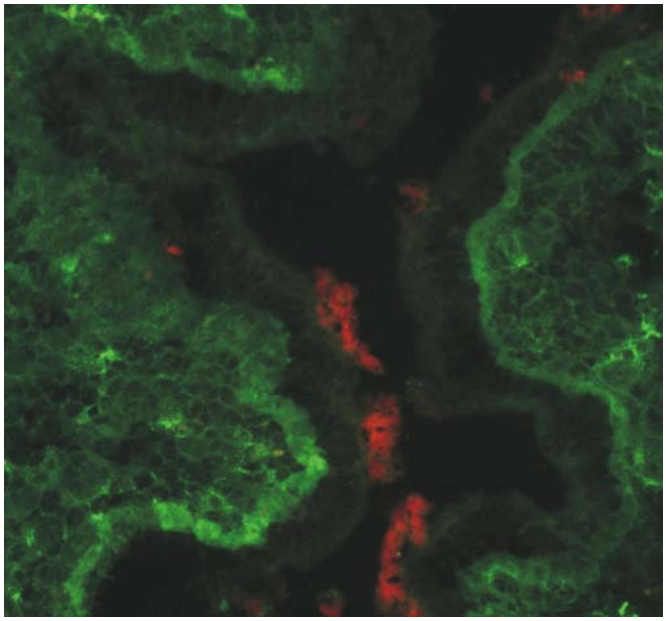
A Novel Technology for In Vitro and In Utero Modification of Endometrial Cells



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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Susannah McIntosh
Auckland,
October, 2009

This thesis is dedicated to my mother, Juliet Louise McIntosh – my first, my greatest and my most loved teacher – and friend.

I miss you Mum.

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This project was governed by ethics approval granted prior to this research by the Animal Ethics Committee, Faculty of Medical & Health Sciences, The University of Auckland, approval number: AEC 10/2003/0183, granted 31 January, 2005.

List of Abbreviations

FSL…..……Functional Spacer Lipid
EEC……...……Endometrial Epithelial Cell
DIC……..……..Direct Interference Contrast
OCT……..……Optimum Cutting Temperature
PBS……Phosphate Buffered Saline
bHABP……..……biotinylated Hyaluronic Acid Binding Protein
FBS……..Fetal Bovine Serum
FITC……Fluorescein Isothiocyanate
BSA……...Bovine Serum Albumin
PVP……..……..…Poly vinyl pyridine
HBSS……..……..……Hanks Balanced Salt Solution
IVF……..……In Vitro Fertilisation
ECM……..Extracellular Matrix
WOI……Window of Implantation
NK……Natural Killer (Cells)
uNK……..……uterine Natural Killer Cells
IL-1……..……Interleukin-1
IL-1R……..……Interleukin-1 Receptor
IL-Rβ……...Interleukin-1 Receptor beta
LIF……..……Leukemia Inhibiting Factor
IL-11……..…Interleukin-11
LE……luminal epithelium
Ley……..Lewis Y (antigen)
HA……Hyaluronic Acid
ICSI……..Intra-cytoplasmic Sperm Injection
ART……..……Assisted Reproductive Technology
LH……..…Luteinising Hormone
HSG……hystosalpingogram
PBS……..……..Phosphate Buffered Saline
IFN γ……Interferon Gamma
IL-10……..……Interleukin-10
OSCM……Oil Soluble Contrast Media
ICM……..Inner Cell Mass

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Abstract

Implantation failure is a major contributing factor to both the diagnosis of unexplained infertility and unsuccessful In Vitro Fertilisation (IVF) outcomes. Research efforts aimed at increasing IVF success rates have largely focussed on improving embryo quality and selection. The reciprocating partner in implantation, the endometrium, has received less attention or at least resulted in fewer useful advances. Difficulties in accessing the endometrium, coupled with the limited methods for bringing about alterations in this organ have left treatment options wanting. KODE™ modification is a proven technology used to impart functional changes to the cell surface by insertion of glycolipid-like constructs into the bilipid membrane of cells, altering antigen exhibition. This investigation set out to explore the ability of KODE™ constructs to modify endometrial epithelial cells (EECs) in order to impart functional changes in the attachment of trophoblastic spheroids in vitro and embryos in an in situ mouse model. Insertion and retention assays have demonstrated that the benign proof principle molecule, FSL A, successfully inserts into the target endometrial epithelial cells in an efficient manner. A decrease in spheroid attachment was unexpectedly demonstrated in an in vitro assay of FSL A-treated EEC monolayers ($p < 0.001$). Similarly, FSL B molecule also decreased the attachment potential in these cells. Results in vivo reflected those of in vitro assays, with decreased implantation rates observed when uterine endometrial cells were modified in situ by administration using a novel lavage technique. This is the first report that KODE™ technology can be used as a research tool to advance the understanding of cellular interactions in the uterine environment and the ultimate development of potential therapeutic applications for IVF.

1 Introduction

If the uterus were able to be engineered to provide peak conditions of receptivity for an embryo, one of the major existing limitations of In Vitro Fertilisation (IVF) technology may be remedied. The manipulation of this sensitive environment may simply require the targeted exposure of a modifying agent delivered directly to the endometrium.

Mammals prioritise the nurturing of a fetus through long phases of development to achieve survival of highly developed progeny. This nurturing is facilitated by the uterus - a unique, complex and dynamic organ which provides accommodation for the developing mammalian young. Female derived oocytes are fertilised in the fallopian tubes of the female reproductive tract by the male derived spermatozoa, marking conception. The resultant embryo is a genetically unique entity able to survive just a few days on nutrients derived from the ovum. It must promptly make its way to the uterus where it must tap into the maternal system to obtain nutritional sustenance. The attachment and implantation of this genetically foreign entity into the maternal system may be regarded as a uniquely natural and tolerated allograft. Hospitable conditions for fetal development must be well maintained, however the host immunity must also remain sufficiently intact so that protection of the host from infection is not significantly jeopardised.

The uterus is a remarkably dynamic and complex organ which, as with most tissues exposed to the external environment, usually acts as a barrier. However, it also provides the ideal environment for hosting an embryo through different stages of development until birth. Uterine tissue allows for the invasion of the embryo into the uterine lining in a precisely timed manner. Embryo attachment to and implantation into the endometrium lining the uterus is facilitated by cell surface expression of molecules which are essential for targeted maternal-embryonic cross talk. As research into human conception is ethically and morally restrained, current knowledge of this integral step is derived mainly from animal studies.

The increasing age of first time parents is a phenomenon which has fuelled the demand for IVF. However, a recognised hurdle in the IVF clinic is the attachment of the embryo to the endometrium and while current practices may soon allow the use of human embryo derived tissues for regulated research, there is a still a real need for robust, human-specific in vitro models to investigate this attachment. It is 25 years since the introduction of IVF into New Zealand (Chism, 2009), and since this technology is still relatively new in terms of generational information, models both in vitro and in vivo are essential with respect to current development of IVF technologies.

Attachment and implantation of an embryo to the uterine wall clearly involves two distinct entities. Previously, research has focussed on the optimisation of embryo attachment potential through improved selection and culture conditions of embryos during critical periods of development. This thesis targets the uterus. The ultimate goal is to modify the surface endometrial epithelial cells to achieve a measurable difference in the attachment of an embryo. In vitro models will be employed to develop appropriate modification parameters and to monitor cell to cell attachment. A novel route of fluid delivery to the uterine environment will be developed which will then be employed to deliver modifying agents directly to the uterine environment in vivo. The potential influence of modification on embryo attachment and implantation will then be ascertained in an in vivo mouse model.

1.1 Mammalian Reproduction

Demonstrative of the versatility of the uterus is its necessity to maintain relatively hospitable surroundings for the passage of sperm, a genetically foreign entity, several days prior to fertilisation. Parameters such as pH, viscosity of mucous and temperature within the female reproductive tract can all influence this initial interaction between sperm and oocyte. In addition to the presence of sperm, the semi-allogeneic blastocyst must be tolerated without compromising the uterine immune surveillance required to protect the host from infectious agents (Lea & Sandra, 2007). Provided fertilisation of the mature oocyte is successful, the resultant embryo makes its passage through the fallopian tube and into the uterus and prepares for attachment to the uterine

wall. Subsequent to attachment of an embryo to the endometrium lining of the uterus, the mammalian embryos implant into the underlying uterine tissue.

The invasive capability of the embryo determines the depth of implantation, which varies significantly across mammalian species (Renfree, 2006). The human trophoblast is unusually aggressive in terms of invasion and while decidualisation occurs spontaneously in humans as part of the menstrual cycles the presence of a blastocyst is required for this to occur in most other species with haemochorial placentation (Dimitriadis et al., 2005; Kimber, 2005). However, in the human, certain molecular changes of the luminal epithelium occur only in the regions in direct contact with the embryo (Dimitriadis et al., 2005). The implanted genetically distinct embryo may be regarded as an allograft within the uterus (Hoozemans et al., 2004). A trophoblast cell of an embryo which attaches to the uterine epithelium must possess two major abilities: i) to perform an adhesive interaction with the apical cell pole of uterine epithelia and ii) the ability to invade the extracellular matrix (ECM), (Hohn & Denker, 2002). Cell to cell adhesion of trophoblast cells with uterine epithelial cells involves two distinct steps: i) easily dissociated, initial contact which is readily reversed and ii) a strengthened adhesion requiring metabolic energy to become stabilised (John et al., 1993).

1.1.1 The Uterus – Structure and Function

The endometrium is the name given to the tissue overlaying the myometrium of the uterus and lining the lumen into which an embryo implants. The surface cells of the endometrium which are exposed to the external environment are known as the endometrial epithelial cells (EECs) and beneath these are the underlying stromal cells, which together make up the functional layer. Beneath this functional layer is the basal layer which is attached to the myometrium. EECs are peculiar to the endometrium in that whilst most epithelial cells in the body function to maintain a strict barrier to the external environment, mammalian endometrial epithelial cells must allow their barrier function to be temporarily breached in order to allow the implantation of an embryo. Further to the barrier function of the epithelial cells, the stromal cells lying beneath the uterine endometrial epithelial cells include a significant number of immune cells which are synchronised across the menstrual cycle.

