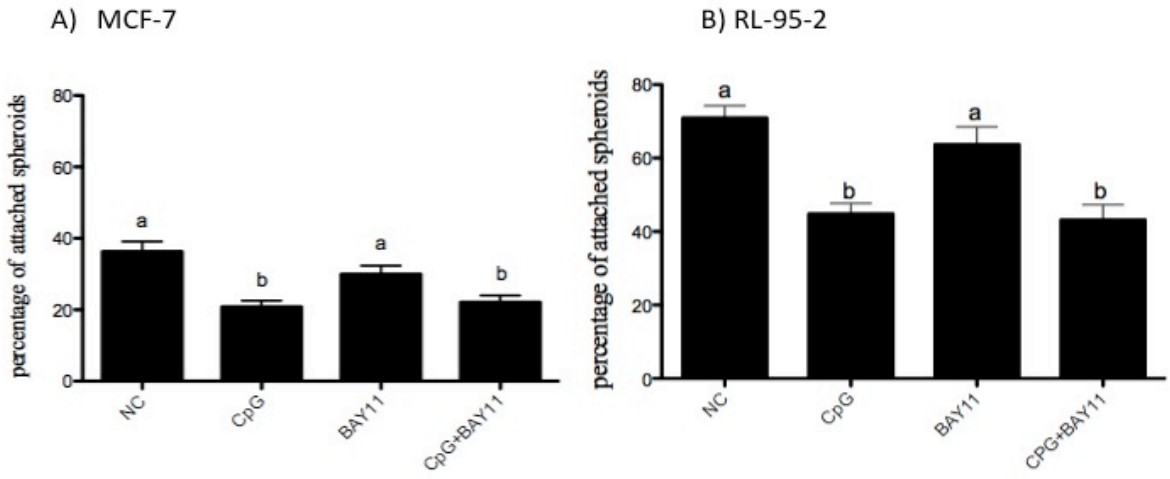


Figure 4.4, **Blocking the NFκB transcription factor could not restore the JAr spheroid attachment to the endometrial epithelial cells.** Pre-treatment of both the MCF-7 (A) and the RL95-2 (B) cells with BAY-11 could not restore the JAr spheroid attachment to endometrial epithelial cells in the presence of CpG. Furthermore, endometrial epithelial monolayers pre-treated solely with NFκB inhibitor showed no significant alteration in the number of attached JAr spheroids compared to the NC. NC: Negative control (untreated cells). The data are the average of three independent experiments (n=9). The results are presented as the mean ± S.E.M. ANOVA was used to compare the percentage of the attached spheroids at each condition. Different letters indicate significant statistical differences. $p<0.05$ was considered to be significant.

4.3.4 No NFκB activation was detected when the endometrial epithelial cells were stimulated with CpG

NFκB activation were not detected when both MCF-7 and RL95-2 cells were stimulated by CpG compared to the negative control (untreated cells). Yet a significant amount of NFκB was detected while the endometrial epithelial cells were stimulated with TLR5 ligand as a positive control (flagellin treated cells) (Figure 4.5).

4.3.5 Blocking the p38 MAPK restored the JAr spheroid attachment to the MCF-7 monolayer but not to the RL95-2

Pre-treatment of MCF-7 epithelial cells with p38 inhibitor restored the JAr spheroid attachment to their monolayer in presence of CpG. Furthermore, epithelial monolayers pre-treated solely with the inhibitor showed no significant alteration in the percentage of attached JAr spheroids compared to the control (untreated cells). Interestingly, blocking the p38 in RL95-2 cells could not suppress the detrimental effect of CpG on JAr spheroid attachment (Figure 4.6).

4.3.6 No AP1 activation was detected when the endometrial epithelial cells were stimulated with CpG

AP1 activation was not detected when both the MCF-7 and the RL95-2 cells were stimulated by CpG compared to the negative controls (untreated cells). Yet a significant amount of AP1 was detected while the endometrial epithelial cells were stimulated with TLR3 ligand as a positive control (Poly I:C treated cells) (Figure 4.7).

4.3.7 No changes in IRF7 gene expression level was seen when the endometrial epithelial cells were stimulated with CpG

The level of IRF7 gene expression did not change significantly in the RL95-2 endometrial epithelial cells when stimulated by CpG over two and over four hours when compared to the untreated cells (Figure 4.8).

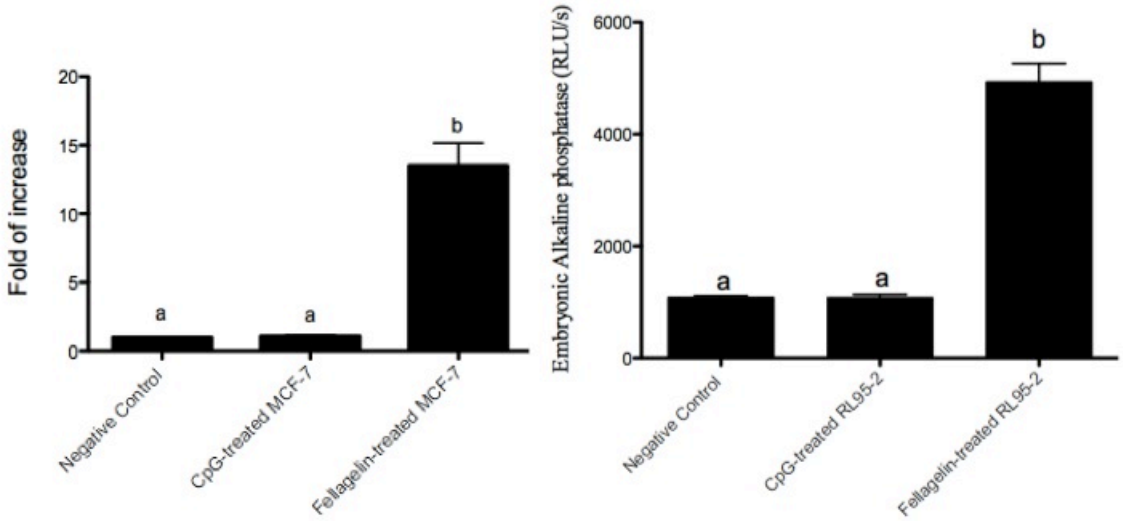


Figure 4.5, **No NFκB Activation was detected when the endometrial epithelial cells were stimulated with CpG.** NFκB activation had not been changed significantly when both the MCF-7 (left) and the RL95-2 (right) cells were stimulated by CpG compared to the negative control (untreated cells). Yet a significant amount of NFκB had been detected while the endometrial epithelial cells were stimulated with TLR5 ligand as a positive control. Data were reported as the fold induction of SEAP activity over the non-stimulated control for the MCF-7 cells and as row data for the RL95-2 cells. The data are the average of three independent experiments (n=9). The results are presented as the mean ± S.E.M. ANOVA was used to compare the amount of NFκB activation in each condition. Different letters indicate significant statistical differences. $p<0.05$ was considered to be significant.

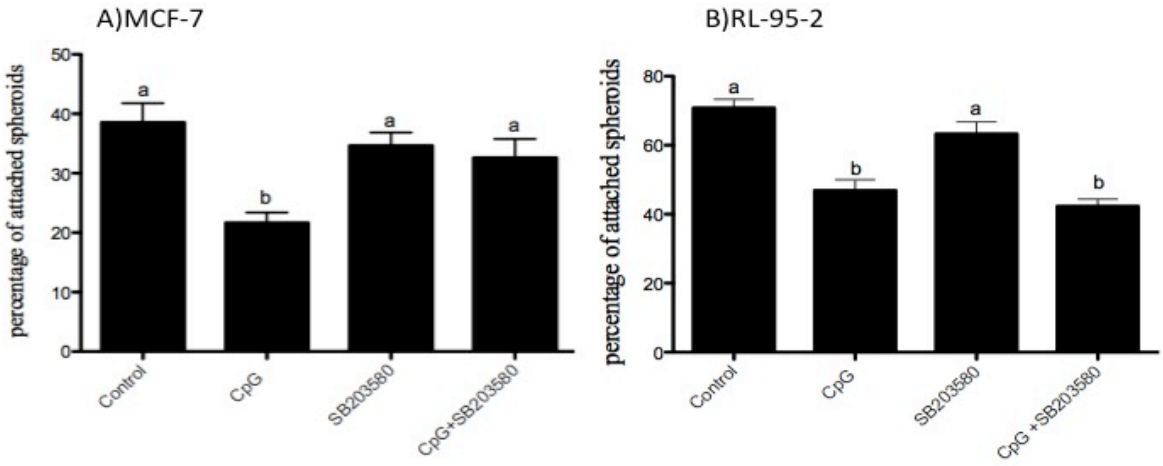


Figure 4.6, **Blocking the p38 MAPK restored the JAr spheroids attachment to the MCF-7 monolayer but not to the RL95-2.** Pre-treatment of MCF-7 epithelial cells (A) with p38 inhibitor restored the JAr spheroid attachment to their monolayer in the presence of CpG. Furthermore, epithelial monolayers pre-treated with the inhibitor solely showed no significant alteration in the number of attached JAr spheroids compared to the control (untreated cells). Interestingly, blocking the p38 in the RL95-2 cells (B) could not suppress the detrimental effect of CpG on JAr spheroid attachment. The data are the average of three independent experiments (n=9). The results are presented as the mean ± S.E.M. ANOVA was used to compare the percentage of the attached spheroids in each condition. Control = untreated cells. Different letters indicate significant statistical differences. $p<0.05$ was considered to be significant.

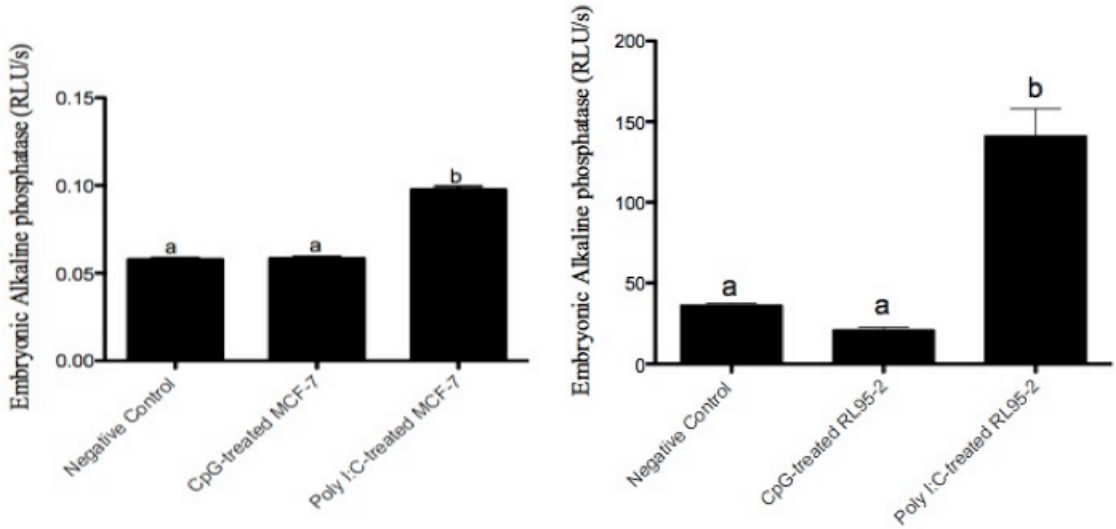


Figure 4.7, **No AP1 activation was detected when the endometrial epithelial cells were stimulated with CpG.** AP1 activation was not changed significantly when both MCF-7 (left) and RL95-2 (right) cells were stimulated by CpG compared to the negative control (untreated cells). Yet a significant amount of AP1 was detected while the endometrial epithelial cells were stimulated with TLR3 ligand as a positive control. The data are the average of three independent experiments (n=9). The results are presented as the mean ± S.E.M. ANOVA was used to compare the amount of AP1 activation at each condition. Different letters indicate significant statistical differences. $p<0.05$ was considered to be significant.

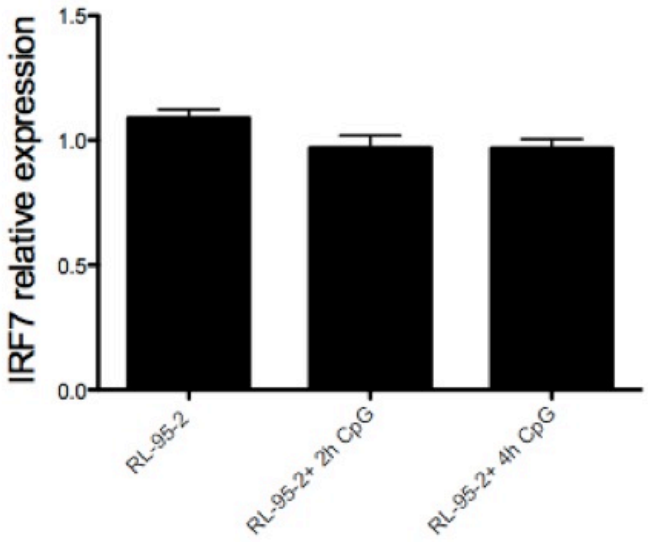


Figure 4.8, **No changes in IRF7 gene expression was seen when the endometrial epithelial cells were stimulated with CpG.** IRF7 gene expression was not changed significantly in the RL95-2 endometrial epithelial cells when stimulated by CpG over two and four hours compared to the non-stimulated RL-95-2. The data are the average of three independent experiments (n=9). The results are presented as the mean ± S.E.M. ANOVA was used to compare the gene expression in each condition. $p<0.05$ was considered to be significant.

4.3.8 IL-8 production in response to CpG is MyD-88 signalling pathway dependent, but it is not dependent on the NFκB and p38 MAPK signalling pathways

IL-8 production in response to 1μM dose of CpG in the RL95-2 cells was suppressed when the MyD88 mediator was inhibited, while when the endometrial epithelial monolayers were pre-treated solely with the inhibitor, no significant alteration in IL-8 production was seen compared to the untreated RL95-2 cells (Figure 4.9). Additionally, IL-8 production through the TLR9 receptor in RL95-2 cells did not cease when both NFκB and p38 MAPK were inhibited (Figure 4.10). Furthermore, IRF7 gene expression was not significantly altered in RL95-2 endometrial epithelial cells when stimulated by high doses of CpG after 24 hours compare to the non-stimulated RL95-2 cells (Figure 4.11).

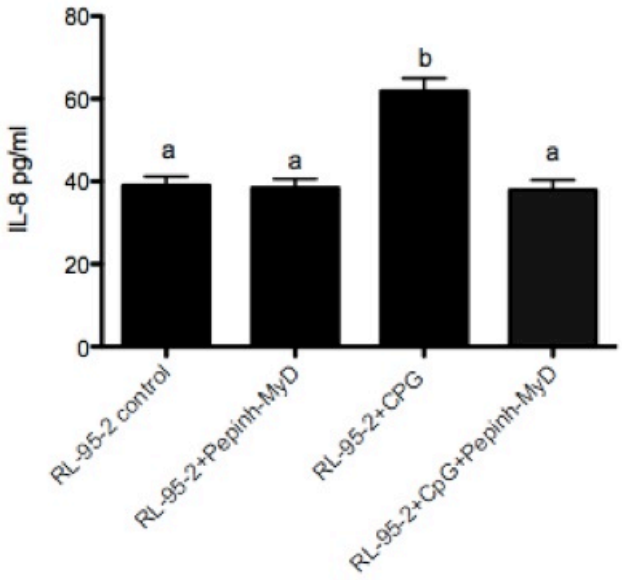


Figure 4.9, **IL-8 production in response to CpG is MyD-88 dependent.** IL-8 production in response to high doses of CpG in RL95-2 cells was suppressed when MyD88 mediator was inhibited, while endometrial epithelial monolayers solely pre-treated with the inhibitor showed no significant alteration in IL-8 production. RL-95-2 control= untreated RL-95-2 cells. The data are the average of three independent experiments (n=9). The results are presented as the mean ± S.E.M. ANOVA was used to compare the amount of IL-8 production at each condition. Different letters indicate significant statistical differences. $p<0.05$ was considered to be significant.