Throughout the menstrual cycle, the basal layer remains attached to the myometrium, while the cells of the functional layer separate and are shed during menstruation (Diedrich et al., 2007). The basal layer then initiates endometrial regeneration. Epithelial proliferation occurs in response to estrogen if its receptor is present in the stroma. Estrogen receptors are also required in the endometrium in order to control the expression of epithelial secretory proteins (Aplin & Kimber, 2004). The basal endometrium is responsible for the regeneration of tissue post menstruation in humans (Aplin & Kimber, 2004). Also present in the layers of the uterine tissue are the immuno-competent cells and blood vessels (Diedrich et al., 2007). During the proliferative phase of the menstrual cycle, 20% of stromal cells are leukocytes, whilst in the early stages of pregnancy, this rises to 30% - 40% (Lea & Sandra, 2007; Vigano et al., 2003). This observation is indicative that these cell populations are under hormonal regulation.

The menstrual cycle, driven by ovarian hormones estradiol and progesterone, prepares the uterus, for potential embryonic attachment and implantation, approximately every 4 weeks (Diedrich et al., 2007; Rombauts, 2008; Strowitzki et al., 2006). Estrogen and progesterone drive the proliferative and secretory phases of the menstrual cycle respectively and it is during the secretory phase that stromal cells of the uterus will differentiate into decidual cells in preparation for implantation of an embryo (Fazleabas & Strakova, 2002; Kennedy et al., 2007; Strowitzki et al., 2006). Decidual cells, important in implantation, provide nutritional support of the embryo, control trophoblast invasion and help to protect the embryo from maternal immune rejection through the production of specific decidual proteins (Fazleabas & Strakova, 2002). Figure 1 shows the changing endometrium throughout the cycle and the stage of potential presence of an embryo. Epithelial and stromal cells both express progesterone and estrogen receptors, and the levels of these receptors together with the concentration of the hormones act in tandem to exert a response (Diedrich et al., 2007). Androgen receptors present in these cells also allow endometrial function to be changed according to androstenedione and testosterone expressed during implantation (Diedrich et al., 2007). During menstruation, immune cells which have been activated release matrix metalloproteinases

which act on the ECM and are important for the endometrial breakdown (Lea & Sandra, 2007).

Figure 1. Hormone and receptor expression of the endometrial cycle. Estrogen receptor expression and proliferation are stimulated early in the cycle by estrogen (E_2). By day 17 there is an increase of secretions in the uterine cavity with secretory activity peaking at around day 19-20 giving rise to decidualisation. The presence of the potentially developing blastocyst is shown early in the cycle. Adapted from Diedrich et al, 2007.

The time of receptivity cessation in the human endometrium is considered to probably be when the corpus luteum is no longer feasible, the uterus is hostile to non-implanted embryos and menstruation is imminent (Aplin & Kimber, 2004). The endometrial cycle is commonly split into two phases: the proliferative phase during which the growth of stromal, epithelial and vascular cells occur and the secretory phase, marked by increased gland secretory activity and stromal decidualisation (Lobo et al., 2004). During the secretory phase a myriad of biochemical, cellular and morphological changes prepare the endometrium for potential embryonic implantation (Lobo et al., 2004). It has been suggested that implantation resembles the process of inflammation, and indeed, mechanical injury or irritation of the endometrium of pseudo-pregnant recipient mice has been reported to induce proliferation of endometrial cells and

facilitate the decidualisation of the stroma, necessary for implantation (Wakuda et al., 1999). Prostaglandin production is integral in the inflammation process important for menstruation and is inhibited when progesterone is maintained during pregnancy (Lea & Sandra, 2007).

A uterus unprepared for embryo attachment is refractory to attachment until conditions are appropriate and optimal for hosting and nurturing a fetus. The phase during which these conditions are optimal and a uterus is receptive to the implantation of an embryo is widely referred to as the window of implantation, (WOI), (Nikas & Psychoyos, 1997; Strowitzki et al., 2006). In the latter part of the menstrual cycle, stromal cells begin to differentiate into endometrial stromal fibroblasts to form the decidua of pregnancy, or decidual cells in conjunction with the influx of uterine natural killer (uNK) cells (Dimitriadis et al., 2005). In most mammals, this reaction requires the presence of the blastocyst, but it occurs spontaneously in the human menstrual cycle (Dimitriadis et al., 2005). The luminal epithelium plays an essential role in decidualisation and if this layer is destroyed or removed, decidualisation cannot occur in response to stimuli which would usually elicit the decidual response (Kennedy et al., 2007). Prostaglandins are thought to be involved in the process of decidualisation (Kennedy et al., 2007). During the secretory phase , the epithelium becomes transiently receptive to an embryo and will slough off and shed via menstruation if implantation does not occur (Rombauts, 2008).

Uterine natural killer cells (uNK) are controlled by estrogen and are critically important in the regulation of the immune response to the semi-allogeneic trophoblast and are responsible for production of a variety of cytokines implicated in trophoblast invasion (Lea & Sandra, 2007; Lobo et al., 2004). These cells increase in number in the pre-implantation uterus due to proliferation together with an influx of natural killer cells from the peripheral circulation and make up to 80% of the immune cells in the pre-implantation uterus (Lobo et al., 2004). Natural killer cells and the cytokines they produce are essential for implantation, contrary to their classic function of being cytolytic to non-self cells (Lobo et al., 2004) and have also been associated with menstruation, decidualisation and vascular remodelling (Dimitriadis et al., 2005; Lea & Sandra, 2007; Lobo et al., 2004).

A blastocyst stage embryo which attaches to uterine epithelium is made up of cells which have begun differentiation into trophoblast cells. Trophoblast cells do not contribute to the embryo proper, but later develop to form the placenta. The inner cell mass becomes the newly developing fetus. The maintenance of the conceptus and establishment of pregnancy is reliant on the trophoblast cells attaching through their apical cell surface to the apical surface of endometrial epithelial cells lining the uterus (Hohn & Denker, 2002). This attachment is regarded as a phenomenon of embryonic attachment, as the normal epithelia do not allow adhesion of any other cells to their apical surface (Hohn & Denker, 2002). Figure 2 shows a blastocyst attaching to a receptive endometrium and the molecular dialogue occurring between the embryo and the endometrium.

A blastocyst involved in molecular dialogue with the maternal endometrium will undergo three phases of attachment before proper implantation into the maternal uterine wall is achieved. Initial aligning, or apposition, occurring most commonly in the upper wall of the uterus, is a relatively unstable, physical interaction between the blastocyst and the uterine epithelium. The more stable adhesion occurs with the blastocyst then oriented with its embryonic pole toward the uterine epithelia. Invasion into the uterine tissue ensues giving rise to implantation of the embryo (Norwitz et al., 2001). Implantation in the human occurs 6-12 days after conception, with the blastocyst finally being embedded in the stromal tissue of the uterus (Norwitz et al., 2001; Wilcox et al., 1999). The synchronisation of the hormonally primed uterus with the presence of the mature blastocyst is essential for successful implantation (Norwitz et al., 2001).

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Figure 2. Blastocyst apposition and adhesion. The diagram shows a pre-implantation-stage blastocyst (approximately six to seven days after conception) and the processes considered necessary for uterine receptivity, blastocyst apposition and adhesion. LIF denotes leukemia inhibiting factor. Adapted from Norwitz et al, 2001.

Molecular dialogue between the embryo and the maternal cells allows the endometrial receptivity to be influenced by the secretion of several factors by the conceptus (Vigano et al., 2003). Figure 3 demonstrates factors which influence the endometrium and trophoblast interaction. Initially, secretion of estrogen stimulates the proliferation and differentiation of uterine epithelial cells and the subsequent progesterone produced by the corpus luteum stimulates distinct cellular and molecular changes in the endometrium (Vigano et al., 2003). These specific hormonal interactions elicit the release of many proteins into the uterine environment (Vigano et al., 2003).

Cytokines are small, glycoprotein mediators with multifunctional actions linked to many bodily processes including immune function and implantation (Dimitriadis et al., 2005). Cytokines facilitate the maternal-conceptus cross talk, important for the establishment of early phases of pregnancy, attachment,

implantation and interaction with the maternal immune cells (Vigano et al., 2003). Redundancy which exists within cytokine families, allows an over-lapping of functions of certain cells and provides a back-up function in individuals lacking particular cytokines or their receptors (Dimitriadis et al., 2005). Many cytokines are expressed by the myriad of cells which make up the uterus and are important at the time of implantation (Dimitriadis et al., 2005).

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Figure 3. Endometrial influences during early stages of human implantation. Adapted from Dimitriadis et al, 2007

Interleukin-1 (IL-1) is one of the first factors involved in embryo-maternal cross talk, the receptor for which (IL-1R) is expressed by the surface epithelia, and increases significantly during the mid-luteal phase of the menstrual cycle (Vigano et al., 2003). This cytokine, after binding to its receptor, induces molecular changes in the endometrium which are necessary for the attachment of the blastocyst (Vigano et al., 2003). However, it has been shown by some researchers that IL-1β is negatively associated with clinical pregnancy. Intrauterine cytokine profiles of women trying to achieve natural pregnancy were investigated by these researchers to elucidate important uterine cytokines at the time of implantation (Boomsma et al., 2009).

A cytokine important for the regulation of trophoblast invasion is IL-11 and is reduced in women with infertility and endometriosis. This cytokine has also been shown to be essential for embryo implantation in the mouse (Dimitriadis et

al., 2006). Many other cytokines are involved in the maternal-conceptus cross talk, initial attraction and attachment of the embryo to the uterine cells and are important contributors to implantation. The preparation of the endometrium for pregnancy by the differentiation of stromal cells to form the decidua is also influenced by cytokines (Dimitriadis et al., 2005; White et al., 2007).

Leukemia Inhibitory Factor (LIF) is one of the most important cytokines in the reproductive tract and is responsible for proliferation, differentiation and cell survival. LIF is particularly essential for blastocyst development and is critical for decidualisation and implantation (Aplin & Kimber, 2004; Dimitriadis et al., 2005; Kimber, 2005; Lea & Sandra, 2007; Norwitz et al., 2001). LIF is a highly glycosylated glycoprotein expressed at particularly high levels in the uterus (Kimber, 2005) and the level of this cytokine rises dramatically in the mid-secretory part of the menstrual cycle (Aghajanova et al., 2003; Norwitz et al., 2001). Uterine LIF appears to be primarily regulated by estrogen however it is influenced by progesterone during the secretory phase when it reaches peak levels in the endometrium (Aghajanova et al., 2003; Lea & Sandra, 2007). LIF mRNA has been shown to be present in the endometrium of women with proven fertility between days 18-28 of the menstrual cycle. Furthermore, whilst LIF is maximal in uterine flushings from women of mid-late secretory phase, at the time of expected implantation, it appears to be significantly reduced in flushings from women with unexplained infertility (Aghajanova et al., 2003; Dimitriadis et al., 2005; Kimber, 2005). Diminished secretion of LIF is also associated with recurrent pregnancy loss (Norwitz et al., 2001), and may be considered as a candidate marker for endometrial receptivity (Boomsma et al., 2009).