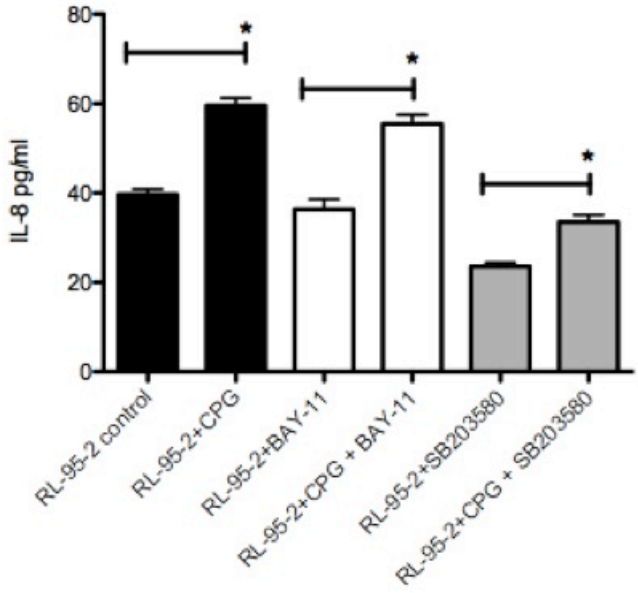


Figure 4.10, **IL-8 production in response to CpG was not dependent on NFκB and MAPK.** IL-8 production through TLR9 in RL95-2 cells did not cease when both NFκB and p38 MAPK were inhibited. RL-95-2 control= untreated RL-95-2 cells. The data are the average of three independent experiments (n=9). The results are presented as the mean ± S.E.M. ANOVA was used to compare the amount of IL-8 production at each condition. * indicates significant statistical differences. $p<0.05$ was considered to be significant.

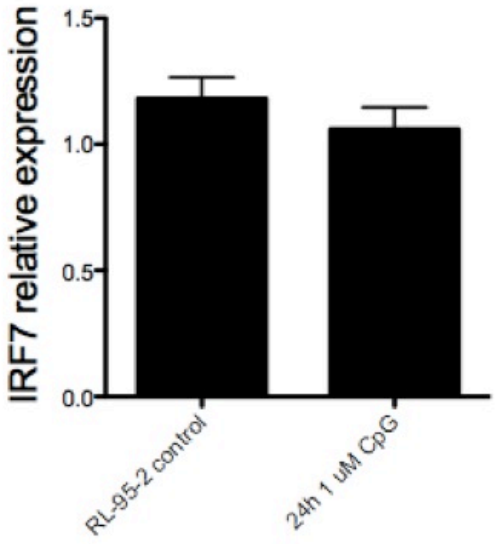


Figure 4.11, **No changes in IRF7 gene expression were seen when the endometrial epithelial cells were stimulated with CpG.** IRF7 gene expression was not changed significantly in the RL95-2 endometrial epithelial cells when stimulated by high doses of CpG after 24 hours. RL-95-2 control= untreated RL-95-2 cells. The data are the average of three independent experiments (n=9). The results are presented as the mean ± S.E.M. The Student t-test was used to compare the gene expression in each condition. $p<0.05$ was considered to be significant.

4.4 Discussion

A growing body of evidence suggests three possible intracellular signalling pathways for the TLR9 receptor that are strongly dependent on the cell type and the amount of the stimuli (Kawai and Akira, 2006). However, all these possible pathways are initiated with the Myeloid differentiation primary response gene 88 (MyD88) as the main mediator (Moynagh, 2005, Ning et al., 2011, Honda et al., 2004, Hirsch et al., 2010). Here we showed that blocking the MyD88 mediator restored the JAr spheroid attachment to the endometrial monolayer in the presence of 0.01 μM CpG. The fact that MyD88 is essential for CpG recognition was discovered in late 2000 (Schnare et al., 2000). Later on, using MyD88 -/- mice macrophages, Kaisho and Akira proved that the TLR9 signalling pathway is totally dependent on MyD88, and the cytokine secretion completely ceases in the absence of MyD88 (Kaisho and Akira, 2001). Inhibition of MyD88 downstream signalling in this study was achieved by the application of a cell penetrating peptide (CPP) inhibitor. CPPs are small peptides that have the ability to enter cells. Once they are linked to an inhibitory molecule, they can be used to target many intracellular processes. An important feature of CPPs is that they can deliver their cargo not only *in vitro* but also *in vivo* (Orange and May, 2008, Bechara and Sagan, 2013). In particular, the peptide used in this study, Pepinh-MYD, was derived from the Toll/interleukin-1 receptor homology (TIR) domain located within the BB (second β-strand and second helix) loop of MyD88. It contained a protein transduction sequence derived from antennapedia, which enables the peptide to translocate through the cell membrane (Loiarro et al., 2005). Loiarro and colleagues have shown that suppression of MyD88 with a specific CPP-inhibitor that prevented MyD88 homodimerization remarkably reduced the amount of NFκB secretion in HeLa cells (Loiarro et al., 2005). Also, the application of Pepinh-MYD in the cardiomyocyte cell culture totally stopped NF-κB translocation from the cytoplasm into the nucleus and reduced the secretion of proinflammatory cytokines and type I-IFN (Pan et al., 2011). Our findings regarding MyD88 confirm that the TLR9 signalling pathway in our 2D endometrial culture system is strongly dependent on the MyD88 mediator.

Blocking the NFκB transcription factor could not restore the JAr spheroid attachment to the endometrial epithelial cells. Furthermore, no NFκB activation was detected

when both MCF-7 and RL95-2 cells were stimulated with 0.01 μM of CpG. Although NFκB was introduced as a universal mediator that is activated in nearly all TLR signalling pathways (Moynagh, 2005), it seems, however, that TLR5, and not TLR2 or TLR2/6, stimulation was able to activate NFκB in both RL95-2 and Ishikawa 3H-12 cell lines (Caballero et al., 2013, Sanchez-Lopez et al., 2014). Furthermore, NFκB activation in response to TLR9 ligation has been found to vary from cell to cell and also to be dependent on the type and quantity of CpG (Li et al., 2007, Li et al., 2004, Takeda et al., 2011, Sathe and Reddy, 2014). In this regard, stimulation of human corneal endothelial cells with CpG resulted in upregulation of NFκB gene's promoter, whereas TLR9 activation in a human colorectal adenocarcinoma cell line (HT-29) resulted in activation of MAPK (Takeda et al., 2011, Akhtar et al., 2003). Our results confirmed TLR9 signalling variation between the cells, since we showed that NFκB was not involved in the process of the reduction of JAr spheroid attachment to both MCF-7 and RL95-2 cells in the presence of CpG.

Blocking the p38 MAPK restored the JAr spheroid attachment to the MCF-7, but not to the RL95-2, monolayer, although no AP1 activation was detected when both MCF-7 and RL95-2 cells were stimulated with 0.01 μM of CpG. MAP kinases are vital elements in intracellular signalling to control various cell behaviours such as proliferation, differentiation, and cell death. Based on the sequence of their activation loops, MAP kinase families have different members in mammalian cells, extracellular signal-regulated kinases (ERK1 and ERK2), c-Jun N-Terminal Kinases (JNKs), and p38 (Pearson et al., 2001). Although it seems that MAP kinase activation is cell-type specific in response to CpG (Hacker et al., 1999, Hacker et al., 1998), p38 is the most common MAPK involved in the TLR9 signalling pathway (Yi and Krieg, 1998, Li et al., 2004, Parilla et al., 2006). Hence an established p38 inhibitor (SB 203580) was used to block the TLR9-induced MAPK pathway (Campbell et al., 2013, Khan et al., 2004). During the human implantation process, when the trophoblast cells try to penetrate into the endometrial epithelium, they emit apoptosis signals to surrounding endometrial epithelial cells. This apoptosis signalling cascade is mediated by MAPKs in endometrial epithelial cells (Scherle et al., 2000, Li et al., 2003). Inhibiting the p38 MAPK in RL95-2 before co-culturing them with trophoblast spheroids suppressed the spheroid outgrowth over the endometrial monolayer. This indicated that trophoblast-

induced apoptosis in RL95-2 cells is mediated by MAPKs (Li et al., 2003). Using the same inhibitor in RL95-2 cells while co-culturing them with trophoblast spheroids at the time of TLR9 stimulation did not suppress the negative effect of CpG on spheroid attachment to epithelial cells. This may indicate that although p38 seems to mediate different signalling pathways in RL95-2 cells, it is not involved in the TLR9 signalling cascade. In contrast to the RL95-2 cells, p38 is found to be involved in the TLR9 signalling cascade, leading to a reduction of JAr spheroid attachment to the epithelial cells in response to CpG in MCF-7 cells. Hence one can speculate that the CpG-induced TLR9 intracellular signalling pathway in MCF-7 cells is initiated from MyD88 and that it continued downstream to the p38 MAP kinase, which results in a detrimental effect on JAr-MCF-7 interaction in our 2D endometrial culture system.

Activating protein 1 (AP1) is another transcription factor downstream of MAPKs. It is suggested to be involved in the TLR9 intracellular signalling pathway. Once activated by MAPKs, AP1 will be translocated into the nucleus and induce inflammatory responses (Kawai and Akira, 2006). Yet in our hands, TLR9 ligation with 0.01 μM of CpG was not able to induce AP1 activation in either MCF-7 or RL95-2 cells.

The IRF7 gene expression level did not change in the endometrial epithelial cells when stimulated with CpG based on qPCR results. Interferon regulatory factor 7 (IRF7) is a transcription factor located downstream of MyD88. IRF7 is activated by Interleukin-1 receptor-associated kinase 1 (IRAK1). After phosphorylation and dimerization, IRF7 is translocated into the nucleus (Kawai and Akira, 2006). IRF7 usually remains latent at the basal level in the cytoplasm of most cell lines in the normal condition. At the time of infection, the latent IRF7 will be changed to a phosphorylated form, translocated to the nucleus, and will induce a small amount of type I interferons, which has a positive regulatory feedback on IRF7 gene expression. Hence more IRF7 means more type I IFNs. According to the short half-life of the IRF7 protein (one hour in Epstein-Barr virus (EBV) stimulated cells and five hours in normal cells) (Ning et al., 2011), we expected an increase in IRF7 gene expression at the time of TLR9 stimulation. Although we have not checked the IRF7 protein levels in response to CpG, there were no changes in IRF7 gene expression levels when RL95-2 cells were stimulated with different concentrations of CpG. We have not

checked the IRF7 inhibitory effect on JAr spheroid attachment to the endometrial epithelial cells, as, to the best of our knowledge, there is no commercial inhibitory protein available for IRF7.

TLR9 ligation with 0.01 μm CpG in both MCF-7 and RL95-2 cells was enough to negatively affect JAr spheroid attachment to the endometrial epithelial cells in the 2D endometrial culture system. However, this amount of CpG was not sufficient for the endometrial epithelial cells to induce IL-8 production. At least 1μm CpG for 24 hours was needed for endometrial epithelial cells to produce IL-8. Type I interferons and IL-8 are the most common inflammatory molecules released at the time of TLR9 stimulation with usually higher doses of ligands and longer periods of pre-treatment (Andersen et al., 2006, Sathe and Reddy, 2014, Greene et al., 2005, Akhtar et al., 2003, El Kebir et al., 2009, Erridge et al., 2008, Li et al., 2004, Gaajetaan et al., 2012, Xu et al., 2012, Steinhagen et al., 2013, Wan et al., 2011, Yu et al., 2011, Rasmussen et al., 2007). While IL-8 is usually known as the end product of the TLR9-MyD88-AP1/NFκB signalling cascade (Andersen et al., 2006, Pedersen et al., 2005, Akhtar et al., 2003), Type I IFNs are usually the end products of the TLR9-MyD88-IRF7 pathway (Kawai et al., 2004, Honda et al., 2004, Xu et al., 2009). Our findings indicated that in contrast to MyD88, neither NFκB nor p38/AP1 are involved in IL-8 production in response to 1 μM of CpG in RL95-2 cells. Therefore, the possibility of another unknown TLR9 signalling pathway in response to different concentrations of CpG can be a point for future investigation.

To conclude, pre-treatment of RL95-2 cells with 0.01μM of CpG was able to inhibit JAr spheroid attachment to the endometrial epithelial cells through none of the known TLR9 intracellular signalling pathways, while pre-treatment of MCF-7 cells with the same dose of CpG was able to inhibit JAr spheroid attachment to the endometrial epithelial cells through the p38 MAP kinase pathway. Furthermore, IL-8 production from RL95-2 cells in response to 1μM of CpG was not dependent on either NFκB or p38, but it was definitely dependent on MyD88 mediation. To sum up, our findings indicated that there may be another TLR9 intracellular signalling pathway that is currently unknown that mediates inhibition of JAr spheroid attachment to the endometrial epithelial cells when RL95-2 cells were stimulated with CpG.

Chapter 5: TLR9 activation affects the outgrowth and invasion of trophoblast spheroids in a 3-dimentional *in vitro* endometrial culture system

5.1 Introduction

Sexually transmitted infections (STI) are a major worldwide health problem. The human innate immune system combats STIs and other pathogens to maintain a healthy environment in the female reproductive tract (FRT) and to support its unique physiological characteristics such as menstruation, fertilization, implantation and pregnancy (Horne et al., 2008). The immune system recognizes microorganisms via pattern recognition receptors (PRRs) such as Toll-like receptors (TLR) (Akira et al., 2006). Up to now, 10 different types of TLRs have been discovered in humans. TLRs 1, 2, 5, 6 and 10 are extracellular, whilst TLRs 3, 7, 8 and 9 are intracellular (Akira et al., 2006). TLR structures are composed of a leucine-rich extra-membranous domain as the pathogen recognition component and a cytoplasmic Toll/interleukine-1 receptor (TIR) domain that is responsible for the initiation of the intracellular signalling cascade. TLRs recognize specific ligands from different microbial agents. In this regard, while TLR4 is stimulated by lipopolysaccharide (LPS) from the gram-negative bacterial cell wall, TLR5 senses bacterial flagellum TLR3 is sensitive to double strand RNA (dsRNA), while TLR7 and 8 are stimulated by single strand RNA (ssRNA). TLR9 is capable of recognizing short DNA sequences containing unmethylated cytosine-guanine nucleotides (CpG), but as of yet, no specific ligand has been detected for TLR10 (Akira et al., 2006, Aflatoonian and Fazeli, 2008). Once TLRs are stimulated by their specific ligands, an intracellular signalling pathway is initiated that leads to inflammatory responses such as the secretion of different cytokines and chemokines at the site of the infection (Kawai and Akira, 2006).

Among the 10 different members of the TLR family, TLR9 is responsible for recognition of DNA viruses such as the herpes simplex viruses (HSV), human cytomegalovirus (HCMV), the adenovirus, and *Chlamydia trachomatis* bacteria (Fredlund et al., 2004, Barton, 2007). Among these pathogens, *Chlamydia trachomatis* is more likely to affect women and is regarded as the main cause of fertility problems such as pelvic inflammatory disease (PID) and fallopian tube and endometrial infections (Fredlund et al., 2004, den Hartog et al., 2006, Paavonen and Eggert-Kruse, 1999). Activation of TLR9 receptors usually results in different responses from cells based on the cell type, the amount of the pathogen, or the dose of

the ligand. For example, it has been reported that stimulation of TLR9 by synthetic CpG in the human colorectal adenocarcinoma (HT-29) cell line triggered an intracellular signalling pathway through mitogen-activated protein kinases (MAPK), while ligation of TLR9 with *E. coli* DNA was able to activate the nuclear factor kappa B (NFκB) in human endocervical epithelial cells (Akhtar et al., 2003, Sathe and Reddy, 2014). Furthermore, it has been shown that stimulation of mouse plasmacytoid dendritic cells with 1μg/ml CpG leads to myeloid differentiation primary response gene 88 (MyD88)-NFκB-dependent interferon α (IFNα) secretion, but a 10-fold increase in the concentration of the ligand resulted in TIR-domain containing adapter-inducing interferon β (TRIF)-dependent type I IFN secretion (Volpi et al., 2013).

Although activation of TLR9 with CpG initiates an intracellular signalling pathway leading to inflammatory responses such as cytokine production at the site of the infection (Kawai and Akira, 2006), CpG is known to be a potential enhancer of epithelialization and the wound healing process (Aplin et al., 2014, Hergert et al., 2013, Sato et al., 2010, Yamamoto et al., 2011, Rose et al., 2012, Koff et al., 2006, Miller et al., 2005). Furthermore, CpG is also used to initiate vascular endothelial growth factor (VEGF) induced angiogenesis (Aplin et al., 2014, Sorrentino et al., 2011, Zheng et al., 2002, Yamamoto et al., 2011, Rose et al., 2012) and is a popular vaccine adjuvant, stimulating the innate immune system and initiating complicated adaptive immune responses (Furi et al., 2013, Li et al., 2015, Silva et al., 2015). Hence a TLR9/CpG combination not only has a role in pathogen recognition and pathogen removal, it may also modulate many other cellular activities.

Studying human implantation *in vivo* is almost unfeasible due to ethical issues. Hence *in vitro* systems and animal studies have been developed to allow detailed investigation of human implantation. However, the knowledge gained from animal studies, including mice and primates, is not always extendible to humans due to differences in their reproductive systems (Weimar et al., 2013). Using two-dimensional (2D) cultures with the help of different human immortalized cell lines or primary cells is the most common *in vitro* method that has been used over the years to study human implantation (Weimar et al., 2013). Although 2D cell cultures are very

easy to use, time efficient and economical, since cells are simply cultured over the plastic tissue culture dishes, they have the disadvantage that they are not able to present what is really going on in real life. To overcome this issue, three-dimensional (3D) cell cultures have been created with the aim of mimicking *in vivo* conditions in more detail (Weimar et al., 2013, Evron et al., 2011, Wang et al., 2012). One important factor for *in vivo*-like behaviour of cells when cultured in 3D systems is the scaffold and matrix used to prepare such cultures. There are four key parameters to be considered when preparing a 3D culture system: (i) dimensionality, the advantages of using 3D versus 2D *in vitro* cultures; (ii) rigidity, the mechanical properties of the matrix to regulate cytoskeleton organization and cellular migration; (iii) fibrillarity in the *in vivo* like structure, such as the fibrillar structure of the collagen that regulates adhesion molecules and cell migration; (iv) chemistry, the two-way signalling between matrix and cells (paracrine activity). When designing a 3D culture system, it is important to choose a model that meets all these criteria. *In vitro* 3D culture systems are mainly divided into two models: 3D gel, in which cells are embedded into the matrix; 3D surface, in which cells are seeded on top of the basal nanofibrillar surface. In this regard, culturing fibroblasts and osteoblasts in a surrounding layer provided by the gel would be the most representative of the *in vivo* environment, while culturing cells with polarity characteristics such as epithelial and endothelial cells on top of the basal surfaces would represent the *in vivo*-like environment (Page et al., 2013, Ahmed et al., 2008, Schindler et al., 2006). The 3D culture system used in this investigation was built based on a mixture of human plasma and agarose type VII. Plasma carries nutrients, hormones and proteins needed for cell growth and differentiation. Plasma also provides rigidity to the culture; therefore, it has been used as the nutrition and stiffness source of the 3D culture. Agarose type VII resembles the collagen-like fibrillarity and is used as the structural source of the 3D culture (Wang et al., 2012, Wang et al., 2013). Figure 5.1 deciphers some of the different 3D culture systems used by scientists.

In the current investigation, we tried to clarify the effect of TLR9 stimulation on human implantation by developing two different (2D and 3D) endometrial culture systems, and demonstrated that stimulation of TLR9 with CpG decreased the percentage of JAr spheroid attachment to the endometrial cells in a dose dependent manner in the 2D culture system. However, TLR9 stimulation with a very low dose of

CpG in the apical part of the 3D endometrial culture system had a supportive effect on trophoblast outgrowth and invasion into the endometrial cells. This further exemplifies the double-edged sword effect of TLR9 simulation observed in other tissue and cell systems and its probable application as an implantation-promoting factor.

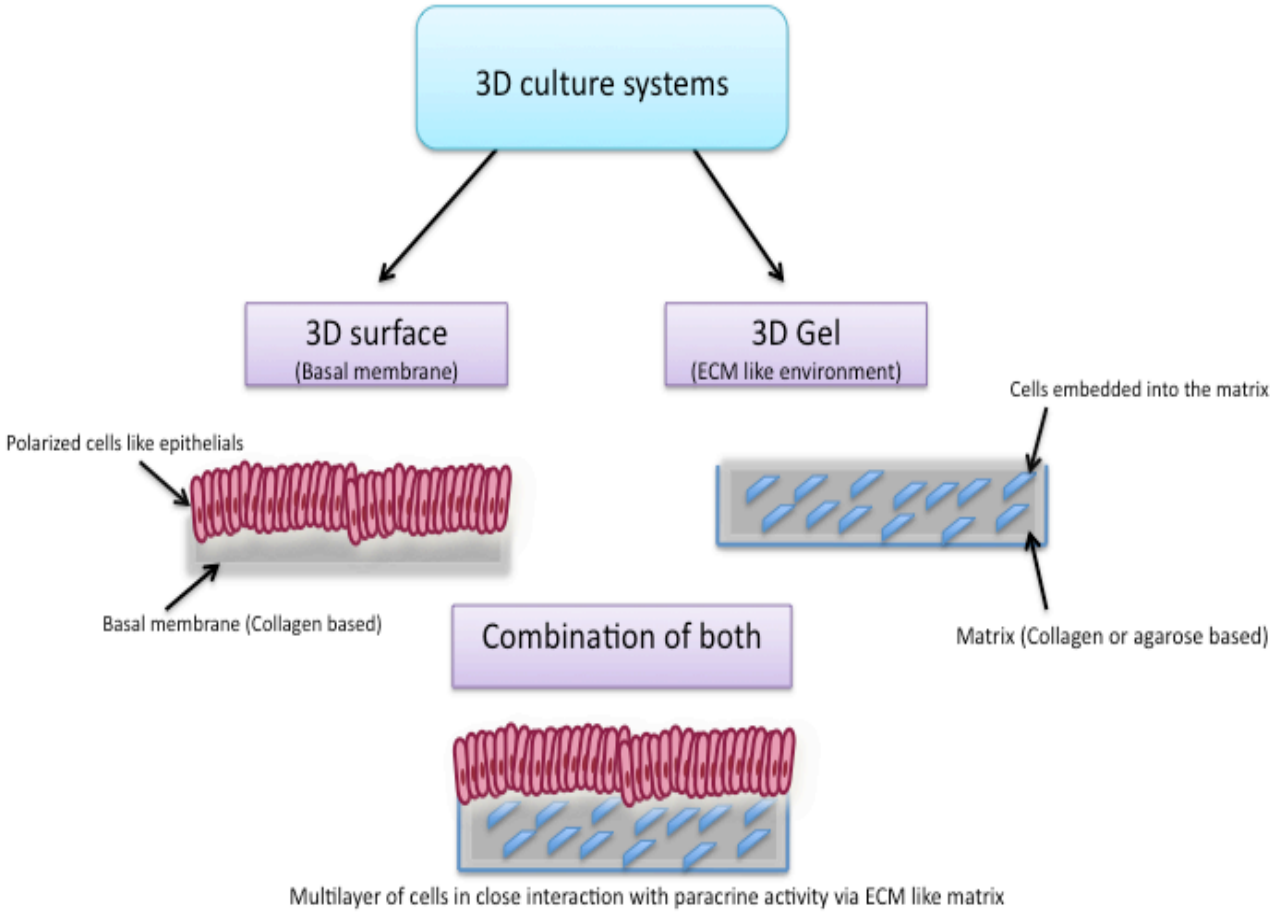


Figure 5.1 Different available 3D culture systems. *In vitro* 3D culture systems are mainly divided into two models, 3D gel, in which cells are embedded into the collagen or agarose based matrix, and 3D surface, in which cells are seeded on top of the basal nanofibrillar surface. Here, in this study, a combination of both methods has been used to prepare an *in vivo*-like human endometrium, where the stromal cells were embedded into the agarose based gel while the epithelial cells were laid over the matrix.

5.2 Material and methods

5.2.1 Cell Culture

A human endometrial adenosquamous carcinoma cell line (RL95-2), a human choriocarcinoma cell line (JAr) derived from the first trimester trophoblast cells, and an immortalized human endometrial stromal cell line (HESC) were purchased from American Type Culture Collection (ATCC, Teddington, UK). RL95-2 cells were used to represent a receptive endometrium, while JArs were used to simulate the human embryo trophoblast.

RL95-2 cells were cultured at 37°C in Dulbecco-modified Eagle medium (DMEM-F12) (Sigma, Dorset, UK), supplemented with 10% Fetal Bovine Serum (FBS) (Sigma), 1% L-glutamine (Sigma), 0.005 mg/ml insulin (human recombinant insulin, Gibco, Life technologies, Paisley, UK) and 1% penicillin/streptomycin (Sigma) in a 5% CO2 atmosphere until confluent. The JAr cells were grown in RPMI (Roswell Park Memorial Institute) 1640 (Sigma) supplemented with 10% FBS (Sigma), 1% L-glutamine (Sigma), and 1% penicillin/streptomycin (Sigma) in T75 flasks in a 5% CO2 atmosphere until confluent. HESC cells were grown at 37°C in a phenol red free DMEM-F12 (Gibco) media, supplemented with 10% Charcoal stripped Foetal Bovine Serum (Sigma), 1% Insulin-Transferrin-Selenium (ITS) (Gibco), and 500ng/ml puromycin (Gibco) in T75 flasks in a 5% CO2 atmosphere until confluent. At confluency, the cells were washed with Ca^{+2} and Mg^{+2} free Dulbecco's phosphate-buffered saline (PBS) (Sigma), harvested using trypsin-EDTA (Sigma), and pelleted by centrifugation at 300 g for four minutes.

5.2.2 Preparation of JAr spheroids

The process of the formation of the JAr spheroids has been described in detail in Chapter 2 (2.2.4.1)

5.2.3 Formation of two-dimensional endometrial culture

The process of the formation of the two-dimensional endometrial culture (monolayer) has been explained in detail in Chapter 2 (2.2.4.2).

5.2.4 Co-incubation of JAr spheroids with 2D endometrial culture

The process of the co-incubation of the JAr spheroids with the endometrial monolayer has been described in detail in Chapter 2 (2.2.4.3).

5.2.5 Removal of unattached JAr spheroids and counting the attached spheroids

The process of the removal of the unattached JAr spheroids and counting the attached spheroids has been explained in detail in Chapter 2 (2.2.4.4).

5.2.6 Preparation of the three-dimensional embryo implantation in the *in vitro* model

The 3D culture system was a modified version of that of (Wang et al., 2013). Figure 5.2 depicts a schematic diagram describing the different steps involved in setting up the 3D embryo implantation *in vitro* model. A confluent T75 flask of HESCs containing nearly 6 x 10^6 cells was washed with PBS trypsinized and pelleted by centrifugation. The pellet was then resolved in 105 μl of phenol red free DMEM-F12 media (Gibco). An aliquot of the re-suspended cells (35 μl, nearly 2.0 x 10^6 cells) was added to 425 μl of human plasma (Sigma) and gently mixed. Thereafter, 7.5ul of 50 mg/ml tranexamic acid (Sigma) was added to the cells mixture. Meanwhile, low gelling temperature agarose (Type VII) (Sigma) that was dissolved in PBS to make a 2% W/V gel was melted and kept in a 38-40° water bath to cool it down. A volume of 25 μl of the agarose gel plus 7.5 μl of 200mM $CaCl_2$ was then added to the cells and the plasma mixture, which was shaken gently. This preparation constituted the base layer of the 3D matrix. The prepared base layer of the 3D matrix was immediately divided among five inserts, 100μl into each insert (12 mm transparent polystyrene inserts for 12 well plate culture dishes with 0.4 μm pores, Greiner Bio-One). The inserts were then placed inside the wells of a 12 well plate culture dish (Greiner Bio-One) and allowed to solidify at 37 ° for 45 minutes. Immediately after solidification of the gel, 500 ml of phenol red free DMEM-F12 media was added to the bottom of the culture well in the basolateral area of the 3D culture system.

After five hours, RL95-2 cells in a confluent T75 flask were washed with PBS, trypsinized, and pelleted by centrifugation. 750,000 of the cells were counted and seeded on top of the gel in each of the inserts in a total volume of 200 μl DMEM-F12

media and co-cultured for 24 hours. The next day, depending on the type of experiments performed, 30 to 50 JAr spheroids were gently transferred to the upper surface of the 3D Endometrial Epithelial-Stromal Cell Culture (3DEESCC).

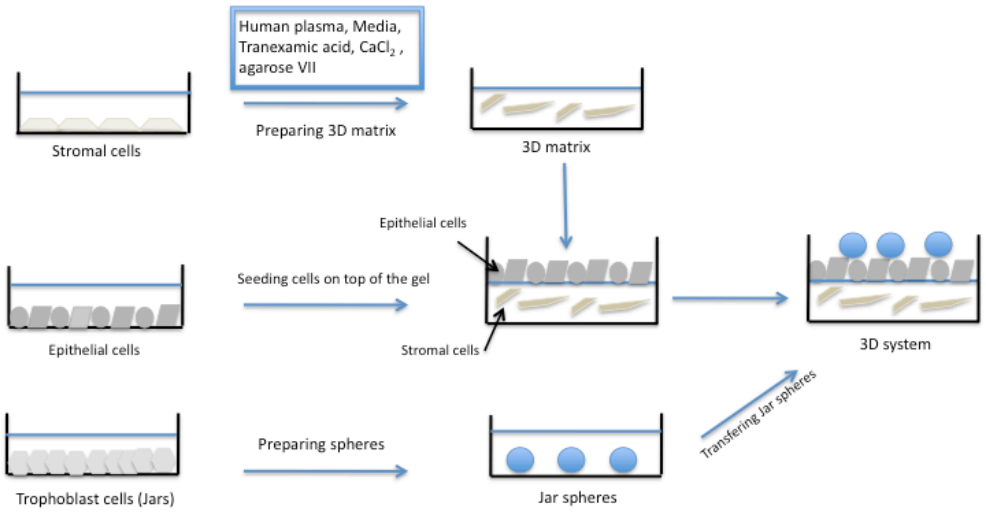


Figure 5.2 Schematic view of different steps of 3D Endometrial Epithelial-Stromal Cell Culture (3DEESCC) preparation. Stromal cells were embedded into the fibrin-agarose matrix, and the endometrial epithelial cells have been seeded on top of the matrix to form a tight monolayer. JAr spheroids were prepared and added on top of the 3D system.

5.2.7 JAr spheroid outgrowth analysis on top of the 2D endometrial culture and/or 3DEESCC

To aid visualization of the JAr spheroids' outgrowth, approximately 3,000 JAr spheroids were suspended in 1ml serum free RPMI media containing 1 ng/ml of Hoechst dye (Hoechst 33342, Life Technologies) for 45 minutes. The excess dye was omitted by washing the spheroids once with BPS. 30 JAr spheroids were then gently transferred on top of the endometrial monolayer and/or 3D culture and incubated for one hour. The stained spheroids were then located and monitored with an inverted fluorescent microscope (Olympus CKX41, Southend-on-Sea, UK) over that time. Pictures were taken with the help of a camera (Nikon DS-Fi1, Surrey, UK) connected to the microscope after 1, 24, 48 and 72 hours of the co-incubation of the spheroids with the endometrial monolayer and /or 3DEESCC using 4x magnification. The area of each spheroid outgrowth was then measured by ImageJ 1.47v software (Wayne Rasband, National institutes of Health, USA).