Carbohydrates also play a critical role in cell to cell interactions of the pre and peri-implantation embryo (Poirier & Kimber, 1997). Cell surface carbohydrates are important for cell recognition and may interact with other carbohydrates or proteins (lectins) (Poirier & Kimber, 1997). In animal cells, saccharide sub-units varying from a single sugar to long chains in excess of 30 residues and which may exist in a branched conformation may attach to either lipids or proteins (Poirier & Kimber, 1997). As the embryo develops, specific carbohydrate antigens appear on the cell surfaces. Considerable evidence suggests that

carbohydrates may be involved in the very early events of implantation. Glycoconjugates making up the apical glycocalyx of luminal epithelial cells are known to extend beyond the cellular projection of proteins and into extracellular space are likely to be the first contact an embryo has with the endometrium (Poirier & Kimber, 1997). This initial interaction may allow the apposition with the luminal epithelial (LE) cells of the endometrium (Poirier & Kimber, 1997). The blood group antigen, H type 1 expressed on the mouse endometrial epithelium at the time of implantation, but absent by day 6 is recognised by its receptor on the trophectoderm of the embryo (Aplin, 1997; Poirier & Kimber, 1997). Another interesting carbohydrate in terms of endometrium receptivity is Le^y, present at the surface of mouse blastocysts and endometrial epithelium. Anti-Lewis antibodies have been shown to inhibit implantation in the mouse when delivered into the uterine lumen prior to attachment, indicative of its importance for implantation (Aplin, 1997; Poirier & Kimber, 1997).

The interaction between epithelial cells and their surrounding stroma is facilitated by transmembrane receptors that interact with extracellular matrix (ECM) molecules such as CD-44 (Afify et al., 2005). Hyaluronic acid (HA), a component of the ECM interacts with CD-44 a transmembrane protein (Afify et al., 2005). CD-44 has been identified on pre-implantation embryos along with a large number of negatively charged sialic acid residues which may have a variety of functions (Poirier & Kimber, 1997). This molecule is present up to the blastocyst stage of development of the embryo and recognises hyaluronan and chondroitin sulphate, abundant on the endometrial apical epithelium (Aplin, 1997). HA is a glycosamino-glycan composed of repeating disaccharides, the chains of which vary enormously and can reach up to 107 kD. The negative charge associated with these molecules allow polymers to take up large volumes of water and create space for cell motility and proliferation (Afify et al., 2005). HA synthesis in the human endometrium increases dramatically around the time of implantation and may support embryo implantation in the uterine cavity, cellular migration aggregation and cell to matrix adhesion (Afify et al., 2005).

Mucins are proteins which exhibit a high sugar content and cause an increase in oligosaccharide receptors on endometrial epithelial cell surfaces

(Norwitz et al., 2001). Mucins are thought to be involved in endometrial receptivity. The glycocalyx surrounds the uterine lumen and is encountered by the attaching embryo as it approaches the epithelial surface (Aplin, 1997). One of the predominant cell surface associated mucins is MUC-1, a plasma membrane molecule which is abundant on the microvilli and cilia extending from endometrial epithelial cells' apical surfaces (Aplin, 1997). The function of MUC-1 in humans is thought to be one of protection, inhibiting cell to cell interactions by extending outwards into the uterine lumen and causing steric hindrance of ligand access to the cell (Aplin, 1997) inhibiting cell adhesion and creating a barrier to implantation (Aplin & Kimber, 2004; Isaacs & Murphy, 2003). The MUC-1 may further act as a barrier to implantation by remodelling the cyto-skeletal network or by down-regulating activity of attachment molecules such as integrins (Isaacs & Murphy, 2003). The apical glycocalyx, of which MUC-1 is an important component, also protects the upper reproductive tract from infectious agents, however the thickness and the overall charge of the glycocalyx is known to decrease prior to implantation (Aplin & Kimber, 2004; Lindenberg et al., 1990). Due to the increased expression of MUC-1 in humans just prior to implantation, it is thought that the balance of cell surface associated and secreted MUC-1 is important, with small areas of low expression thought to define areas of receptivity in the uterus (Aplin, 1997). It has been suggested that the initial weak attachment of an embryo to the endometrium allowing apposition, may be fortified by carbohydrate-lectin binding (Aplin & Kimber, 2004).

Integrins are potential participants in fertilisation, implantation and placentation as they respond to both intracellular and extracellular signalling and mediate cell to cell and cell to substrate attachment (Bowen & Hunt, 2000; Sharkey, 1998). Integrins are made up of a large family of heterodimeric transmembrane receptors composed of α and β subunits each with an extracellular domain, a transmembrane domain and a short cytoplasmic domain (Bowen & Hunt, 2000). Three integrins of particular interest appear to be important for implantation in the human endometrium: $\alpha 9\beta 1$, $\alpha v\beta 1$, and $\alpha v\beta 3$ and are all members of either fibronectin or vitronectin receptors (Bowen & Hunt, 2000). The integrin $\alpha v\beta 3$ has been implicated in human implantation and is capable of binding to a wide variety of extracellular matrix (ECM) components (Bowen & Hunt, 2000;

Sharkey, 1998). It has been suggested that integrins expressed on both the embryo and on endometrial epithelial cells may bind to ECM components in the intercellular space. The multi-dimensional nature of these molecules and the redundancy compensating the loss of any sub-unit by another and makes them potentially important participants in reproductive processes (Bowen & Hunt, 2000; Sharkey, 1998)

1.1.2 Assisted Reproduction and In Vitro Fertilisation

Infertility, generally defined as the inability to conceive after unprotected sexual intercourse for one year (OBGYN., 2009a) may arise from a male infertility problem (35%), a female problem (35%) or a combination of the two (20%). Unexplained infertility accounts for around 10% of cases (OBGYN., 2009a).

Assisted reproduction includes techniques such as artificial insemination and administration of super-ovulatory drugs, while in vitro fertilisation (IVF) involves more specific, technically demanding and invasive techniques such as oocyte retrieval, and intra cellular sperm injection (ICSI). Fertilised ova are maintained in culture and choice embryo(s) are chosen for transfer into the synchronised uterus. Days 20-24 of a regular menstrual cycle are recognised to be the optimal period for implantation to occur (Norwitz et al., 2001), and may be targeted for embryo transfer of cultured embryos. Excess embryos may then be cryogenically frozen and stored for potential future transfers. By targeting specific phases of development of the embryo many causes of natural infertility may be circumvented.

Attachment of the healthy embryo is a fundamental requirement for implantation and the establishment of clinical pregnancy. This hurdle may account for a high proportion of the “unexplained infertility” diagnoses concluded by clinicians and remains the major rate limiting step for IVF success (Boomsma et al., 2009). As few as 50-60% of all natural conceptions advance beyond 20 weeks gestation, with 75% of lost pregnancies representing a failure in implantation, and therefore not regarded as clinical pregnancies (Norwitz et al., 2001). Implantation failure remains a major problem for assisted reproduction techniques and along with poor oocyte quality or delayed implantation may be caused by diminished uterine receptivity (Norwitz et al., 2001;

Simon et al., 1999). By investigating implantation rates in oocyte donation, it is observed that implantation rates are higher than compared with IVF. This may be due to the IVF individuals being subjected to heightened physiological steroid levels which may affect implantation (Simon et al., 1999). It has been contended by researchers that the implantation rate of IVF cycles is actually comparable to natural cycles. However, the percentage of continuing viable pregnancies may be higher in natural cycles as frequency of early pregnancy loss in assisted reproduction (ART) has been shown to be approximately 40% (Simon et al., 1999). Implantation of chromosomally abnormal embryos which appear normal (19.3%) when graded during the ART process may be hampered due to disruption of adhesion molecule expression and cytokine production. (Simon et al., 1999). An abnormal endocrine milieu in IVF may also be responsible for lower implantation rates seen (Simon et al., 1999). Implantation has been observed in individuals as early as 6 days post ovulation and as late as 18 days post ovulation based on a ratio of estrogen metabolites to progesterone metabolites in urine samples (Wilcox et al., 1999). There appears to be a high risk of early pregnancy loss with later implantation. This evidence may provide a potential area for improving uterine receptivity by artificial manipulation (Wilcox et al., 1999). However, most successful pregnancies exhibited implantation of the conceptus between days 8 to 10 after ovulation, with no successful pregnancies maintained from implantations which occurred after day 12 post ovulation (Wilcox et al., 1999).

The number of cycles carried out for assisted reproduction is continuing to increase, with a 3 % rise in the year from 2005 to 2006, and a doubling since 1997 exhibited in Europe (ESHRE, 2009). Intra-cytoplasmic sperm injection cycles have appeared to rise in preference to standard IVF. This is perhaps surprising given that there is no evidence that ICSI is beneficial in cases other than severe male infertility. Furthermore, it is more time consuming and expensive. Also, due to its relatively novel nature, the long term implications of male mitochondria present in the conceptus is yet to be conclusively elucidated. However, this practice appears to be emerging as the preferred approach in some countries (ESHRE, 2009).

While the proportion of all human conceptions that fail to implant is uncertain, studies of sperm donation programmes indicate that the maximal chance of achieving implantation under optimal conditions is around 40% (Diedrich et al., 2007). Within Australia and New Zealand in 2006, 10,000 babies were born to women who had fertility treatments, with 78% being single births (Illingworth, 2008). This single birth rate is the highest number recorded for IVF recipients and is good news in an industry where the practice of transferring multiple embryos to a recipient is increasingly controversial. Multiple embryo transfers introduce all of the potential obstetric complications associated with carrying and maintaining multiple fetuses. This practice raises concerns with respect to both the optimal health of the developing fetus and the mother. Resultant twin delivery has recently fallen below 20% for the first time following the introduction of Assisted Reproductive Technology (ART), with 79% of all ART deliveries being singleton deliveries (ESHRE, 2009). Pregnancy rates throughout Europe have increased with fewer embryos being transferred, providing evidence that this single embryo transfer is the most successful approach (ESHRE, 2009).