5.2.8 JAr spheroid invasion analysis into the 3DEESCC

Confocal microscopy was used to investigate the invasion of JAr spheroids into the 3DEESCC system. HESC cells were cultured in T75 flasks till confluent; the cells were then harvested and pelleted by centrifugation. The pellet was re-suspended in 1ml serum free DMEM-F12 (Gibco) media containing 50 μM of Blue cell tracker (Violet BMQC, Life Technologies) and incubated for 45 minutes. The cells were then washed once with PBS to omit the excess dye. Stained HESC cells and endometrial epithelial cells were used to prepare the 3DEESCC, as described before. Approximately 3,000 JAr spheroids were suspended in 1ml serum free RPMI media containing 10 μM Deep Red cell tracker (Life Technologies) for 45 minutes. The spheroids were then washed once with PBS to omit the excess dye. Stained JAr spheroids were transferred on top of the 3DEESCC and the depth of the spheroids' invasion into the 3DEESCC was monitored with Z-images captured by 710 Laser Scanning Microscope (LSM 710) (Carl Zeiss, Cambridge, UK). Blue fluorescent was excited in 405nm wavelength with a diode laser; deep red fluorescent was exited in 630nm He-Ne laser and finally green fluorescent (from GFP) was exited in 488nm wavelength with an argon laser.

5.2.9 Creation of stable TLR9 knocked-down RL95-2 endometrial epithelial cells

In brief, the psiRNA-hTLR9 and psiRNA-lucGL3 (control) plasmids (InvivoGen, Toulouse, France) were amplified and transformed into the E. coli GT116 strain provided with the kit, following the protocol of the manufacturer (PsiRNA KIT, InvivoGen). To increase the quantity of DNA, transformed bacteria was cultured in Fast-Media Zeo provided with the kit in large scale (100ml) for 24 hours on a gyratory surface (≈300 rpm) at 37°. Plasmids were then extracted using a plasmid DNA purification kit (Plasmid Plus Midi Kit, QIAGEN, Manchester, UK) based on the manufacturer's protocol. The final concentration of each plasmid was measured using a nanophotometer (GENFLOW, Lichfield, UK). Both the control and the siRNA-hTLR9 plasmids were digested by XbaI restriction enzyme (Biolabs New England, Ipswich, MA, USA). This allowed linearization and random insertion of vectors into the genome of the cells for the purpose of creating stable transfected cell lines. RL95-2 cells were cultured in 12 well plate culture dishes (Greiner Bio-One) till 70% confluency. The cells were then transfected by 500 ng/ml of linearized control or siRNA-hTLR9 plasmid in separate wells. 48 hours post transfection, confluent RL95-2 cells were trypsinized and transferred into100x20 mm tissue culture dishes (Greiner Bio-One) with 8ml DMED-F12 media (Sigma) containing 500μg/ml of Zeocin antibiotic (InvivoGen) as a selective marker to obtain RL95-2 cell colonies that carried the linearized plasmids. Colony selection was performed using cloning cylinders (Sigma). Briefly, colonies of transfected cells were placed in the centre of the cylinders, trypsinized, harvested, and transferred into T25 cell culture flasks (Greiner Bio-One) to grow. Transfected RL95-2 cells were also visible under the fluorescent microscope, since both psiRNA-hTLR9 and control plasmids contained green fluorescent protein (GFP) coding sequence. Stably transfected cells, Toll-like receptor 9 knocked down RL95-2 cells (TLR9KD-RL95-2), and control plasmid transfected RL95-2 cells (CP-RL95-2), were kept in liquid nitrogen or in the culture for further experiments (detailed in Chapter 3).

ODN Type B CpG oligonucleotide-human TLR9 ligand (5'-tcgtcgtttgtcgttttgtcgtt-3') (Invivogen) was used to stimulate TLR9 in different experiments.

5.2.10 Experimental design

5.2.10.1 Determination of the effect of different concentrations of CpG on the JAr spheroids attachment to the 2D endometrial culture

To determine the effect of TLR9 activation with different concentrations of CpG on trophoblast attachment to the endometrium, RL95-2 cells were grown in 12 well plates to 70% confluence. The media was then replaced with serum-free media containing CpG at different concentrations (0, 0.001 and 1 μM) and incubated for four hours. Thereafter, 50 JAr spheroids were delivered to each well and co-incubated with the RL95-2 monolayer for one hour. The spheroids were then washed and counted, as described elsewhere. The experiment was repeated three times on different days.

5.2.10.2 Determination of the effect of TLR9 ligation on JAr spheroids outgrowth on top of the 2D endometrial culture

To investigate the effect of TLR9 ligation on the JAr spheroid outgrowth on top of the 2D endometrial culture, three different concentrations of CpG, 0 (control), 0.001, and 1 μM, were added to the RL95-2 monolayer. After four hours, 30 stained JAr spheroids were gently transferred to the upper surface of the 2D endometrial culture, and the amount of each spheroid outgrowth was monitored after 1, 24, 48 and 72 hours of co-incubation of the spheroids with the 2D endometrial culture. The experiments were repeated three times on different days.

5.2.10.3 Determination of the kinetics of the JAr spheroid outgrowth on the 3DEESCC over time

To determine the kinetics of the JAr spheroid outgrowth on the 3DEESCC over time, JAr spheroids were stained with Hoechst dye as described earlier. Thirty JAr spheroids were gently added to the upper part of the 3DEESCC. The spheroids were then located and monitored under the inverted fluorescent microscope (Olympus) over time. Pictures were taken after 1, 24, 48 and 72 hours of co-incubation of the

spheroids with the 3DEESCC. The area of each spheroid outgrowth was measured as described before. The experiments were repeated three times on different days.

5.2.10.4 Determination of the effect of TLR9 ligation on JAr spheroid outgrowth on top of the 3DEESCC

To investigate the effect of TLR9 ligation on the JAr spheroid outgrowth on top of the 3DEESCC and the dependency of this effect on the presence of TLR9 in endometrial cells, RL95-2, CP-RL95-2 and TLR9KD-RL95-2 cells were used to prepare different 3DEESCC systems. Three different concentrations of CpG, 0 (control), 0.001 and 1 μM, were added to the apical, basolateral and both the apical and basolateral regions of the 3DEESCC. After four hours of pre-treating the 3DEESCC with CpG, 30 stained JAr spheroids were gently transferred to the upper surface of the 3DEESCC, and the amount of each spheroid outgrowth was monitored after 1, 24, 48 and 72 hours of co-incubation of spheroids with the 3DEESCC. In the case of TLR9KD 3DEESCC, the co-incubation time of the JAr spheroids was limited to 1, 24 and 48 hours only. The experiments were repeated three times on different days.

5.2.10.5 Confocal analysis of JAr spheroids invasion into the 3DEESCC over time

To determine the kinetics of the JAr spheroids invasion into the 3DEESCC over time, the amount of JAr spheroid invasion into the 3DEESCC was analysed using confocal microscopy. Approximately 50 stained JAr spheroids were transferred on top of the 3DEESCC. A stack of images (Z-stack) was captured at optimal distances (set automatically by the microscope) from the very bottom of the 3DEESCC to the top along the z-axis using a 10x Plan-Neofluar objective. Microscope settings were optimized using Zen 2010 software (Carl Zeiss, Cambridge, UK). The JAr spheroids were monitored at different time points (2, 10, 20, 30 and 40 hours post co-incubation of the spheroids with the 3DEESCC), and the exact amount of the invasion of each spheroid into the 3DEESCC was measured by analysing the z-stack images at each time point using the Zen software (2010 full version, Carl Zeiss).

Finally, the percentage of the invasion of each JAr spheroid was calculated by using the following formula:

Invasion distance from the surface of endometrial cells / Spheroid thickness **X** 100.

The experiment was repeated three times on different days (Figure 5.3).

5.2.10.6 Determination of the effect of TLR9 ligation on JAr spheroid invasion into the 3DEESCC

To investigate the effect of TLR9 ligation on the kinetics of JAr spheroids invasion into the 3DEESCC and the dependency of this effect on the presence or absence of TLR9 in the endometrial cells, two different 3DEESCC systems were prepared. One contained CP-RL95-2; the other contained TLR9KD-RL95-2. Three different concentrations of CpG, 0 (control), 0.001, and 1μM, were added to the apical, basolateral, and both the apical and basolateral regions of the 3DEESCC. After four hours of pre-treating of the 3DEESCC with CpG, 50 stained JAr spheroids were gently transferred to the upper surface of the 3DEESCC, and the amount of invasion of each spheroid into the 3DEESCC was analysed, as describe before. The experiment was repeated three times on different days.

5.2.11 Statistical analysis

Results were expressed as mean ± S.E.M. The statistical analysis was performed by SPSS software (IBM, Portsmouth, UK), using repeated measures ANOVA with Sidak Adjustments or Bonfferroni post hoc tests and regression coefficient analyses. $P<0.05$ was considered to be significant.

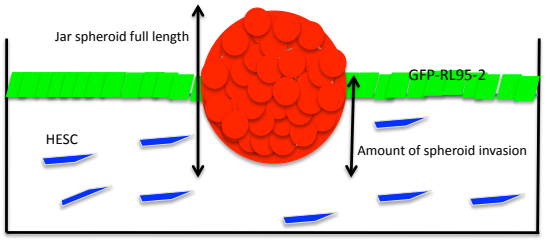


Figure 5.3 Schematic view of a JAr spheroid invasion into the 3DEESCC. The percentage of invasion of a JAr spheroid was calculated using a formula: invasion distance from the surface of epithelial cells (short arrow) / spheroid thickness (tall arrow) **X** 100.

5.3 Results

5.3.1 CpG presence in high doses decreased JAr spheroid attachment to the endometrial cells in the 2D culture system

Treatment of the RL95-2 cells with 1 μM of CpG significantly decreased the number of JAr spheroids attached to the endometrial cells ($p<0.05$). The inhibitory effect of CpG on JAr spheroid binding to the endometrial monolayer was not observed with lower doses of CpG (0.001 μM) (Figure 5.4).

5.3.2 CpG effect on the JAr spheroid outgrowth on top of the 2D endometrial culture

To investigate the effect of TLR9 ligation on JAr spheroid outgrowth on top of the endometrial monolayer, RL95-2 cells were cultured as a monolayer with different concentrations of CpG (0, 0.001 and 1 μM). Pre-treatment of the endometrial monolayer with 0.001 and 1 μM CpG significantly suppressed JAr spheroid outgrowth on top of the RL95-2 monolayer, especially after 48 hours, compared to the control (untreated RL95-2 monolayer) (Figure 5.5).

5.3.3 JAr spheroids expanded on top of the 3DEESCC over the time

The JAr spheroid surface area was significantly expanded on top of the 3DEESCC as the co-incubation progressed. The average area of the spheroids outgrowth increased from 76,000μm±2800 μm after one hour to 104,000±6016 μm after 24 hours (around a 1.3 fold increase) and 176,000±13355 μm after 48h hours after initiation of the JAr spheroid co-incubation with 3DEESCC. Figure 5 shows the significant changes in the area of the spheroids' outgrowth over time (figure 5.6A and B).

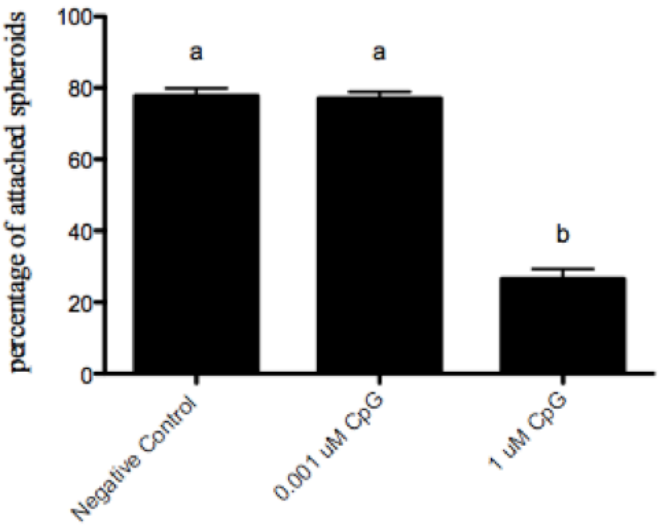


Figure 5.4 The effect of different CpG concentrations on the percentage of spheroids attached to endometrial cells. RL95-2 cells pre-treated with 1 μM of CpG for four hours significantly reduced the percentage of JAr spheroids attaching to the endometrial cells. Applying a lower dose of CpG did not alter the percentage of JAr spheroids attaching to the endometrial cells compared to the negative control (untreated cells). The data are the average of three independent experiments (n=9). The results are presented as the mean ± S.E.M. ANOVA was used to compare the percentage of the attached spheroids at each CpG concentration. Different letters indicate significant statistical differences. $p<0.05$ was considered to be significant.

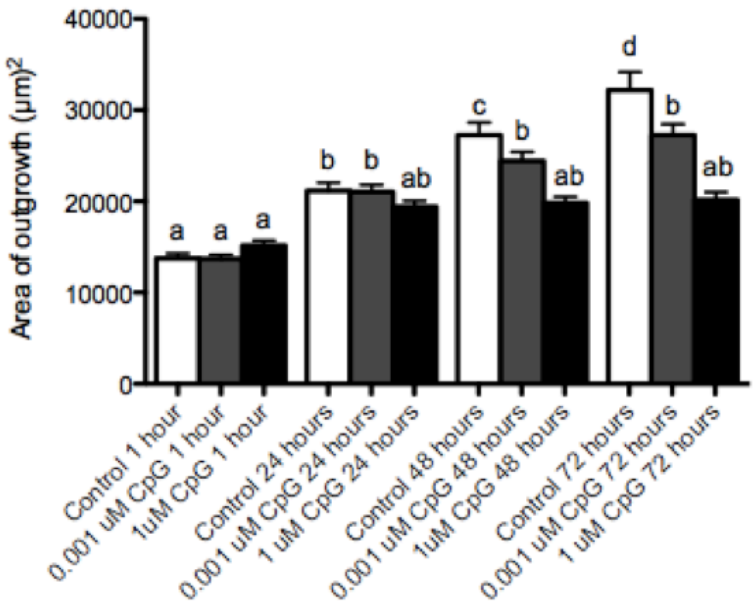
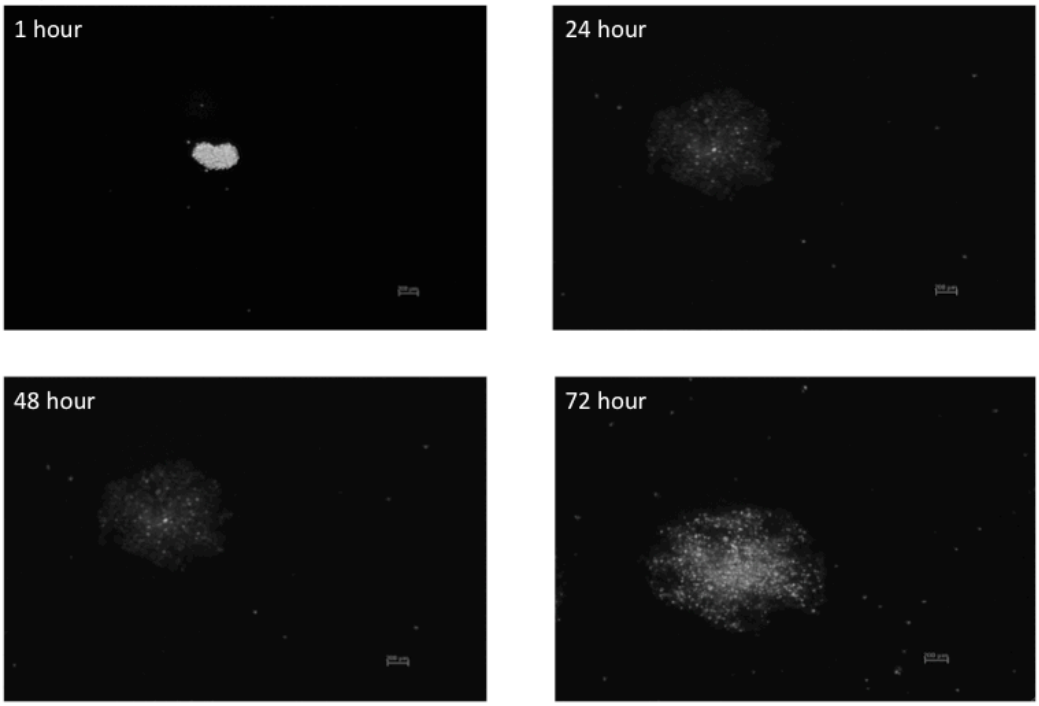


Figure 5.5 The effect of CpG on the JAr spheroids outgrowth on top of the 2D endometrial culture. RL95-2 monolayers were pre-treated with different concentrations of CpG (0, 0.001 and 1 μM) for four hours. 30 JAr spheroids were then added to the culture, and the area of outgrowth of each sphere was monitored at four different times points, from 1 to 72 hours post co-incubation. The data of the graph are the average of three independent experiments. The results are presented as the mean ± S.E.M. ANOVA was used to analyse the results. Different letters indicate significant statistical differences. Control = untreated RL95-2 monolayer. $P<0.05$ was considered to be significant.

A)



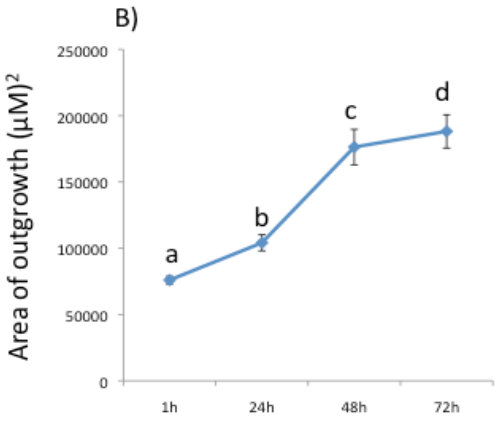


Figure 5.6 JAr spheroid outgrowth on top of the 3DEESCC A) JAr spheroid outgrowth on 3DEESCC over time. **B)** 10 JAr spheroids were added to the upper surface of the 3D culture, and the area of outgrowth of each sphere was monitored at four different time points, from 1 hour to 72 hours post co-incubation. The data of the graph are the average of three independent experiments. The results are presented as the mean ± S.E.M. ANOVA with Sidak's adjustment used to analyse the results. Different letters indicate significant statistical differences. $P<0.05$ was considered to be significant.

5.3.4 CpG effect on the JAr spheroids outgrowth on 3DEESCC

To investigate the effect of TLR9 ligation on JAr spheroid outgrowth on top of the 3DEESCC and the dependency of this effect on the presence of TLR9 in the endometrial cells, RL95-2, CP-RL95-2 and TLR9KD-RL95-2 cells were used to prepare three different 3DEESCC systems containing each of these endometrial cells. Three different concentrations of CpG (0, 0.001 and 1μM) were added to the apical, basolateral, and both the apical and basolateral regions of the 3DEESCC.

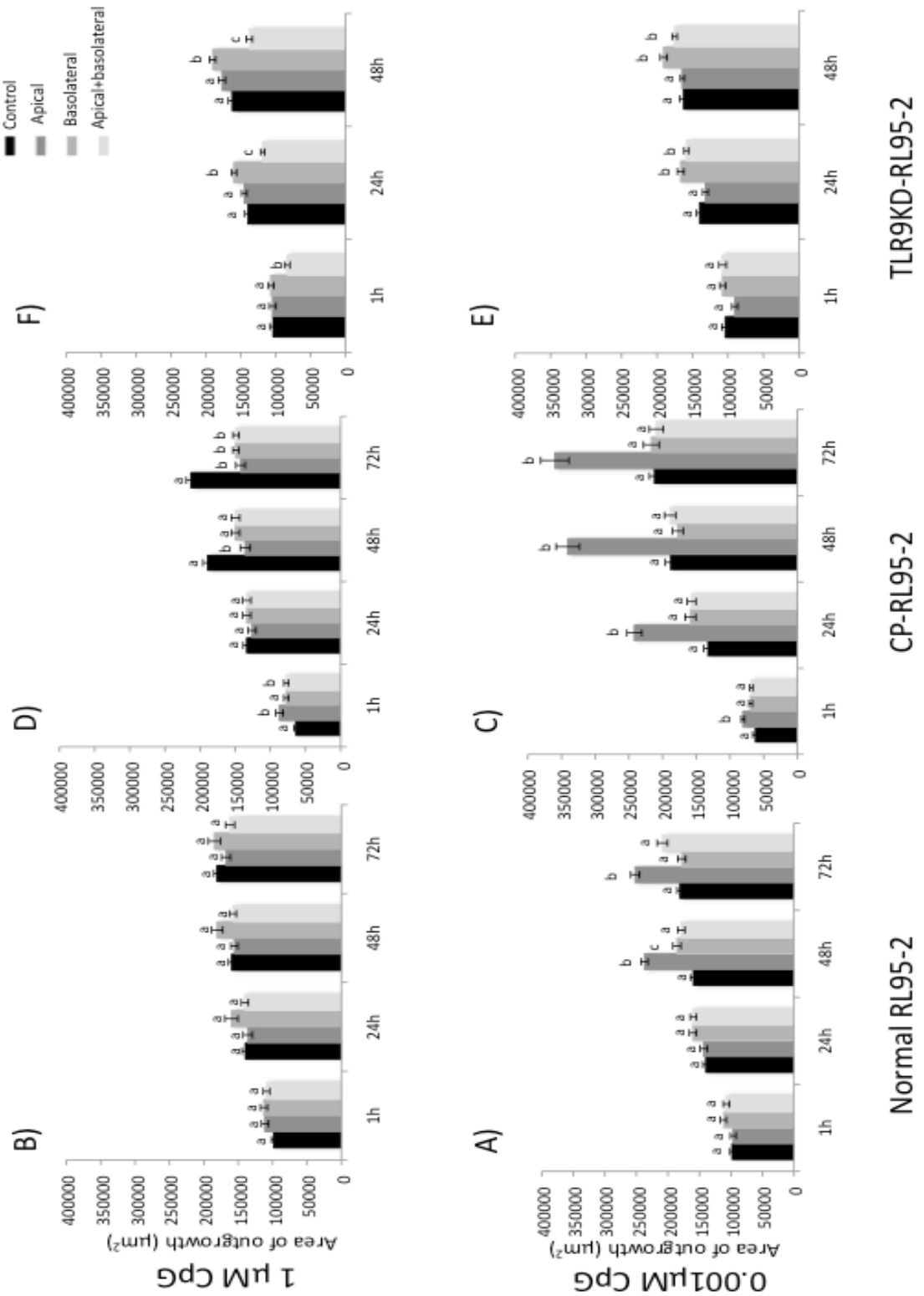
Pre-treatment of the apical region of the 3DEESCC containing RL95-2 cells with 1μm CpG did not significantly affect the amount of the JAr spheroid outgrowth (Figure 5.7B). However, a significant suppression of JAr spheroid outgrowth was seen after 48 hours when CP-RL95-2 cells were used to prepare the 3DEESCC ($P<0.023$) (Figure 5.7D). Surprisingly, the detrimental effect of applying a high dose of CpG on the apical part of the 3DEESCC completely disappeared when TLR9KD-RL95-2 cells were used to prepare the culture ($P<0.271$) (Figure 5.7F). Furthermore, using the same CpG concentration, TLR9 ligation of the basolateral and of both apical and basolateral regions of the 3DEESCC containing CP-RL95-2 cells had a significant negative effect on JAr spheroid outgrowth compared to the control after 72 hours (Figure 5.7D). Using TLR9KD-RL95-2 cells to prepare the 3DEESCC, 1 μm CpG significantly suppressed the JAr spheroid outgrowth when added to both the apical and basolateral regions of the 3DEESCC (Figure 5.7F).

Pre-treatment of the apical part of the 3DEESCC containing RL95-2 and CP-RL95-2 cells with 0.001μM CpG significantly increased the amount of JAr spheroid outgrowth over time ($P<0.001$). No significant alteration of JAr spheroid outgrowth was seen when 0.001 μM CpG was added to either the basolateral part or to both parts of the culture (Figure 5.7A and 5.7C).

Pre-treatment of the apical part of the 3DEESCC that was prepared with TLR9KD-RL95-2 cells with 0.001μM CpG did not cause a significant alteration in the JAr spheroid outgrowth over time compared to the control (Figure 5.7E).

Statistical analysis also indicated that applying 0.001 μM CpG in the apical region of the 3DEESCC distinctly stood out when both RL95-2 and CP-RL95-2 were used. This implies that the cluster of the cells stimulated with 0.001 μM CpG in the apical region were behaving differently than the clusters receiving other treatments (1 μM CpG or 0.001 μM CpG in regions other than the apical) (Figure 5.8).

The statistical analysis also revealed that stimulation of the apical part of the 3DEESCC (prepared with CP-RL95-2 and RL95-2) with 0.001 μM CpG significantly increased the speed of JAr spheroid outgrowth on top the 3D culture. Furthermore, using the higher dose of CpG (1μM) in all three regions of the 3DEESCC (apical, basolateral, and both) significantly decreased the speed of JAr spheroid outgrowth. This particular effect was not seen when the 3DEESCC contained TLR9KD-RL95-2 cells.



gure 5.7 Determination of the effect of TLR9 ligation on JAr spheroid outgrowth on top of the 3DEESCC. Three different 3DEESCC re prepared. Different concentrations of CpG (0.001 and 1µM) were added to apical, basolateral, and the both apical and the basolateral parts the 3D EESCC. After four hours, 30 JAr spheroids were added on top of the 3DEESCC, and the amount of the JAr spheroid outgrowth was essed over time. A and B: 3DESCC with RL95-2 cells. C and D: 3DESCC with CP-RL95-2 cells, and finally, E and F: 3DEESCC with R9KD-RL95-2 cells. The data of the graphs are the average of three independent experiments. The results are presented as the mean ± S.E.M NOVA with Sidak adjustment used to analyze the results. Different letters indicate significant statistical differences. $P<0.05$ was considered to significant.

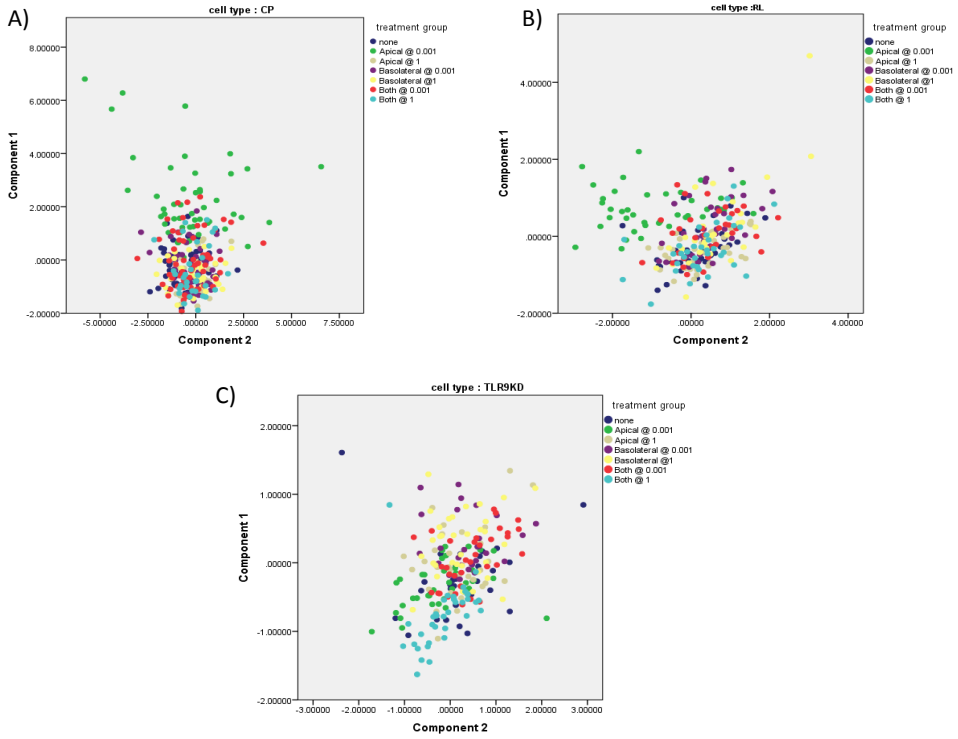


Figure 5.8 Scatter plot analysis of JAr spheroids outgrowth on top of different 3DEESCCs. Principal components on the covariance matrix used as a method of summarizing the data into two dimensions. A) CP-RL95-2, B) RL95-2 cells, and C) TLR9KD-RL95-2 cells were used to prepare the 3DEESCC.

5.3.5 JAr spheroid invasion into the 3DEESCC over time

JAr spheroids significantly invaded into the 3DEESCC as the co-incubation progressed over time. The percentage of the JAr spheroids invading into the 3DEESCC significantly increased from 40%±3.25 after 1 hour to 48%±2.14 after 10 hours and to 61%±2.81 after 30 hours post incubation of the JArs with 3DEESCC (Figure 5.9). Figure 5.9A shows the actual 3DEESCC under the confocal microscope.

5.3.6 CpG effect on JAr spheroid invasion into the 3DEESCC

To investigate the effect of TLR9 ligation on the percentage of JAr spheroids invading into the 3DEESCC (prepared with CP-RL95-2 cells and/or TLR9KD-RL95-2cells), three different concentrations of CpG (0, 0.001 and 1μM) were added to the apical, the basolateral, and both the apical and basolateral regions of the 3DEESCC.

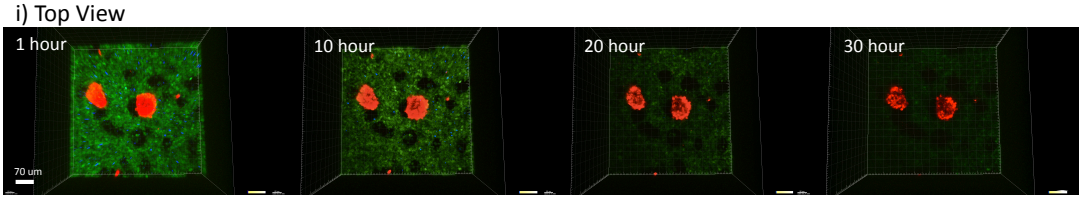
Pre-treatment of the apical, basolateral and both parts of the 3DEESCC containing CP-RL95-2 cells with 1μM of CpG had a significant detrimental effect on the percentage of JAr spheroids invading into the endometrial cells compared to the control after 20 hours of co-incubation (Figure 5.10B). However, applying the same amount of CpG (1 μM) to stimulate the apical and both the apical and basolateral parts of the 3DEESCC containing TLR9KD-RL95-2 cells did not significantly affect the percentage of the JAr spheroids that invaded, but stimulation of the basolateral region of the 3DEESCC significantly suppressed the percentage of JAr spheroids that invaded ($P<0.001$) after 30 hours of incubation of the spheroids with the 3DEESCC (Figure 5.10D).