Despite huge advances in the field of ART, particularly in quality, safety and efficacy, in the last 30 years, implantation rates are still widely considered by clinicians to be inefficient (Diedrich et al., 2007). In Europe, the average pregnancy rate per embryo transfer in 2006 was 32.8% after IVF (ESHRE, 2009), compared with 26.1% in 1997. In New Zealand, in 2006, 28% of treatment cycles resulted in clinical pregnancy and 22% in live birth (Illingworth, 2008). A potential cause of implantation failure may be due to the failure of the blastocyst to be released from the zona pellucida (Diedrich et al., 2007). Ovarian hyperstimulation, undertaken to recover ova for assisted reproductive purposes is known to have an adverse effect on the endometrial receptivity in the mouse, (Aplin & Kimber, 2004).

Peak uterine conditions must be targeted for optimal embryo transfer resulting in a successful implantation into the endometrium. In the 1950s a histological technique was developed by Noyes et al to distinguish particular time points during the menstrual cycle (Noyes et al., 1975). Biopsies of endometrial tissue stained using histological techniques, provided a snap-shot of the state of the endometrium and enabled overt abnormalities to be evaluated. While there are

clearly limitations of this technique, it has remained the standard for endometrial analysis with morphology and cell function both able to be assessed (Bourgain et al., 1994; Diedrich et al., 2007). The major limitation of such a technique is due to the subjective interpretation of histological data both inter- and intra-observer. Furthermore, variability is observed when considering the inter- and intra- cycle variations, the particular area chosen for biopsy and any influence of ovarian stimulation on the maturation of the endometrium (Acosta et al., 2000; Bourgain et al., 1994; Diedrich et al., 2007; Murray et al., 2004).

Hormonal changes may be assessed, such as the luteinising hormone (LH) surge predictive of ovulation however this too has inter- and intra-subject variability. Hormonal activity within the endometrium is dominated by the key modulators, estrogen and progesterone. Receptors for both estrogen and progesterone, their metabolism and any co-activators or repressors all influence the endometrial development (Diedrich et al., 2007; Punyadeera et al., 2003). Epithelial and stromal cells both express estrogen and progesterone and the expression of their receptors in a cyclical fashion is essential for endometrial receptivity (Diedrich et al., 2007). Immunohistochemistry also suffers the same variability problems as histological assessment. However some molecules appear to be showing promise with respect to being markers of the implantation window (Diedrich et al., 2007; Hoozemans et al., 2004).

1.2 Modification of the Uterine Environment

1.2.1 General Modification

In the natural environment, specific changes occur in the uterus, both in terms of cellular architecture and cell surface molecular expression. These changes are critical for the successful attachment and implantation of an embryo. Endometrial epithelial cells (EECs) make the initial contact with embryonic tissue. For this reason, their barrier function must be circumvented in order that the endometrium may become receptive to attachment of an embryo. Murphy et al have suggested that the “plasma membrane transformation” is a common and necessary process of change in all aspects of uterine epithelial cell membranes (Murphy, 2004).

Prior to implantation, the apical membrane of the uterine epithelial cells flattens and loses the regular microvilli present in the resting uterine epithelia. During the receptive period, the endometrium becomes vascularised, the endometrial glands increase their secretory potential and pinopodes develop on the surface epithelia (Aghajanova et al., 2003; Norwitz et al., 2001). These bulbous protrusions of cellular cytoplasm are more correctly referred to as uterodomes as they do not have the pinocytotic function implied by the term pinopode. Uterodomes replace the microvilli on luminal epithelial cells around day 5-6 after ovulation (Aplin & Kimber, 2004; Lindenberg et al., 1990). Their progesterone driven appearance is confined to the implantation window, and it appears to be co-expressed with leukaemia inhibitory factor (LIF) during the secretory phase (Aghajanova et al., 2003).

Receptive EECs exhibit a loss in polarity during this plasma membrane transformation (Aplin & Kimber, 2004; Murphy, 2004), tight junctions change shape and gap junctions in the luminal epithelium also change with respect to their distribution and complexity in several species (Aplin & Kimber, 2004). Tight junctions are composed of intra-membrane proteins arranged in a network of strands separating the apical and baso-lateral domain of the plasma membrane of the endometrial epithelial cells (Orchard & Murphy, 2002). The tight junctions found at the lateral surface of the endometrial epithelial plasma membrane above the adherens junctions and desmosomes, control fluid movement across the uterine epithelium and impart polarity to endometrial epithelial cells. Studies in the rat have shown these junctions to become deeper in receptive cells, decrease the permeability and preserve uterine luminal fluid (Orchard & Murphy, 2002; Preston et al., 2006). Desmosomes, also located on the lateral surface of the EECs are the main junctional structure between epithelial cells. Further studies in the rat have shown these are down-regulated during the period of receptivity (Preston et al., 2006). Furthermore, adherens junctions are completely lost at the time of implantation. The observed increase in the protein occludin in tight junctions at the time of implantation may compensate for the loss of the other junctions and at the same time act to decrease para-cellular permeability to control the uterine luminal environment (Orchard & Murphy, 2002). Figure 4 shows two cells: one non-receptive (A) and the other receptive (B) with lateral desmosomes, tight

junctions and microvilli at their apical surface. The terminal web, made up of actin filaments is clearly visible in the non-receptive cell, but lost from the receptive cell. Also shown in Figure 4 are the tortuous plasma membrane of the receptive endometrial epithelial cell and the thickened basal plasma membrane (B).

Figure 4. Diagram of non-receptive and receptive uterine epithelial cells. Non receptive (left) and receptive (right) epithelial cells show lateral desmosomes (d) and tight junctions (tj) with microvilli (mv) at the apical surface of the non-receptive cell on the left. The terminal web (tw) is prominent in the non-receptive endometrial epithelial cell but lost in the receptive cell (Murphy, 2004).

The major morphological changes undertaken naturally in EECs during this period are indicative of their sensitivity to the environmental conditions and their adaptability to specific environmental changes. These features provide an opportunity for these cells to be targeted and artificially manipulated in order to alter certain cellular characteristics such as embryo receptivity. Furthermore, as these cells are themselves sloughed off monthly with the menstrual cycle in the human, they may potentially provide the ideal system for the temporary modification in an environment which is inherently tolerant of change.

Many methods have been used historically to artificially alter the endometrium, usually to provide a contraceptive function to the uterus. Examples of non-specific alterations include: physical disruption of the endometrium by intrauterine devices, cellular changes due to systemic hormones administered orally, or intra muscular injection of slow release medium containing hormones in order to alter the conditions of the uterus conducive to successful implantation. The uterine environment may be artificially modified by the simple systemic administration of hormones, or administration of modifying agents directly to the uterus. These methods however, provide a general alteration of uterine conditions rather than a specific modification of target cells.

Liposome mediated transfection of the murine uterus has also been used successfully in the mouse reproductive tract to transfect the uterus with a plasmid containing a bacterial gene. Viruses may also be used as vehicles for the transfer of genes and may be used to engineer the host tissue to produce high levels of the gene product (Daftary & Taylor, 2003). This technology allows high levels of protein to be targeted directly to the appropriate tissue, circumventing the host immunological system. Despite a change being efficiently produced in the uterine epithelia using this technology, it is questionable whether this approach would be acceptable for manipulation of the female reproductive tract in the clinical setting. ART is tightly bound by ethical constraints and safety concerns which may prove prohibitive to such technology.

Embryo receptivity of the uterine EECs may be manipulated by artificially altering the molecular presentation at the cell surface directly. A cell's plasma membrane provides a canvas for the expression of many different molecules. These molecules impart cell specific characteristics which provide functionality to the cells which is often highly tissue specific. This functionality facilitates the interaction between cells and provides a means for a dialogue system between many different cells.

Glycolipids are common components of membrane lipid bilayers and are composed of a carbohydrate chain and in the case of sphingolipids, a ceramide tail. The sphingolipid tail, composed of a long chain amino linked to a fatty acid is hydrophobic and can thereby insert itself into the lipid bilayer of cells

(Gilliver & Henry, 2003). Characteristics of the sphingolipid tail may be imparted by the length of the hydrocarbon chain, the presence of double bonds and the number of hydroxyl groups present (see Figure 5). Glycosphingolipids carry the Lewis and ABO blood group determinants, and glycolipid antigens such as Lewis antigens are acquired from plasma (Frame et al., 2007). Lewis glycolipids are found either circulating as complexes with lipoproteins in blood plasma, or inserted into cell membranes, with the carbohydrate exposed to the extracellular environment (Henry, 1996). Lewis antigens of blood cells, platelets and macrophages are acquired from the plasma (Henry, 1996) and while it has been postulated that the antigens may originate from the gastro-intestinal tract, it has been shown these antigens are much more likely to have originated from the liver, spleen or other organs (Gustavsson et al., 1999).

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Figure 5. Schematic diagram of natural blood group A glycolipid and FSL A. The synthetic constructs have dioleoyl-phosphatidylethanolamine bilipid tails which insert into lipid bilayers of plasma membranes of cells. Adapted from Frame et al, 2007

Red blood cells may be modified by naturally occurring glycosphingolipids as the bilipid tail inserts into their cell membrane and imparts antigenicity based on the sugar moiety which it carries. KODE™ technology has exploited this phenomenon in order to purposefully manipulate red blood cells and embryonic trophoblast cells in vitro using natural glycolipids. The limitations of this technique however are that these natural glycolipids must be isolated and purified from a natural source and the insertion characteristics of these

molecules are considerably limited by their poor solubility (Frame et al., 2007). Synthetic molecules have been engineered which overcome these limitations. Functional Spacer Lipid (FSL) technology employs the synthetically assembled three component Functional Spacer Lipid, and has evolved from the original KODE™ technology which employed naturally derived glycolipids such as gangliosides (Williams, 2008). Water solubility is introduced to the molecule by the incorporation of a linker onto the synthetic dioleoyl-phosphatidylethanolamine bilipid tail in place of the nervoic acid nonsaturated fatty acid ceramide tail of the natural molecule (see Figure 5). This important feature increases the diversity of applications for these molecules (Frame et al., 2007). Not only is insertion temperature lowered, but the insertion efficiency is enhanced (Frame et al., 2007). Furthermore, the requirement to utilise solvents or detergents in the preparation of the synthetic molecules, making them difficult to use in living cell applications, is circumvented (Williams, 2008).

The modification of embryos using naturally occurring glycolipids of early KODE™ technology has been extensively studied. This work suggested that potential adhesion molecules could be conjugated to lipid molecules and inserted into the embryonic cells (Blake, 2003). This modification may be achieved without causing harm to the embryo, the development of the fetus, or the recipient female (Blake, 2003). Synthetic molecular constructs have been shown to insert into embryos and positively influence embryo adhesion (Carter, 2007). The outer membranes of the embryo trophoblast cells were transformed with natural, semi-synthetic and completely synthetic molecular constructs. In vitro experiments confirmed that modification was harmless to embryos and these embryos displayed a significantly increased adhesion capacity for a range of cells and mucus (Carter, 2007).