Pre-treatment of the apical part of the 3DEESCC containing CP-RL95-2 cells with 0.001μM of CpG significantly enhanced the percentage of JAr spheroids invading over time ($P<0.001$). This particular effect of TLR9 ligation was not seen when 0.001 μM CpG was added to either the basolateral or to both parts of the 3DEESCC (Figure 5.10A) or when 0.001 μM CpG was added to the apical part of the 3DEESCC containing TLR9KD-RL95-2 cells (Figure 5.10C). Furthermore, pre-treatment of the basolateral and both the apical and basolateral parts of the 3DEESCC containing TLR9KD-RL95-2 cells with 0.001 μM CpG did not significantly affect JAr spheroid invasion into the 3DEESCC (Figure 5.10C).

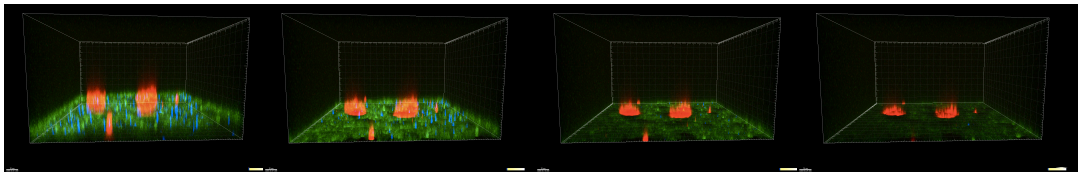
Statistical analysis also indicated that the cluster of 3DEESCC cells prepared with CP-RL95-2 stimulated with 0.001 μM CpG in the apical region of the culture was behaving differently from the clusters receiving other treatments (1 μM CpG or 0.001 μM CpG in other regions than the apical) or when TLR9KD-RL95-2 cells were used to prepare the 3DEESCC (Figure 5.11). Furthermore, the results showed that the apical application of 0.001 μM CpG enhanced the speed of JAr spheroid invasion very quickly, after just two hours. Figure 5.12 shows the differences in JAr spheroid invasion between 3DEESCC containing CP-RL95-2 and 3DEESCC containing TLR9KD-RL95-2 at two hours. Statistical analysis also revealed that the speed of JAr spheroid invasion was distinctly dependent on the concentration and location of the CpG used in the system (Figure 5.13).

A)

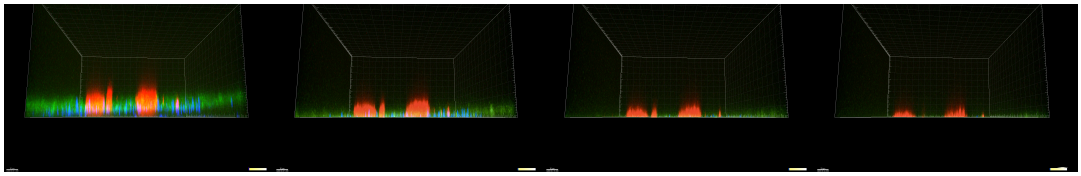
i) Top View



ii) Tilted View



iii) side View



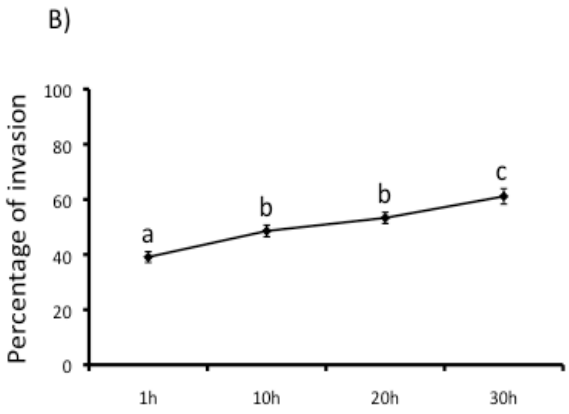


Figure 5.9 Confocal analysis of the three-dimensional endometrial culture and visualization of JAr spheroids invasion. A) JAr spheroids were added to the upper surface of the 3D culture and the invasion of each spheroid was monitored at four different time points (1, 10, 20, 30 hours), and B) the percentage of the invasion of the spheroids was calculated. The data of the graph are the average of three independent experiments. The results are presented as the mean ± S.E.M. ANOVA with Sidak adjustment used to analyse the results. Different letters indicate significant statistical differences. $P<0.05$ was considered to be significant.

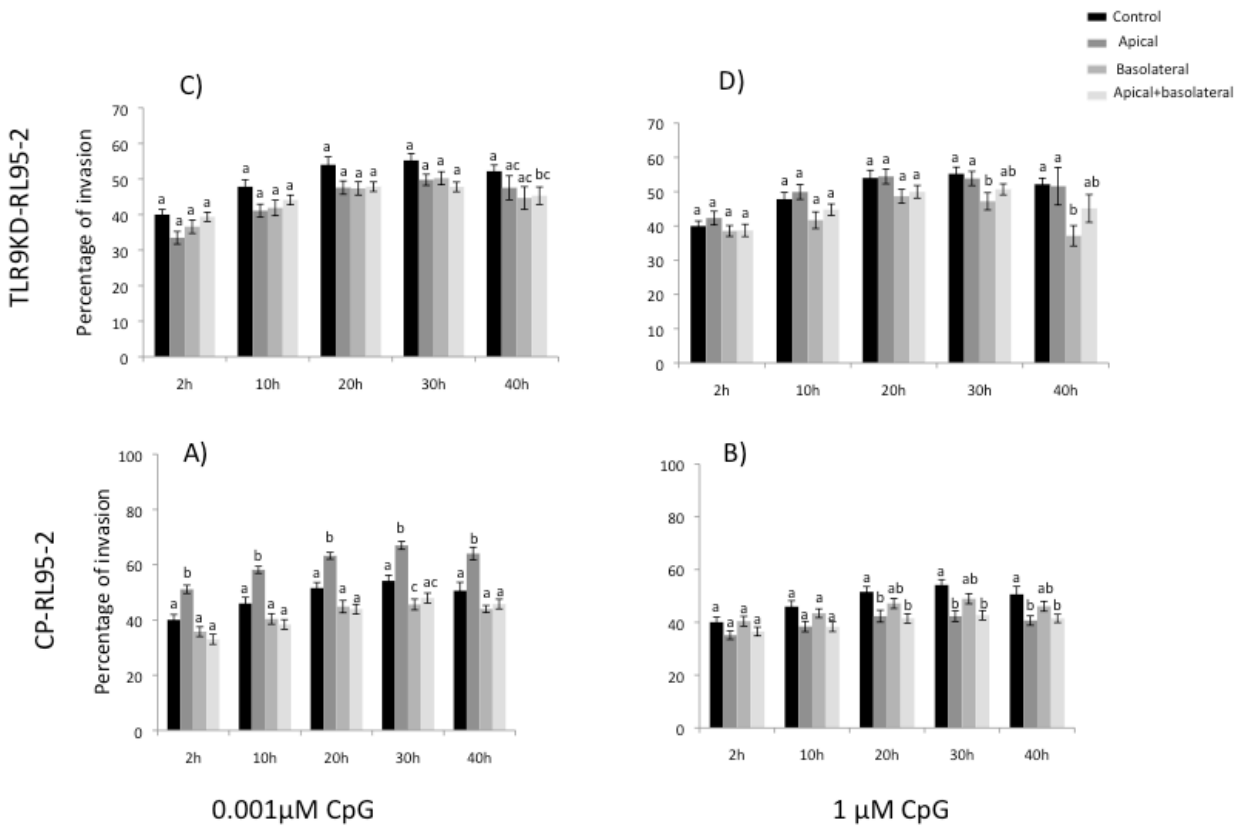


Figure 5.10 Determination of the effect of TLR9 ligation on JAr spheroid invasion into the 3D EESCC. Two different 3DEESCCs were prepared, with CP-RL95-2 cells and with TLR9KD-RL95-2 cells. Different concentrations of CpG (0, 0.001 and 1µM) were added to the apical, basolateral and both the apical and basolateral parts of the 3DEESCC. After four hours, 50 JAr spheroids were added on top of the 3DEESCC, and the percentage of the JAr spheroid invasion was assessed over time. A and B: 3DESCC with CP-RL95-2 cells. C and D: 3DESCC with TLR9KD-RL95-2 cells. The data of the graphs are the averages of three independent experiments. The results are presented as the mean ± S.E.M. ANOVA with Sidak's post hoc test used to analyse the results. Different letters indicate significant statistical differences. $P<0.05$ was considered to be significant.

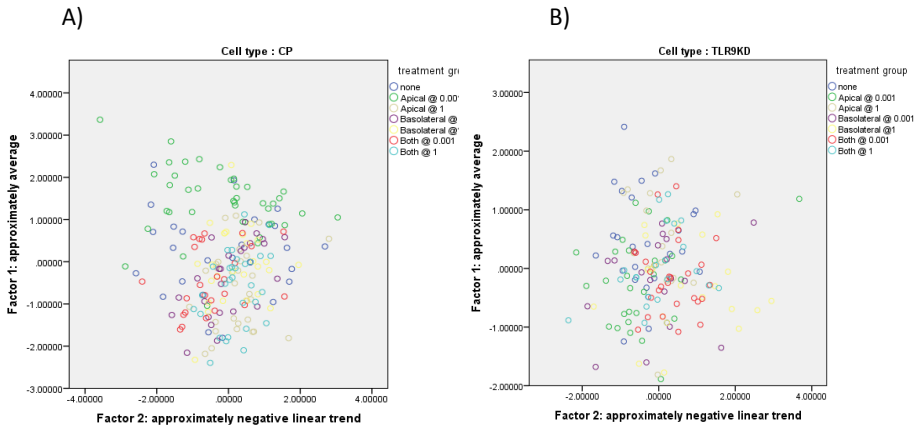


Figure 5.11 Scatter plot analysis of JAr spheroid invasion into the 3DEESCC. Principal components on the covariance matrix was used as a method of summarizing the data into two dimensions. CP-RL95-2 (A) and TLR9KD-RL95-2 (B) cells were used to prepare the 3DEESCC.

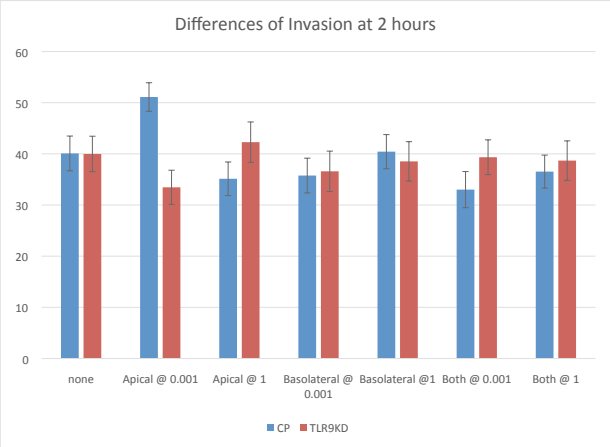


Figure 5.12 Differences in JAr spheroid invasion between 3DEESCC containing CP-RL95-2 and 3DEESCC containing TLR9KD-RL95-2 at two hours. A significant difference was only seen when 0.001 μM CpG was added to the apical area of the 3DEESCC. Regression coefficient analysis was used to analyse the differences in JAr spheroid invasion between the two 3DEESCC at two hours of co-incubation.

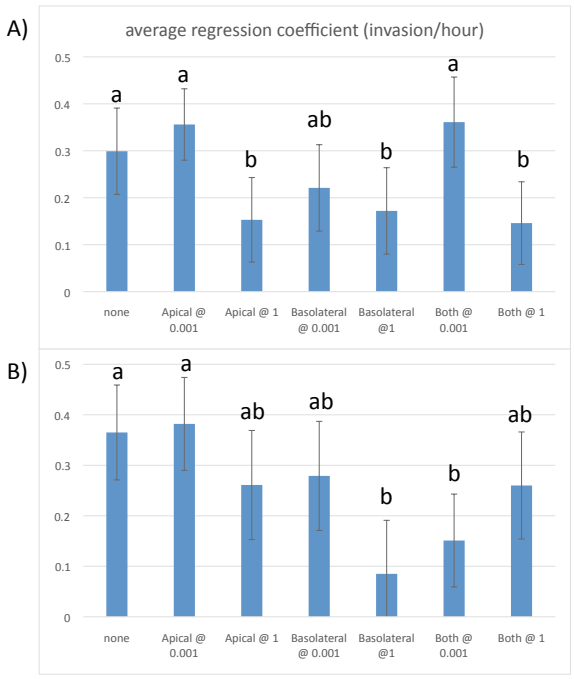


Figure 5.13 Regression coefficient analysis of JAr spheroids invasion/hour. A) CP-RL95-2 and B) TLR9KD-RL95-2 cells were used to prepare the 3DEESCC. The speed of JAr spheroid invasion was distinctly dependent on the concentration and the location of the CpG used in the system.

5.4 Discussion

There is a growing body of evidence suggesting that TLRs play an important role in maternal-foetal interactions (Mor, 2008). Multiple TLRs have been reported in various parts of the female reproductive tract, such as the endometrium and the placenta (Fazeli et al., 2005, Aflatoonian and Fazeli, 2008). Studies have shown that stimulation of some of these TLRs could lead to pregnancy loss or preterm birth. In this regard, exposure of the cervicovaginal epithelium to bacterial lipopolysaccharide, a specific ligand for TLR4, induced implantation failure in mice (Meter and Horton, 2007). It has also been demonstrated that stimulation of TLR3 with poly (I:C) in C57BL/6 wild type mice resulted in preterm labour, while poly (I:C) treatment did not show any effect in TLR3 knockout mice (Koga et al., 2009b). Considering the fact that TLR9 is known to be involved in the recognition of STIs, one of the causes of infertility (Fredlund et al., 2004), this present research study aimed to clarify the importance of TLR9 in the trophoblast-endometrium interaction during the early stage of pregnancy by applying two different (2D and 3D) *in vitro* endometrial culture systems.

Activation of TLR9, with its specific ligand CpG, did not necessarily have a negative effect on the activities and behaviours of cells and tissues. It seems that CpG has several therapeutic applications (Silva et al., 2015). It has been found that a synthetic TLR9 agonist has an anti-tumour activity by affecting the DNA repair genes in colorectal tumour cells (Furi et al., 2013). TLR9 agonist therapy has been clinically tested not only in colon cancer but also in other cancers, such as pancreatic and breast cancer (Furi et al., 2013, Sommariva et al., 2011, Hofmann et al., 2008). It has also been reported that TLR9 activation with CpG enhances epithelialization and the wound healing process (Miller et al., 2005, Rose et al., 2012, Sato et al., 2010, Yamamoto et al., 2011). Hence based on the dual action of CpG via TLR9, we hypothesized that activation of TLR9 with CpG in an *in vivo*-like environment (3DEESCC) positively affects trophoblast spheroid invasion and outgrowth. To test the validity of our hypothesis, different concentrations of CpG were added to the different regions of the 3D culture system, and the amount of the trophoblasts' outgrowth and invasion was monitored over time.

Both the 2D and the 3D culture systems used in this study were valid models for research purposes; both were classified *in vitro* culture systems for human implantation (Weimar et al., 2013). The 2D endometrial culture system used in this study had been previously developed in our laboratory (Aboussahoud et al., 2010b) (Sanchez-Lopez et al., 2014, Montazeri et al., 2015a), while the 3D culture system was a modified version developed by another group (Wang et al., 2012). In the latter, the endometrium was represented using human endometrial stromal cells (HESC) seeded within a fibrin-agarose matrix with human endometrial epithelial cells (RL95-2) overlaying the matrix as a flat monolayer. Multi-cellular human trophoblast cell (JAr) spheroids were added to the system to represent the embryo. The JAr cell line is the most commonly used trophoblast cell line to create multicellular spheroids employed as embryo surrogates (John et al., 1993, Hannan et al., 2010, Sanchez-Lopez et al., 2014, Montazeri et al., 2015a). The glandular epithelial cell line RL95-2 is commonly used to mimic receptive endometrial epithelium (Galan et al., 2000, Liu et al., 2011, Xiong et al., 2012). Finally, endometrial stromal cells have been found to play a supportive role in promoting endometrial epithelial growth and differentiation even when they were embedded into matrigels to create a 3D cell culture system (Arnold et al., 2001). Hence all of the experiments performed in this investigation were designed using valid and well-known *in vitro* culture systems.

Preliminary results of the current investigation performed using the 2D culture system demonstrated that CpG stimulation of TLR9 in RL95-2 cells significantly decreased the percentage of JAr spheroids attaching to the endometrial cells in a dose-dependent manner. This is a strong indication of the involvement of TLR9 activation in the process of implantation failure, consistent with other TLR types; stimulation of TLR2/6, TLR3 and TLR5 in human endometrial epithelial cells leads to failure of attachment of JAr spheroids to the 2D endometrial culture (Aboussahoud et al., 2010b, Montazeri et al., 2015a, Sanchez-Lopez et al., 2014). Furthermore, it has been reported that stimulation of TLRs in the first trimester trophoblast cells, in addition to endometrial cells, can induce different immune responses such as inflammation and apoptosis (Koga and Mor, 2008). In our study, as the CpG remained in the co-culture of endometrial monolayers with JAr spheroids for 1 hour, it is probable that the

inhibitory effect on binding originates from either the trophoblast, the endometrial cells, or a combination of both cell types.

We have shown that the surface area of JAr spheroids significantly expanded on top of the 2D endometrial culture and 3DEESCC over time. This indicated that JAr spheroids grow dynamically over the endometrial monolayer, representative of cytotrophoblasts at the time of human implantation (Johnson, 2000a, Fisher et al., 1989). These results are in agreement with Evron *et al.* showing that JAr spheroid outgrowth on 3D culture systems significantly increased over time in the presence of the hormone progesterone (Evron et al., 2011). There are numerous studies regarding trophoblast attachment and outgrowth in 2D and 3D endometrial cultures (Li et al., 2002, John et al., 1993, Bentin-Ley et al., 2000, Li et al., 2003). However, to the best of our knowledge, this is the first time that trophoblast attachment, outgrowth, invasion and also the speed of trophoblast outgrowth as well as the speed of trophoblast invasion have been studied in response to TLR9 stimulation *in vitro*.

Regarding JAr spheroid invasion into the 3DEESCC, our results showed that trophoblasts are able to invade dynamically into the 3DEESCC over time. Successful implantation is highly dependent on the capability of cytotrophoblasts to digest through the epithelial layer of the uterus and invade into the stroma. Cytotrophoblasts also migrate further into the myometrium, where they would be in direct contact with the mother's blood circulation (Staun-Ram et al., 2004, Fisher et al., 1989). Therefore, JAr cells with invasive ability and a strong proteinase secretion profile, similar to cytotrophoblasts, are regarded as a reliable cell line used in human reproductive studies (Staun-Ram et al., 2004, Evron et al., 2011, Zhang et al., 2005, Montazeri et al., 2015a, Sanchez-Lopez et al., 2014).

We have found that the higher dose of TLR9 ligand used in this study, 1μM CpG, had a detrimental effect on JAr spheroid attachment and outgrowth in the 2D culture system and also on the speed of JAr spheroid outgrowth and invasion in the 3DEESCC. This indicates that high doses of CpG may trigger an intracellular signalling pathway from both the endometrial epithelial and trophoblast cells that results in a negative trophoblast-endometrial interaction in both 2D and 3D culture systems. This is comparable to other findings showing that high concentrations of

CpG results in different inflammatory responses compared to low concentrations (Aplin et al., 2014, Volpi et al., 2013, Platz et al., 2004). In this regard, it has been found that stimulation of TLR9 in human dendritic cells with 0.3 μg/ml CpG resulted in MyD88-dependent interleukin (IL)-23 production, while using 3 μg/ml CpG to stimulate TLR9 in the same cells resulted in TIR domain-containing adaptor-inducing interferon β (TRIF) dependent transforming growth factor (TGF) β secretion (Volpi et al., 2013). Our results are in agreement with these findings, since we have shown that 1μM CpG, in contrast with 0.001 μM CpG, had a suppressor effect on trophoblast-endometrial interactions.

Application of a low dose of CpG (0.001 μM) in the 2D endometrial culture suppressed JAr spheroid outgrowth on the RL95-2 monolayer. However, applying the same dose of CpG in the apical part of the 3DEESCC enhanced the JAr spheroid outgrowth. Applying the same dose of CpG in both the 2D culture and the apical part of the 3DEESCC culture ended in two different consequences. A possible explanation is that the 2D endometrial culture, in contrast to the 3DEESCC, is not an *in vivo*-like environment where the cells are in a close interaction. Hence pre-treatment of the endometrial monolayer with a low dose CoG could provoke a robust inflammatory response that negatively affected trophoblast outgrowth. The apical part of the 3DEESCC is the only region where CpG is in direct contact with both the epithelial monolayer and the JAr spheroids. This may explain why a supporting effect has not been observed when the same concentration of CpG was applied to the basolateral part of the 3DEESCC. It is probable that TLR9 stimulation with a low dose of CpG triggered a reduced inflammatory response that was not strong enough to stop or decrease the trophoblast-endometrial interaction, but was good enough to boost the trophoblast-endometrial interactions in the 3DEESCC. Recently, in the literature, there are other examples indicating that dampened endometrial inflammatory responses have a beneficiary effect on embryonic implantation. Similarly, scratching the endometrium in the *in vitro* fertilization (IVF) cycle just before the embryo transfer improved embryo implantation and pregnancy rates (Coughlan et al., 2014, Gibreel et al., 2013, Dain et al., 2014). Endometrial scratching can act as a low-level stimulant of the innate immune system in the same way that the low CpG doses acted here. Hence one can speculate that administration of a very low dose of CpG may

prepare a favourable environment to support embryo implantation, similar to the beneficial effects of the endometrial scratch on implantation.

Applying a low dose of CpG (0.001 μM) in the both the apical and the basolateral regions of the 3DEESCC did not enhance JAr spheroid outgrowth. Primary stromal cells have a supportive effect on the percentage of trophoblast spheroids attaching to the RL95-2 cells when both stromal and RL95-2 cells were in interaction in a 3D culture system, although this effect is highly dependent on the type of stromal cells used in the culture (Evron et al., 2011). Stromal cells taken from patients during the "implantation window" phase have significantly more potential to increase the number of trophoblasts attached to the 3D culture (Evron et al., 2011). This indicated that interactions between the cells are very important to their responsiveness and behaviour with the outside environment, representative of *in vivo*. Therefore, HESC cells may have a supportive role in the attachment and outgrowth of the JAr spheroids in 3DEESCC. Assuming that stromal cells in the basolateral area of the culture are in close interaction with cells located in the apical region, we can speculate that the addition of CpG to the basolateral media of the 3DEESCC would affect the trophoblasts-endometrium interaction via HESCs. More studies are still needed to clarify the role of stromal cells in trophoblast-endometrium interactions in the presence of CpG.

Results from our 3DEESCC also showed that applying a low dose of CpG (0.001 μM) in the apical region of the 3DEESCC accelerated the speed of JAr spheroid invasion into the endometrium. Therefore, this particular concentration of CpG boosted the trophoblast-endometrial interactions *in vitro* by supporting both trophoblast outgrowth and invasion. It was found that JAr spheroids were able to invade into the extracellular matrix (ECM) (Glass et al., 1983) with the help of trypsin-like enzyme activities (Fisher et al., 1989). Metalloproteinases-2 (MMP-2) and metalloproteinases-9 (MMP-9) (Zhang et al., 2005) are two key enzymes involved in trophoblast invasion during the implantation process. Both MMP-2 and MMP-9 are expressed in JAr cells as well as in endometrial epithelial cells, but MMP-2 is the main enzyme modulating JAr spheroid invasion (Zhang et al., 2005, Staun-Ram et al., 2004). Human chorionic gonadotropin (hCG) was also found to induce

MMP production in trophoblasts and to increase their invasion (Fluhr et al., 2008). Hence it can be speculated that TLR9 activation by CpG in the apical part of the 3DEESCC would affect both JAr spheroids and endometrial epithelial cells directly, inducing changes in the balance of MMPs involved in the invasion process. The effects of CpG were dose dependent. Results showed that CpG in lower doses enhanced trophoblast spheroid invasion, while CpG in a higher dose suppressed trophoblast invasion. Clarifying the effect of CpG/TLR9 on the production and activation of MMPs in both trophoblast and endometrial epithelial cells and investigating the role of MMPs in embryo-endometrium cross talk could be considered for future research studies.

JAr cells are able to produce MMPs (Staun-Ram et al., 2004), yet MMPs need plasminogen to be activated (Zhang et al., 2005). In the 3DEESCC model, plasminogen is sourced from the human plasma used to prepare our base layer of the 3D matrix. Considering this, JAr spheroids were expected to digest the endometrial epithelial cells and invade into the 3D matrix where the stromal cells are embedded. However, the confocal microscopy data revealed that JAr spheroids only invaded into the endometrial epithelial monolayer, not deeper into the 3D matrix. The sphere height decreased over time, suggesting simultaneous invasion and flattening, hence cells were not able to invade deeper into the 3D matrix. This is in contrast to literature showing JAr spheroid invasion into the agarose based 3D matrix (Wang et al., 2012, Wang et al., 2013). One of the reasons that might explain why the JAr spheroids were not able to digest and invade into the matrix in our experiments is that, to the best of our knowledge, JAr MMPs are not capable of digesting the agarose type VII used to prepare the base layer of the 3D matrix in our investigation. A second possible explanation is the duration of the co-culture; JAr spheroids on the 3DEESCC were monitored for a maximum of 40 hours. They might need more time to produce a sufficient amount of enzymes and other elements to be able to further digest the agarose-based matrix.

In conclusion, the concentration of CpG and its mode of application seems to determine whether TLR9 promotes or suppresses trophoblast invasion *in vitro*, evidencing a double-edged sword effect of TLR9 stimulation in human implantation.

It remains to be investigated whether such a favourable effect in low dose application of CPG can affect the implantation process *in vivo*.

Chapter 6: General discussion

6.1 Thesis major findings

During the course of the investigations that led to the production of this thesis, this researcher has tried to study human implantation in detail and to clarify the role of innate immunity at the time of trophoblast attachment, outgrowth, and invasion. This researcher has focused on innate immunity primarily because the embryo as a semi-allograft creature that by presenting paternal antigens and other foreign elements acts as an invader to the mother that should arouse innate immune responses at the site of implantation and during pregnancy. But normally, there is maternal immune tolerance of the embryo (Aagaard-Tillery et al., 2006). On the other hand, the presence of any infection in the female reproductive tract at the time of pregnancy may provoke innate immune responses and lead to implantation failure or miscarriage (Aagaard-Tillery et al., 2006). The main objective of this study was to clarify the effect of TLR9 (one of the most investigated innate immune receptors, involved in the recognition of sexually transmitted infections) activation on trophoblast-endometrium interactions and communication. Different approaches were used in this thesis to broaden the current knowledge about this topic.

During the course of this investigation, due to the limitation of studying human implantation and pregnancy *in vivo*, two different *in vitro* culture systems were used to simulate trophoblast-endometrium interaction: i) a 2-dimentional culture system that contained trophoblast spheroids added on top of the endometrial epithelial monolayer, and ii) a 3-dimentional culture system that contained an endometrial epithelial monolayer laid over agarose-gel embedded endometrial stromal cells, with the trophoblast spheroids on top of them. siRNA gene silencing technique, gene expression analysis, and confocal microscopy were the other major approaches used in this thesis.

Among all the TLRs involved in the recognition of sexually transmitted infections in the female reproductive tract, this researcher was interested in TLR9 particularly because of the therapeutic applications of both TLR9 agonist and antagonist in different diseases such as cancers and autoimmune diseases respectively (Kanzler et

al., 2007). There is a growing body of evidence indicating that activation of TLR9 by its specific ligand CpG accelerated some of the cellular activities such as vascularization and epithelialization (Hergert et al., 2013, Rose et al., 2012, Sato et al., 2010, Yamamoto et al., 2011). Considering this positive and supportive role of the TLR9/CpG complex, this writer decided to check whether TLR9 activation had such a positive role in trophoblast-endometrium interactions *in vitro*. According to what has been found in this thesis, this researcher can state that TLR9 acts as a double-edged sword that can have both a positive and a negative impact on the trophoblast-endometrium interaction based on the dose of the ligand and the location where it has been added to the culture system.

In Chapter 2 of this thesis, it was shown that TLR9 activation in endometrial epithelial cells had a detrimental effect on trophoblast attachment to the 2D culture system in a dose dependent manner. Using a 2D culture system, there was only one location available to introduce the CpG effect into the culture system, directly onto the media of the endometrial epithelial and trophoblast cells. Considering that there were no other endometrial cells, like stromal cells, in the system to interact with epithelial cells to possibly soothe the TLR9 activation in 2D cultures, CpG in a dose dependent manner may have provoked a strong inflammatory response from both the trophoblasts and the endometrial epithelial cells that negatively affected their interactions.

Using the siRNA technique, a plasmid containing TLR9 gene specific shRNA was introduced into the endometrial epithelial cells (RL95-2 cells) (Chapter 3). The present researcher managed to create a stable TLR9 knocked-down RL95-2 cell line. Transfecting the endometrial epithelial cells with linear plasmid allowing the plasmid to randomly insert into the genome of the cells. Therefore, no re-transfection was needed during each experiment. This technique is a well-established, simple and very popular method of genome modification in pigs. Expanding this method from pig studies to human *in vitro* cultures, TLR9 knocked-down endometrial epithelial cells were able to grow in the culture for long periods of time with normal appearance and behaviour but no TLR9 functionality. Using the same approach, this thesis writer has tried to create a stable TLR9 knocked-down trophoblast cell line (JAr cells). Since JAR cells are not culture friendly and are very sensitive to every little change in their

environment, they were not able to survive in the culture long enough to grow in colonies. Hence, twice there was a failure to create TLR9 knocked-down JAr cells. It was frustrating, and time was running out. Therefore, this researcher decided to use a well-established TLR9 antagonist for stopping the TLR9 function in the trophoblast cells during the experiments rather than trying to knock it down.

In Chapter 4, the writer investigated TLR9 intracellular signalling pathways in two human epithelial cell lines used in our 2D culture system, RL95-2 and MCF-7 cells. Three possible signalling pathways are suggested for TLR9 in the literature that are all initiated with MyD88 as the main mediator and continue toward NFκB and/or MAPKs and/or IRF7 (Akira et al., 2006). It has also been found that the TLR9 intracellular signalling pathway is cell specific, which means that it varied from one cell type to another and was highly dependent on the dose of the CpG and even the molecular structure of the ligand (Volpi et al., 2013). The writer observed that using a low dose of CpG, which was enough to negatively affect trophoblast attachment to the epithelial cells in the 2D culture system, was p38 dependent in MCF-7 cells, but none of the known pathways were found to be involved in the reduced trophoblast attachment to the RL95-2 cells. Using a high dose of CpG to stimulate RL95-2 cells resulted in significant production of IL-8 that was MyD88 dependent but was independent of the suggested downstream mediators. These findings indicated that the TLR9 signalling pathway is cell specific, but probably an unknown signalling pathway is involved in TLR9 stimulation leading to failure of trophoblast attachment and IL-8 production in RL95-2 cells. There is another possibility, that an unknown receptor exists for CpG that is activated with high doses of ligand and that initiated an intercellular signalling pathway independent of TLR9.