The synthetic glycolipids are modifiable in that both carbohydrate and, more recently, peptide antigens may be specifically chosen to be conjugated to the synthetic tails. The insertion of these molecules into biological plasma membranes will consequently alter antigenic expression of target cells at their surface. Furthermore, the level of this altered expression may be tailored and finely tuned dependent on the concentration of synthetic molecules to which the target cells are exposed (Frame et al., 2007). Red blood cells have been stably

modified with a range of concentrations of synthetic molecules to produce both strongly antigenic and weakly antigenic expression of modifying molecules (Frame et al., 2007; Gilliver & Henry, 2003)

Modification of cell membranes has previously been shown in the membranes of various mammalian tissues (Frame et al., 2007). KODE™ modification of group O red blood cells, which contain neither A nor B blood group antigens, to express either one or both of these antigens has been successfully achieved. By changing their classic ABO blood type, a weak or strong expression of A or B blood cell types may be engineered to provide controls in the clinical setting, (Gilliver, 2005). By using synthetic KODE™ glycolipids, specific A or B antigen expression was targeted and achieved without affecting other important cellular characteristics such as solubility or reactivity with other relevant antibodies. The ability to control the level of modification enabled stable and highly reproducible results (Frame et al., 2007; Gilliver, 2005).

Modification of target cells may occur very rapidly – there is evidence that modification occurs within minutes, with optimal insertion of molecules into the membranes of target cells observed after one to two hours exposure to molecules. In the presence of the modifying molecule, this insertion will continue over time. The anuclear red blood cells for instance, may remain modified for some six weeks (Frame et al., 2007), while some metabolically active cells exhibit retention times of less than 24 hours. This retention depends not only on the individual properties of the molecules, but also on the various different cell types with elimination dependent in large part to the metabolic activity of the cell (Frame et al., 2007; Kovalenko et al., 2004; Schwarzmann, 2001). Elimination from the cell surface may occur by shedding into the inter-cellular medium, or by uptake into the cells via endocytosis (Kovalenko et al., 2004; Schwarzmann, 2001).

Since the advent of KODE™ technology, other groups have used a similar technology to modify cultured cell lines with differing sensitivities to Natural Killer (NK) cells (Kovalenko et al., 2004). They reported an increased cytotoxicity of NK cells towards K562 (human erythroleukemia cells) and Raji (Burkitt's lymphoma) cells which were modified with a Le^x conjugated glycolipid.

The concept of bathing target cells in a fluid containing dissolved FSL molecules may have potential in vivo in the appropriate environment. The uterine environment, whilst not easy to access, may provide an ideal environment, to expose surface endometrial epithelial cells to modifying molecules in situ. Maximal fluid retention in this environment may be targeted in order to maximise the efficiency of modification after administration of the fluid into the uterus. It is accepted that infertility due to failure of implantation of an embryo may arise from a break-down of appropriate molecular expression of either the embryo or the endometrium of the uterus. While the embryo has been the subject of extensive modification utilising KODE™ technology, the endometrium has remained largely unexplored in terms of modification using this technology.

1.3 Lavage

The concept of lavage includes a washing of fluid over a surface to recover certain components from the washed area. Alternatively, the irrigation and soaking of target cells with fluid to introduce certain components via the fluid instillation is another function of lavage. The definition of the lavage concept for the purpose of this project is described as the irrigation or instillation of fluid into the uterine environment in order to impart modifying FSL agents to the uterine environment.

Data has shown that treatment of embryos with glycoconjugates may enhance the adhesive properties of an embryo for various other cell types (Carter, 2007). Endometrial epithelial cells may be targeted in a similar manner in order to manipulate the receptivity of uterine cells for embryo attachment by influencing the maternal-embryonic cross talk. A major difference in the practicalities of this is that modification in the clinical setting would be performed in situ, rather than outside of the body as other cells have been.

Endometrial epithelial cells (EECs) may be infused with a solution of FSL glycoconjugate molecules to target a passive transfer of molecules into their membranes. While access to the human female reproductive tract is already clearly established for use in ART techniques including IVF, access to the uterine lumen in the mouse model, remains a challenge which must be met for

this proposal. Ideally, a non-surgical delivery of lavage fluid to the murine uterine lumen would allow investigations of potential lavage components to be carried out with ease and minimal disruption to the research animal. A transvaginal route of administration may provide an efficient mechanism by which all EECs may be treated simultaneously in vivo.

1.3.1 Precedence in the Clinical Setting

Previous investigations and clinical practice have both employed lavage based approaches. Uterine fluid sampling by infusion of fluid through the cervix and over the uterus, and collection of the eluted fluid enables uterine secreted molecules to be accurately sampled in a non invasive manner. Analogous methods have been employed in order to introduce fluids to the uterine environment in clinical procedures such as hystero-salphingogram (HSG) to introduce a dye to the reproductive tract, and IVF and intra-uterine insemination.

Perturbations in reproductive tract immune defence have been measured by employing cervico-vaginal lavages. Antimicrobial gels have been administered with diaphragms (Anderson et al., 2009) and lavage fluid, collected prior to and during diaphragm use, was analysed for mediators of vaginal inflammation (Anderson et al., 2009). In this manner cervico-vaginal fluid collected by a relatively non-invasive technique using existing or easily adapted tools, may be analysed for a variety of factors to provide a snapshot measurement of uterine conditions. Valuable information about lower genital tract inflammation and immunity, otherwise difficult to measure may be retrieved from vaginal secretions collected in this manner (Anderson et al., 2009). Lavage of uterine luminal fluid proteins has been carried out previously in order to sample uterine luminal fluid proteins from the human endometrium (Bush et al., 1998). Irrigation of phosphate buffered saline (PBS) over the uterine cavity of a surgically removed uterus and collection of the run-off provides intra-luminal fluid which may be analysed for presence of any molecules.

An altered pattern of intra-uterine cytokine concentration and metalloproteinase was observed in IVF patients exhibiting recurrent implantation failure (Inagaki et al., 2003). Higher levels of IL-1b and metallo-proteinase activity was measured, coupled with lower concentration of interferon gamma (IFNγ) and IL-10

(Inagaki et al., 2003). Sterile saline was instilled into the uterine cavity and aspirated in order to sample the intrauterine fluid (Inagaki et al., 2003). Such endometrial secretion analysis is a novel approach for sampling and assessing the uterine milieu and may be useful for obtaining information regarding uterine conditions for embryo transfer in a non-disruptive manner (Boomsma et al., 2009). Following IVF procedures, embryos are commonly transferred to the uterine cavity at day 2-3 of development, and typically spend 3-4 days in the uterine cavity prior to implantation. For this reason, it is of interest to know what the uterine conditions are in order to transfer an embryo into an optimally hospitable uterus.

A similarly simple method may be employed to administer fluid reagents to the uterine environment. Embryo transfer catheters have been used to deliver peripheral blood mononuclear cells directly to uteri of patients with repeated failure of IVF embryo implantation (Yoshioka et al., 2006). The delivery of these cells was shown to impart a significantly higher pregnancy, implantation and live birth rate compared with non-treated individuals exhibiting repeated implantation failure (Yoshioka et al., 2006). As the implantation of an embryo into the endometrium has been considered to involve inflammation-like circumstances, the administration of self mononuclear cells may provide fertility therapy. The maternal immune system may be manipulated to positively influence the endometrium to facilitate implantation of an embryo (Yoshioka et al., 2006). Due to the positive effects seen on implantation, and the simple application prior to embryo transfer, this technique shows promising potential for the improvement of conventional infertility therapy (Yoshioka et al., 2006).

1.3.2 Trans-vaginal Lavage in a Mouse Model

Previous studies (Johnson et al., 2005; Johnson et al., 2004) have indicated that a lavage may be possible in the murine model. Development and optimisation of such a technique would be useful to administer a fluid bolus through the cervix directly into the lumen of the uterus in the mouse. The development of a trans-vaginal murine lavage technique is complicated by the bicornate uterus of the mouse and the inability to visualise fluid being administered. However, the successful development of a non-surgical lavage in a murine model will provide the means for a wide range of agents to be

administered, in a minimally invasive manner, for their effects to be analysed in vivo. Fortunately, access to the human uterus has been well established for ART purposes previously. Oil soluble contrast media (OSCM) used traditionally for hysterosalpingograms (HSGs) in women with unexplained fertility, comprises iodized poppy seed oil and has been confirmed as being effective as a treatment of unexplained infertility in couples (Johnson et al., 2005). Due to its historical use for enhancing pregnancy outcomes of IVF patients, Lipiodol (poppy seed oil) has been investigated in the murine model. Lipiodol was added directly to the uteri of Swiss white mice by performing either a laparotomy, or vaginal instillation (Johnson et al., 2005). Vaginal instillation of 0.5mL oil was relatively non-invasive, and required no surgery. The lipiodol infused into the uterine environment influenced the particular type of uterine dendritic cells, powerful immune cells, present in the uterus. Dendritic cells play a major role in immune suppression and are of utmost importance for the maintenance of the semi-allogeneic fetus during pregnancy. Clinically, lipiodol treatment offers a minimally invasive treatment with a low risk of complications and results of live births per procedure compare favourably with results for IVF treatments in women over 40 years of age (Brent et al., 2006).

The development of a lavage technique in the mouse model to administer significant volumes of fluid in order to manipulate the uterine environment is an application of this novel technique allowing fluid infusions of the uterus to be administered and their effects monitored. This technique may provide a useful tool for the study of early embryonic attachment in vivo, for which the human setting could not be analysed due to the obvious ethical constraints.

1.4 Models of Embryo Attachment

Models are extremely important and powerful tools in the research of implantation and pregnancy. The ethical implications of investigating early embryonic activity in vivo mean that obtaining human data is virtually impossible. In vitro models using cultured cells of human origin allow the basic activities of cells of reproductive relevance to be studied. To gain complete information from the complex in vivo setting, animal models must be employed. By combining data from these models, extrapolation may provide a closer approximation to the human context. The combination of in vivo with in vitro

investigations provides a more robust justification for the extrapolation of data with relevance to the clinical setting. However, the huge diversity in reproductive processes across mammalian species must be appreciated when using in vivo models to analyse data.