The 3D endometrial culture system used in this study (Chapter 5) was a modified version of the culture that had been established by Wang *et al.* (Wang et al., 2012). In this model, endometrial stromal and epithelial cells are in close interaction, which is close to the real endometrium. One of the benefits of using a 3D culture system was that the present researcher was able to stimulate the cells with CpG in three different locations, from above the culture (the apical region), from beneath the culture (the basolateral region), and from both the apical and basolateral regions simultaneously. This provided an opportunity to study the indirect effect of CpG on the trophoblast-

endometrium interaction when the ligand was added to the basolateral region of the culture. The other advantage of using a 3D endometrial culture was that rather than attachment, two other features of trophoblast interaction with endometrium, trophoblast outgrowth and invasion, could also be studied. Applying a low and a high dose of CpG in three different locations of the 3D culture, this researcher has seen that only the addition of a low dose of CpG in the apical region of the 3D culture enhanced trophoblast outgrowth and invasion, while the application of a high dose of CpG hindered trophoblast outgrowth and invasion, regardless of where it was applied. This was strong evidence of a double-edged effect of TLR9/CpG at the time of implantation. This researcher can conclude that, based on the dose of the infection, TLR9 can either reject or support embryo implantation. Figure 6.1 summarises the entire results obtained in this investigation. Figure 6.2 summarises different aspects of the effect of TLR9 activation on implantation and highlights the details that have been covered in this PhD project, and also shows different options for future investigations.

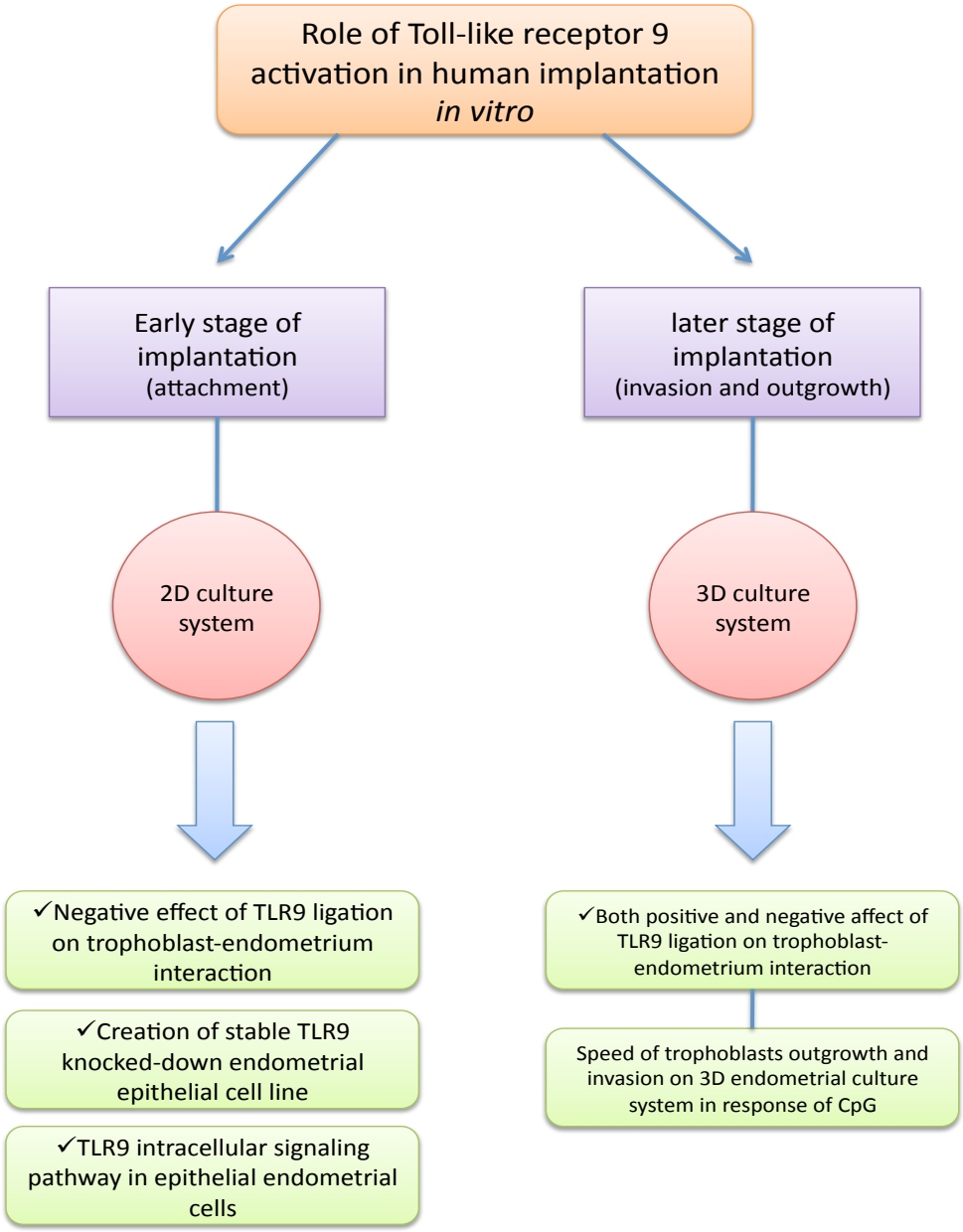


Figure 6.1 Summarized findings of the thesis. The early stage of human implantation (attachment) has been investigated *in vitro* using a 2D culture system, where the effects of TLR9 stimulation on trophoblast attachment and the TLR9 intracellular signalling pathway in trophoblast-endometrium interaction were investigated. The later stage of human implantation has been studied using a 3D culture system, where the effect of TLR9 ligation on trophoblast outgrowth and invasion was investigated. TLR9 activation based on the type of the culture (2D vs 3D) had either a dose-dependent positive or a negative effect on trophoblast-endometrium cross talk.

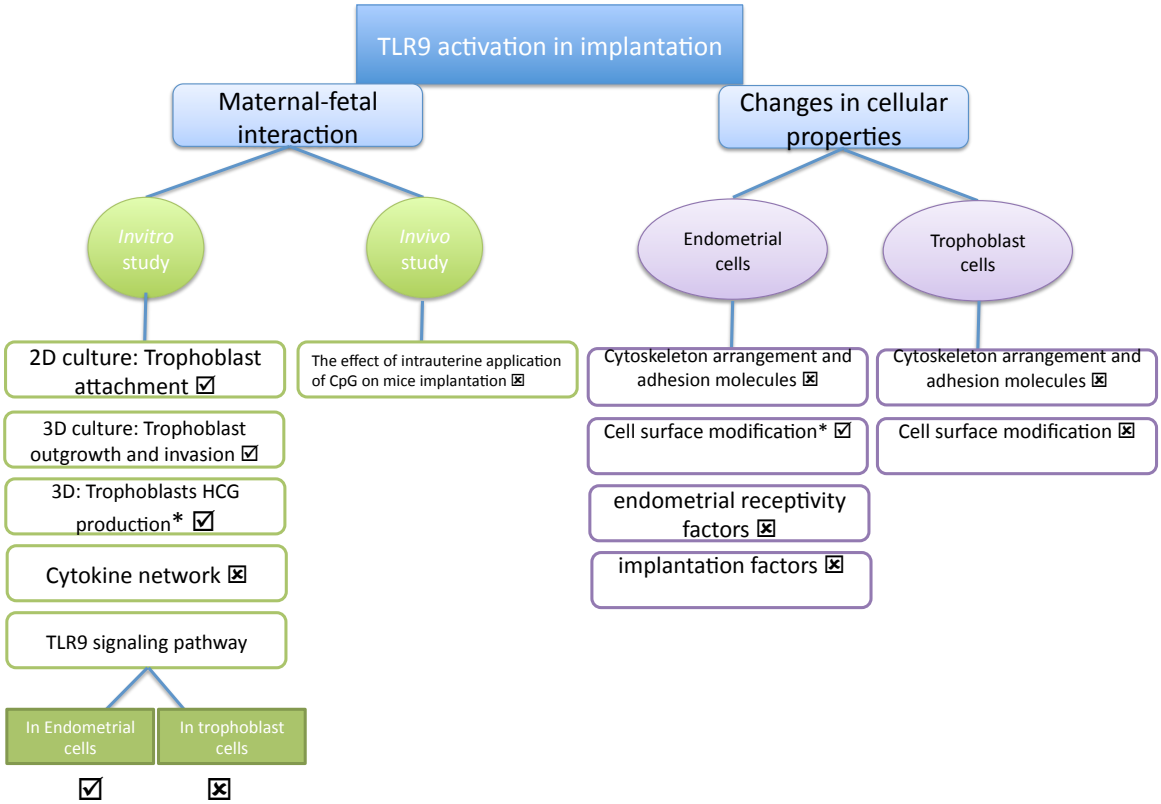


Figure 6.2 The effect of TLR9 activation on implantation. TRL9 activation at the time of implantation could mainly affect the maternal-foetal interactions at the time of implantation and pregnancy and cellular properties such as cytoskeleton rearrangements and cell surface modifications in both endometrial and trophoblast cells. This PhD project is mainly focused on the effect of TLR9 activation on maternal-foetal interactions using *in vitro* culture systems. A black tick symbol indicates the research areas that have been covered in this PhD project. A black cross symbol indicates the research areas that have not been covered by this PhD project and may be considered as future studies. An asterisk plus a black tick symbol indicates the research areas that have been studied by other researchers in our laboratory.

6.2 Implication of thesis findings

Infertility is a significant problem in different societies around the world. Various factors, such as sexually transmitted disease, can cause infertility in women. Depending on the cause of the infertility problem, different assisted reproductive technologies (ART) are available to help increase the rate of successful pregnancies (Ross et al., 2011). Scientists are continuously trying to optimize and improve the quality of ART. However, there are still several risk factors associated with it, such as multiple births, prematurity, low birth-weight and psychological distress that can influence both parents and baby (Ross et al., 2011). Hence there is still room for expanding human knowledge about the nature of fertility problems and ART. Nowadays a substantial amount of money is being invested in reproductive research projects, but finding a way to apply their findings in real life is a matter of concern. Here this researcher has tried to explain some ideas about future application of these research findings in real life.

The 2D model for investigation of the early stages of human implantation used in most of the experiments of this thesis is a reliable and valid method to study human implantation *in vitro* and is widely used by different people in our laboratory and around the world (Aboussahoud et al., 2010b, Montazeri et al., 2015a, Sanchez-Lopez et al., 2014, Weimar et al., 2013). This simple method of simulating the early stage of human implantation has a potential to be used as a pre-pregnancy test to diagnose an unhealthy endometrium. If one could prepare a 2D culture system using the patient's endometrium biopsy, by comparing the percentage of the attached trophoblasts to the healthy endometrium and to the patient biopsy, the capability of the patient's endometrium to receive an embryo *in vivo* might be recognized. However, relating the *in vitro* findings to the *in vivo* conditions is farfetched because of the vast differences in these systems. Additional investigation is needed to expand the *in vitro* knowledge into real life.

Clarifying the functional pathways of TLR9 and other TLRs intracellular signalling pathways involved in the innate immune responses in the female reproductive tract helps experts design medicine that can locally suppress inappropriate immune

activation against the embryo at the site of implantation and during pregnancy. This is not impossible to achieve, since developing drugs to manipulate innate immune responses as a therapy for various cancer types, autoimmune diseases and viral infections has been a concern of clinical programs for many years. Different TLR ligands are now used as a vaccine or vaccine adjutants against various diseases such as hepatitis B, influenza, and anthrax. There are some clinical developments regarding therapeutic applications of both TLRs agonists and antagonists in a number of inflammatory and autoimmune diseases (Kanzler et al., 2007). Hence more studies are needed to clarify and discover the unknown TLR signalling pathways in response to infection in the female reproductive tract to be able to locally suppress any unwanted immune responses at the time of the pregnancy to protect the developing foetus.

In the fifth chapter of this thesis, the writer has shown that using a 3D endometrium culture system, which is close to the *in vivo* condition, and applying a very low dose of CpG to stimulate TLR9 had a supportive effect on trophoblast outgrowth and invasion. Although the details of the TLR9 supportive effect have yet not been found, this can be used as a method to encourage the endometrium to be more receptive to the embryo. It has been reported that endometrium scratching could significantly increase the rate of successful implantation in patients who did not have a favourable condition for the embryo to implant (Karimzadeh et al., 2009, Gibreel et al., 2013). At the time of endometrium scratching, inflammation alongside the wound healing process (epithelialization and vascularization) probably results in production of different factors with positive effects on implantation or in the receptivity of the endometrium (Gibreel et al., 2013). This present researcher personally believes that an attenuated inflammation in the uterus might produce a beneficiary effect on embryo implantation. This immune response is not strong enough to reject the embryo and result in miscarriage, but it is enough to support the receptive endometrium to hold the embryo. Therefore, initiating a very weak and controlled immune response through TLR9 by intrauterine injection of the ligand could be used as an infertility treatment to increase the rate of implantation, similar to the way that endometrium scratching does. More investigation is definitely needed to clarify this issue.

6.3 Ideas for future studies

Scientists have tried for many years to understand the molecular details of the implantation phenomenon not only in humans but also in other mammals. Nevertheless, there is still a lack of knowledge in this field of study. Hence, to help infertile couples experience the joy of parenthood, more investigations are needed.

The preliminary experiments of this thesis showed that CpG has a detrimental effect on trophoblast attachment to the endometrial epithelial cells. Considering the fact that several different factors such as cytokine production are involved in trophoblasts-endometrium interaction, whether TLR9 stimulation with CpG dysregulates the cytokine production or any other factors at the site of implantation that lead to inadequate uterine receptivity could be a matter for future study. Hence recognition of the production of any cytokines and chemokines in response to different doses of CpG that may affect the trophoblasts-endometrium interaction in our 2D culture system is also suggested.

We have seen that the TLR9 intracellular signalling pathway is involved in the trophoblast-endometrium interaction and is also involved in IL-8 secretion. We have also found that the TLR9 intracellular signalling pathway in RL95-2 cells is not through the possible mediators IRF7, NFκB, and p38/MAPK suggested in the literature. Therefore, we speculate that the TLR9/CpG signalling pathway in the RL95-2 cells is through an unknown signalling cascade. Discovering a novel TLR9 signalling pathway in RL95-2 cells as a valid model of a receptive endometrium could help us get one step closer to designing a drug that might locally suppress immune responses at the time of the pregnancy. In this regard, different types of fluorescent-marker expression vectors are now commercially available with various signalling mediators (NFκB, different cytokines, JAK-STAT, and many more) coding sequences. One can generate a lentivirus with the vectors and stably transfect RL95-2 cells with them. Changes in the expression and location of each of these signalling mediators in response to CpG could be easily chased under the time-lapse confocal laser-scanning microscope.

Using a 3D endometrial culture system, in addition to attachment, trophoblast outgrowth and invasion have been studied in this thesis. The writer has proposed another approach, measuring the human chorionic gonadotropin (hCG) secretions from trophoblast spheroids, as a differentiation marker over time when co-cultured with a 3D endometrial culture and analysing changes in hCG gene expression in trophoblasts in contact with the endometrium. This proposal has been accepted as a MSc project by the university last year. Research has been done in our laboratory by one of our talented MSc students, Miss Abigail Moriss. In addition, this thesis writer would like to suggest investigation of the effect of TLR9 activation in endometrial and trophoblast cells on hCG production levels in the 3D culture system as well.

6.4 Conclusion

Human implantation is a complicated phenomenon controlled by several factors, from sex hormones to cytoskeleton rearrangements to innate immune responses. Evidence from this research study indicated that TLR9 stimulation can affect the trophoblast-endometrium interaction in both a positive and a negative way that is highly dependent on the culture condition (2D or 3D) and the dose of the ligand. This is a confirmation of the double-edged sword effect of TLR9 stimulation in human implantation.

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