1.4.1 In Vitro Endometrial Cell Model

The use of cultured cells derived from animal tissue relevant to study provides in vitro models and allows a simple overview of cell interactions to be analysed. In vitro investigations are generally very efficient compared with in vivo studies and are regarded as ethically responsible, adhering to the three Rs of clinical research involving animals: reduce animal numbers utilised, refine assays requiring animal usage and replace animal investigations with in vitro studies where possible, provided equivalent data is obtained. Despite providing a greatly advanced understanding of the precise mechanisms occurring at implantation, animal models always have short comings in terms of the specific behaviour of human embryonic cells attaching to human endometrial tissue. Not only may timing of integral mechanisms be altered between species, but specific attachment molecules may be more or less important between species. For this reason, it is important to qualify any conclusions drawn from animal models with investigations involving human specific tissue. To overcome the major ethical constraints of studying human embryo attachment to and implantation into human tissue, in vitro culture of cells derived from both human embryonic tissue and human endometrial tissue may provide a simple picture of cell-cell interactions which may occur specifically in human embryonic attachment and implantation.

Endometrial epithelial cells (EECs) line the lumen of the uterus and are the first maternal cells with which the embryonic tissue intimately interacts. Human endometrial epithelial cells derived from isolated cancer cells, inherently reproduce themselves continuously under appropriate culture conditions and with sufficient nourishment. The capability of cultured cells to continuously divide, make them extremely useful for studying cell characteristics. There are very many types of endometrial epithelial cells available commercially, mostly developed from endometrial carcinoma cells (Hannan et al., 2009). The type of endometrial cells to be chosen must be determined by and relevant to the stage

of endometrial maturation under investigation. Each cell line has different phenotypic properties, and whilst being useful for a given parameter or response, may be inappropriate for the study of another. RL-95 cells are commonly used to model receptive epithelial cells, due to their highly adhesive nature. By contrast, HEC-1-A cells, exhibit low levels of adhesion, and their derivative cell line, HEC-1 have widely been used in implantation research as the model of non-receptive epithelial cells (Hannan et al., 2009). AN3-CA cells on the other hand are completely refractory to attachment by trophoblastic cells. Previous studies with choriocarcinoma cell lines indicate that it is the state of differentiation of trophoblast cells that influences their competence to establish adhesion to uterine epithelial cells (Hohn & Denker, 2002; Hohn et al., 2000). Stimulation of hCG is commonly used as an indicator of trophoblast differentiation.

Table 1. Characteristics and origin of endometrial epithelial cell types. Adherence characteristics may be used to model a fertile and sub-fertile endometrium using in vitro assays. Adapted from Hannan et al 2009.

Cell Type	Origin	Polarity	Implantation/ Function
Ishikawa	Adenocarcinoma	Moderate	Adhesive
HEC-1-A	Adenocarcinoma	Highly polarised	Poorly adhesive
RL-95	Adeno-squamous carcinoma, moderately differentiated	Non-polarised	Highly adhesive
ECC-1	Adenocarcinoma	Moderate	-
HES	Adenocarcinoma	-	Embryo-trophic

Table 1 shows implantation characteristics of a variety of endometrial epithelial cell lines commercially available for the study of cellular interactions. Ishikawa cells, are considered to demonstrate properties of a normal endometrium, expressing many of the same enzymes and proteins found in normal functioning endometrium (Hannan et al., 2009) whilst, ECC-1 cells and HES cells have been recognised to closely represent luminal epithelial cells, with ECC-1 expressing characteristic cytokeratins, estrogen and progesterone receptors.

MUC-1, an important and complex uterine polysaccharide, is expressed by both Ishikawa cells and HES cell types, making each useful for the study of early interactions between the endometrium and the embryo (Hannan et al., 2009).

There are also many human trophoblast cell lines available, reflective of the vast amount of development occurring in trophoblast cells during growth and migration through the endometrium. These cells are mostly derived from placental choriocarcinomas during the first trimester and retain many characteristics of the trophoblast such as hormone production and invasive properties (John, 1993). For example, expression of certain antigens, expressed by the trophectoderm in the pre-implantation conceptus is retained in BeWo cells. Chorionic gonadotropin production may also be stimulated in this cell type, making them useful for models of extra-villous trophoblast. This cell type is recognised as the usual choice for the study of adhesion and invasion of trophoblastic cells, and also for more specific functions such as differentiation (Hannan et al., 2009). BeWo cells have exhibited an inverse correlation between invasiveness and differentiation – similar to that observed with normal trophoblast cells (Hohn & Denker, 2002)

Monolayers of RL-95 cells, HEC-1-A cells and AN3-CA cells exhibit a range of adhesive properties and may be used to model the differences in surface epithelia which may be encountered in the uterus by an embryo prior to implantation. Whilst RL-95 cells and HEC-1-A cells may exhibit the same adhesion molecules and proteins, they differ in the organisation of their cellular components (Thie et al., 1998). RL-95 cells permanently express the non-polar epithelial phenotype, characteristic of receptive cells in the uterine lining (Heneweer et al., 2002) and thus provide an in vitro model for the receptive human endometrium (Heneweer et al., 2002).

An embryo may be mimicked by the engineering of cultured trophoblastic cells into tiny spheroidal balls (see Figure 6). Examples of trophoblastic cell lines used in assays employing spheroids are JAR, JEG-3 and BeWo cells (Heneweer et al., 2002). Initial attachment of an embryo to the mother's uterine lining may be mimicked by the co-culture of monolayers of endometrial epithelial cells with multicellular spheroids of trophoblastic derived cells. This spheroid-on-monolayer model allows the study at the cellular level of complex

cascades and bond formation at the apical surface of the epithelial cells of the endometrium during adhesion of an embryo (Heneweer et al., 2002). Figure 6 shows a JAR cell spheroid sitting upon a monolayer of RL-95 cells. Spheroids, or sometimes even mouse blastocysts will be added to the monolayered epithelial cells and incubated together for a set period to encourage attachment, after which, the strength of attachment is challenged. This challenge distinguishes true attachment from non-specific association (John, 1993; Li et al., 2002; Simon et al., 1999). The method of challenge is perhaps the most significant variable feature of the attachment assay design. Attachment integrity may be challenged by movement within culture media, by the flushing of medium across an embryo (Simon et al., 1999). Specific binding forces may be precisely measured by employing a highly sensitive cantilever and measuring the force of cell to cell interactions under the atomic force microscope (Thie & Denker, 2002; Thie et al., 1998).

A simple and efficient method of measuring the overall adhesion of spheroids to endometrial cell monolayers to approximate embryo – uterine attachment is by challenging attachment using the centrifugal force of a centrifuge (Hohn & Denker, 2002; John et al., 1993; John, 1993; Li et al., 2002). During the establishment of this method, it was discovered that mechanically robust spheroids were most reliably produced from choriocarcinoma cell lines, such as BeWo, JEG 3 and JAR (Hohn & Denker, 2002). The arrangement of cells in spheroids preserves the apicobasal polarity which is also preserved in monolayered endometrial epithelial cells. This format of attachment assay mimics the polarity exhibited by both trophoblastic cells of a blastocyst stage embryo, and the epithelial cells lining the uterus (Hohn & Denker, 2002; John, 1993), whilst the use of single cell suspensions lose this polarity. Furthermore, the high degree of cell to cell contacts of trophoblast cells within spheroids may influence cell behaviour and differentiation (John, 1993).

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Figure 6. SEM of a JAR spheroid on RL-95-2 cell monolayer. From Thie & Denker, 2002.

1.4.2 In Vivo Fertile Murine Model

Whilst in vitro studies are invaluable for efficiency and ease of manipulation, they do remain rather rudimentary in terms of cellular interactions able to be investigated in any given assay. In vivo studies provide a more complete picture of influences within an entire organism subject to the many influences of that complex biological system. An appreciation of many mammalian species is necessary when extrapolating patterns seen with the use of in vivo animal models.

Due to the impossibility of investigating early implantation in the human model, there are very few examples in existence which document the first few weeks of embryonic development (Norwitz et al., 2001). In vivo investigations must adopt animal models to make sound estimations of molecular and mechanical events associated with implantation (Lee & DeMayo, 2004). The vast majority of investigations have historically been performed in the mouse model. Their small size and rapid reproductive capability they make them the ideal choice for efficient and cost effective in vivo studies. Furthermore, they exhibit a similar mode of implantation particularly relevant for investigations on their reproduction. Mammalian implantation is categorised as centric, eccentric or interstitial dependent on the degree of blastocyst-endometrium interaction. Formation of an invagination of epithelial cells surrounding the trophoblast of the blastocyst describes the eccentric type of implantation exhibited in the mouse. In the human however, Interstitial implantation occurs by the

trophoblast passing through the epithelium to invade the stroma and become embedded into the uterine wall (Lee & DeMayo, 2004). Many aspects of embryonic development have been studied in the mouse model, including metabolism, effects of growth factors, rate of development, cell division and cell death (Lee & DeMayo, 2004). In mice, the epithelium undergoes apoptosis with embryo attachment, whereas in the human, the trophectoderm cells intrude between epithelial cells which are retained. The epithelial cells in humans also express many implantation related molecules otherwise seen only in decidua (personal communication, L Salamonson, December, 2009).

The mouse and human blastocysts comprise an outer layer of trophoblast cells, which give rise to placental tissue, and an inner cell mass (ICM) which becomes the embryo proper. Blastocysts have a thick protein coat surrounding them termed the zona pellucida. The mouse exhibits a bicornuate uterus leading to the vagina, and oocytes may ovulate simultaneously from both ovaries and transit the oviduct, where fertilisation occurs. The multiple embryos may then be accommodated by the two uterine horns. The human on the other hand has a simplex uterus, with a single cervix connecting it to the vagina (Behringer et al., 2006). When successful fertilisation occurs in the human, usually a single ovulation will result in fertilisation in one of the fallopian tubes, with the resulting embryo making the transit to the uterus, where usually just that one embryo will implant into the endometrium.

The gestation of course is very different in mouse and humans, with a 20 day gestation in the mouse compared with 37-42 week gestation typical in humans. However, interestingly, blastocyst development rate is fairly similar, and at day 4 of pregnancy, this equates to 20% of the entire gestation in the mouse, while just 1.5% of the total human gestation period. Mouse embryos implant into the endometrium at day 4 after fertilisation compared with approximately 9 days post fertilisation in the human (Lee & DeMayo, 2004). Cell proliferation, differentiation and later cell death drive the specific stages of embryo development: cleavage of the fertilised egg, compaction and morula formation, and formation of the blastocyst (Hardy & Spanos, 2002).

As mentioned previously, implantation of an embryo involves apposition of the blastocyst to the uterine luminal epithelium, adhesion, penetration through to

the basal lamina and invasion into the stromal vasculature to bring the conceptus into contact with the maternal blood supply (Lee & DeMayo, 2004). Embryo receptivity in the mouse model is enhanced by the presence of embryos in the oviduct which increases implantation in endometria of recipients which may otherwise be non-receptive (Wakuda et al., 1999). Limitations of using the mouse model to estimate conditions of the human uterus include the speed of apposition, attachment and implantation and for this reason, some physiological mechanisms of implantation remain difficult to elucidate. Given this, assumptions about human development should not be based solely on observations from mouse studies (Hardy & Spanos, 2002). However, since the decidual response is important for receptivity and can be elicited easily in the absence of an embryo in the mouse, this phenomenon may be exploited for study of this process, and also for the production of receptive recipients for embryo transfers (Lee & DeMayo, 2004). Furthermore, the use of the vast amount of information obtained from mouse genetic studies with over-expressed or knocked out genes provides a powerful tool in elucidating important genes and the function of their products in implantation (Lee & DeMayo, 2004). While there are clear differences in mouse versus human implantation and certain limitations of their use as with any mammalian model, the similarities between humans and mice are considered significant enough for the mouse to be used as a good model for implantation.

The aim of this project is to modify the cells lining the uterus of the mouse by the administration of FSL proof-of-principle molecules directly to the uterus via a novel trans-vaginal lavage technique developed for use in the mouse model. In vitro investigations using cultured endometrial epithelial cells and trophoblast spheroids will be carried out to assess the influence of modification molecules on the adhesive potential of trophoblast cells. The in vivo effect of this FSL molecule on embryo attachment will be investigated using embryos transferred into FSL lavage treated recipient mice. Finally, any long term effects on natural fertility in the treated mouse will also be considered.

2 Results

2.1 Insertion and Retention

Previous studies had shown that insertion of blood group antigens had been used to safely and efficiently modify blood cells (Gilliver, 2005) and embryos (Blake, 2003; Carter, 2007). The aim was to develop a harmless and reproducible method of modifying endometrial epithelial cells in vitro to provide information on assay parameters for in vivo modification. Assays were carried out to determine the optimal concentration of FSL molecules to effectively modify endometrial epithelial cells and to assess the persistence of this modification. Insertion molecules with the blood group antigens A and B were used as these were relatively abundant compared with other 'functional head group' options available. The optimal exposure time to the FSL molecules for efficient insertion into cell membranes was elucidated and further investigations then determined how long the resulting modification was likely to last for. Due to the developmental nature of these assays, parameters varied slightly between assays to allow for the continual optimisation of insertion conditions and detection using immuno-fluorescent staining. Culturing cells in wells of a Terasaki plate (20µL capacity), provided ideal conditions for monolayered endometrial epithelial cells, whilst minimising quantities of reagents to treat these monolayers. Cells were easily viewed within the Terasaki culture trays, directly under the fluorescent microscope. Three distinct endometrial epithelial cell lines derived from endometrial carcinomas were used which varied according to their attachment characteristics towards an embryo. RL-95 cells are known to have an optimal adherence towards trophoblastic cells, HEC-1-A, are sub-optimally adherent, and AN3-CA are considered to be refractory with respect to attachment of trophoblastic cells (Hannan et al., 2009; Heneweer et al., 2002).

2.1.1 Preliminary Insertion into Adherent Endometrial cells with FSL A and FSL B

A preliminary investigation of FSL A insertion into monolayered RL-95 endometrial epithelial cells (EECs) was carried out to estimate appropriate concentrations for insertion and appropriate incubation times. Retention of the

inserted molecules was then ascertained using immuno-labelling. A semi-quantitative measurement of molecular expression was achieved by grading the emitted fluorescence under fluorescent microscopy by a standardised grading system. This assay was initially carried out using the adherent endometrial epithelial cells (RL-95) monolayered on poly-l-lysine coated cover slips cultured in 24 well culture plates. The assay was developed using cell suspension seeded into plastic Terasaki plates to conserve insertion molecules and staining reagents, and to simplify the technique. Not only did monolayers adhere with greater integrity to the plastic Terasaki plates, but the smaller volumes used facilitated carrying out a concentration gradient of molecule to determine the most appropriate concentration of insertion molecules for endometrial epithelial cells. These plates also provided further versatility as monolayers were viewed directly with fluorescence microscopy in the same wells in which they were grown, treated and stained.

Method Overview:

In the initial assay, RL-95 cell monolayers were prepared in 24 well plates containing cover slips (Protocol 4.9 version 2) to a confluence of approximately 70%, ideal for evaluating fluorescent staining of cell membranes. After monolayer preparation, media was carefully removed and replaced with either fresh media containing 0.1, 0.5, 1.0 and 2.0 mg/mL FSL A or FSL B or media alone. Monolayers were then incubated with the insertion molecules for 4 or 18 hours at 37°C. This incubation time of 18 hours was considered to be an upper limit in terms of clinical applications for the molecule and had been used successfully with this technology previously (Blake, 2003; Carter, 2007; Williams, 2008). Monolayered RL-95 cells on cover slips required removal from wells and were gently rinsed three times with PBS. Monolayers in Terasaki plates required sampling rinsing with pre-warmed PBS using a transfer pipette and a micro-pipette to carefully remove media. Two cover slips were replaced into the wells of the 24 well plate with fresh, pre-warmed culture media. The cover slips or Terasaki plate wells were stained to visualise the insertion of FSL A (Protocol 4.2). Monolayers were fixed for 10 minutes with the addition of 3.7% formaldehyde and then rinsed. Primary antibody, Bioclone anti-A was added directly to the fixed cells and incubated for 30 minutes or 1 hour at room temperature. Blank monolayers which were incubated with 2% BSA in PBS

were included as a control for the staining technique. Secondary antibody, anti-mouse Ig-FITC was added to washed cover slips and incubated at room temperature for 30-45 minutes in a humidified dark box. Monolayers were then washed and cover slips mounted onto microscope slides with glycerine mountant. Phosphate buffered saline (10μL) was left in contact with monolayers in Terasaki plates. Stained cells were viewed under WIB filter of fluorescence Olympus BX51 microscope and images were recorded and graded for fluorescent intensity at 1.9 seconds exposure, (Protocol 4.3). Corresponding DIC images were also recorded for every fluorescent image in order to track cells which did not fluoresce. After two hours post removal of the molecule from growth media, the remaining two cover slips were stained in the same manner. This gave an approximation of molecule retention after two hours by comparison of fluorescent signals with cells stained immediately after FSL A treatment.

Results

Monolayers prepared on cover slips and treated with 0.5 mg/mL overnight elicited a fluorescent intensity after staining and viewing of 2+. Non-treated counterparts elicited an intensity of 0-1+. Fluorescence was exhibited uniformly around the periphery of cells. Two hours after removal of the FSL molecule from media however the staining pattern of treated cells differed. Cells exhibited highly intense patches of fluorescence when viewed under WIB fluorescence. Blebbing of cell membranes was observed in some DIC images recorded after 24 hour exposure to 0.5mg/mL FSL A which may be suggestive of cellular stress. With increasing concentrations of both FSL A and FSL B there was an increase observed in the intensities of WIB fluorescence after staining (Table 2 and Table 3). Untreated endometrial epithelial cells demonstrated fluorescence of 0-1+ on staining. All blanks stained without primary antibody were invisible under WIB fluorescence despite evidence of intact monolayers visible under DIC. Monolayers treated with 0.1 mg/mL FSL A and FSL B demonstrated equivalent fluorescent intensities compared with non-treated counterparts which exhibited a fluorescent intensity of 0-1+. Monolayers treated with 2 mg/mL FSL A or FSL B showed optimal fluorescent intensities with 2-3+ recorded for each of the FSL treated monolayers.

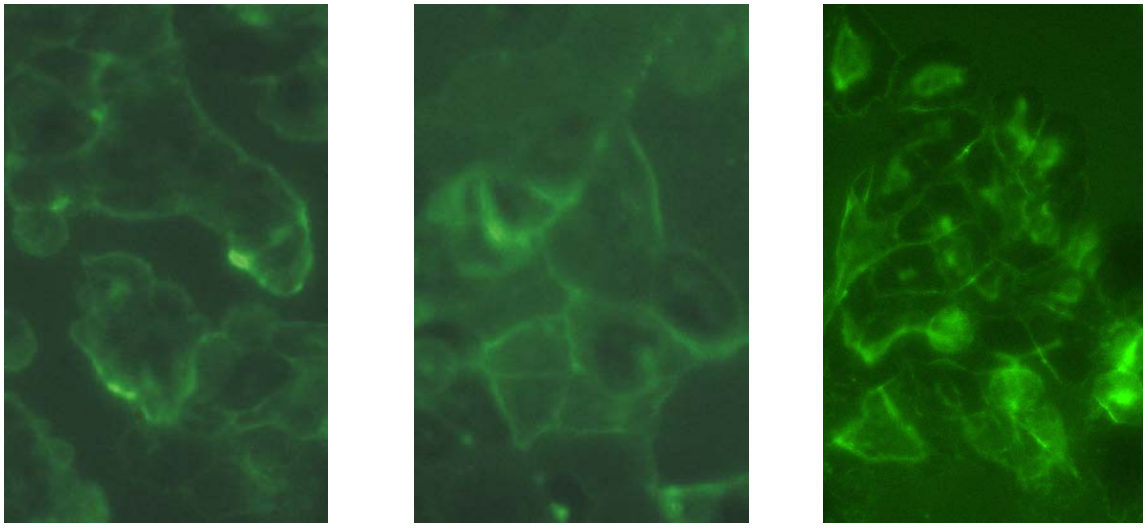
The characteristic fluorescent staining pattern varied according to the particular cell type stained (Figure 7).

Table 2. Measurement of FSL A insertion by fluorescent grading. Fluorescence was graded under WIB incident light of fluorescence microscope and viewed at an exposure time of 1.9 seconds

Concentration FSL A (mg/mL)	Endometrial Cell Type		
	RL-95	HEC-1-A	AN3-CA
0	0-1+	0-1+	0-1+
0.1	0-1+	1+	1-2+
0.5	2+	2+	2+
1.0	2+	2-3+	2+
2.0	2-3+	3+	2-3+

Table 3. Measurement of FSL B insertion by fluorescent grading. Fluorescence was graded under WIB incident light of fluorescence microscope and viewed at an exposure time of 1.9 seconds

Concentration FSL B (mg/mL)	Endometrial Cell Type		
	RL-95	HEC-1-A	AN3-CA
0	0-1+	0-1+	0-1+
0.1	2+	0-1+	1-2+
0.5	2-3+	1+	1-2+
1.0	2-3+	2+	2+
2.0	3+	3+	2-3+



RL-95 HEC-1-A AN3-CA

Figure 7. Comparison of FSL A insertion into three endometrial cell lines. Cells were treated with 1mg/mL FSL A for four hours. Images show cells at magnification 200× with 1.9 seconds exposure.

Interpretation

It should be noted that appropriate negative controls should include serum, or either IgG or an irrelevant monoclonal antibody from the same species as the primary antibody. This ensures that there are no irrelevant antibody molecules or other components of serum other than the specific antibody within the antibody solution that bind to the target tissue, which would result in a false positive stain. The complete elimination of the primary antibody in the blank controls using BSA only, controls only for the non-specific binding of the secondary antibody to the target tissue. Accordingly, IgG, serum or an irrelevant monoclonal antibody from same species as primary antibody should be used in further analyses to confirm results of all immuno-histochemical assays. The initial assay indicated that insertion of FSL A was attained with the overnight treatment of RL-95 with 0.5 mg/mL FSL A. Two hours after removal of FSL A from contact with monolayered cells however, staining appeared patchy and irregular suggestive that the FSL A molecule may be actively excluded from the cells. While there is the possibility that low level modification is observed as an accumulation of the molecules at discrete parts of the cell membrane, this staining pattern may be expected prior to expulsion of the molecules from the cell. (Kovalenko et al., 2004; Schwarzmann, 2001).

Treating the cell monolayers with maximal concentrations of molecule for a limited length of time may both target a higher modification by saturation of cells and avoid prolonged exposure to the molecule which may stress cells. Single operator grading of fluorescence under identical conditions between assays minimised the subjectivity of the fluorescent intensity measurement. The grading system allowed the distinction of degrees of fluorescent signal (Protocol 4.3) making this assay semi-quantitative. Subsequent to removal of media for an investigation of retention of molecules, despite the omission of a washing step in order to maintain monolayer integrity, the dilution factor of replacement media containing no molecule rendered further exposure to the FSL molecule negligible. From the concentration gradient investigation it was observed that 2mg/mL FSL A was optimal for efficient modification of endometrial cells within 4 hours. The compact RL-95 cells provided a robust monolayer which was easily modified and stained by immuno-fluorescent techniques to detect modification. In comparison the more spread out, AN3-CA and HEC-1-A cells had less robust monolayers which were inclined to wash off on staining. Extracellular components of cells and the confluent monolayer may hinder access of molecules to the cell membranes, causing difficulty with insertion of FSL A and FSL B. By using 70% confluent cell monolayers, access to the cell membrane external surfaces was maintained while giving an ideal cell density for viewing. Each of the cell types investigated exhibited an intriguing variance of modification, with the larger, more spread out cells AN3-CA and HEC-1-A showing less intense fluorescence with staining compared with the more compact RL-95 cells.

2.1.2 Insertion of FSL A into Adherent Endometrial Epithelial Cells

RL-95 cells were chosen to further investigate insertion as the monolayers prepared from these cells were the most robust for staining purposes. These cells were also known to be optimally adherent and would be used for screening purposes and controls in later investigations. FSL A was chosen as the most appropriate proof of principle molecule to be utilised in this project due to its simple structure, its relative abundance compared with other candidate molecules available, the extent of previous investigations by other researchers indicating its expected behaviour and its safety in biological systems.

Also, the detection system for FSL A was more sensitive and more robust than the FSL B detection reagents. From the previous assay, it was decided to treat cells with 1 mg/mL FSL A for 2 hours. This concentration was chosen, as it was believed to give close to optimal insertion into endometrial epithelial monolayers, whilst still conserving the expensive molecule. It was considered that 2 hours would be sufficient time to allow for insertion using 1mg/mL.

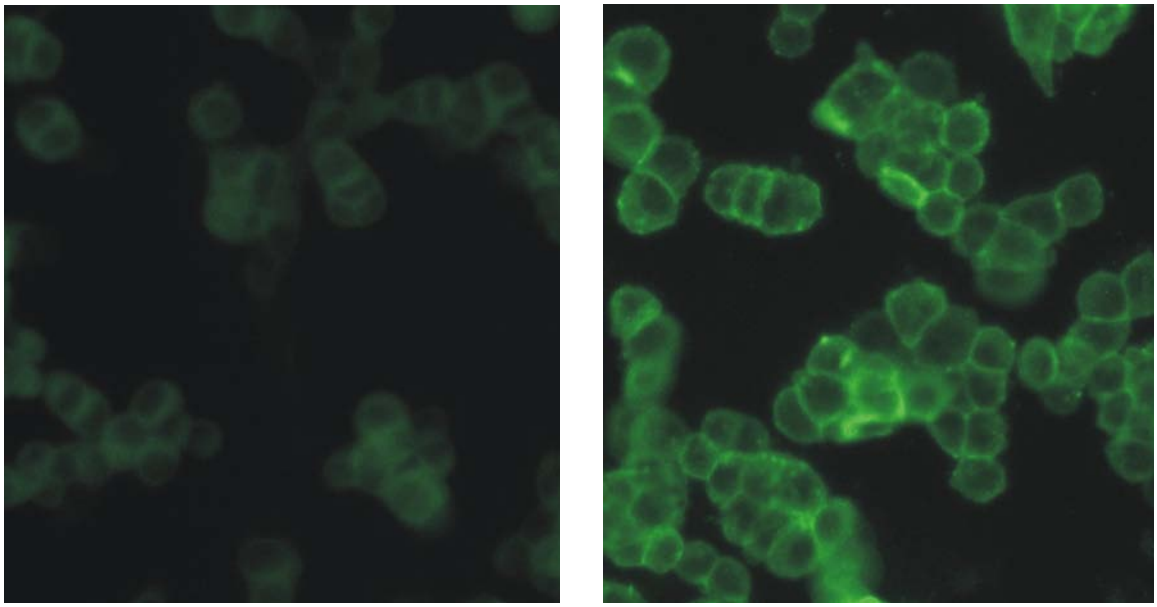
Method Overview

RL-95 cells were seeded into Terasaki trays (Protocol 4.9) to give a cell density of 1.3 x 10^6 cell/ cm^2. Plates were incubated for approximately 18 hours at 37°C in order to prepare monolayers of approximately 70% confluence. Growth media was removed after incubation and media was replaced with pre-warmed fresh media containing 1mg/mL FSL A. Cells were incubated with insertion media for 2 hours at 37°C, after which media was removed and cells were fixed by placing 20µL 3.7% formaldehyde per well at room temperature for 10 minutes. Cells were washed (Protocol 4.9) using PBS, and Terasaki plates blotted gently with paper towels. Primary antibody, anti-A Bioclone was added, undiluted to duplicate wells while 2% BSA was added to cells as a non-primary antibody blank control for specificity of the secondary reporter antibody. Plates were incubated in a humidified box at 37°C for 1 hour and were washed again before secondary antibody was added. Secondary antibody, anti-mouse IgFITC was added (20µL) to all wells and the plate was incubated in a humidified dark box for 30 minutes. Plates were washed and PBS (13µL) was added to each well to aid viewing under the fluorescent microscope. DIC images of each treatment were recorded, and WIB fluorescence was graded and recorded at 1.9 s exposure (Protocol 4.3).

Results

RL-95 cell monolayers at a confluence of 70% treated with 1mg/mL FSL A for two hours displayed an immuno-fluorescent intensity of 3+, while cells which were not treated with FSL A displayed a fluorescent intensity of 1+ (Figure 8). Whilst DIC images provided evidence of intact healthy monolayers, the no primary antibody blank controls were completely invisible under WIB fluorescence. Furthermore, DIC micrographs revealed that RL-95 cells monolayered in Terasaki plates and treated with 1mg/mL FSL A exhibited a

small degree of blebbing on some cell membranes, and a grainy appearance of cells which was not apparent in blanks.



Non-treated FSL A 1 mg/mL

Figure 8. Fluorescent stain of treated and untreated RL-95 cells. Treated cells were exposed to 1mg/mL FSL A for 2 hours prior to staining. Images were taken at 1.9 seconds exposure at 200× magnification. Non-treated RL-95 cells elicit a fluorescence grading of 1+ while cells treated with 1 mg/mL FSL A exhibit fluorescence of 3+.

Interpretation

Efficient modification of RL-95 cell presentation of FSL A was achieved by treatment of monolayered cells with 1mg/mL FSL A for 2 hours. Cell membrane specific insertion was detected by immuno-fluorescent staining which demonstrated that cells were tolerant to 1mg/mL FSL A molecule for 2 hours. Cell blebbing observed was thought to be artefacts of staining, as this blebbing was not apparent in antibody control blanks. Since monolayers all remained intact and individual cells remained well adhered to the plastic plate, cells were considered to be live and resilient to any cell stress to which they may have been exposed.

2.1.3 Retention of FSL A in Endometrial Epithelial Cells

Assays were carried out to determine the persistence of FSL A in RL-95 and HEC-1-A cells. The relative amounts of introduced FSL molecule retained in cell membranes of RL-95 and HEC-1-A cells were compared after a fixed period of time. The persistence of the introduced molecule within the cell membrane of

modified cells gives an indication of the way in which this technology may be targeted in a clinical setting.

Method Overview

RL-95 and HEC-1-A cells were seeded into Terasaki trays and incubated overnight, to give approximately 70% confluence on the surface of the well (Protocol 4.9). FSL A (1mg/mL) was added to replicate wells (20µL), with diluent alone (cell media) added to further replicate wells, as non-treated control wells. Cells were incubated at 37°C for 2 hours to allow FSL A to insert into cells. Insertion media was removed after incubation and cells were replenished with fresh media containing no FSL A and cells were re-incubated at 37°C. Two hours after insertion the replenished media was removed, and RL-95 cells were fixed at room temperature with 3.7% formaldehyde for 10 minutes. Insertion media was removed and cells were washed and blotted gently with paper towels. Primary antibody, anti-A Bioclone (20µL) was added to non-treated wells, and replicate FSL A treated wells. Antibody diluent (2% BSA) was added to wells as a no primary antibody control. Plates were incubated for 1 hour at 37°C in a humidified box and then washed again as before. Secondary antibody, sheep anti-mouse (1/50) was added (20µL) to all wells of the assay plate and incubated for 30 minutes at room temperature in a humidified dark box. Wells were washed, and PBS (20 µL) was added to all wells for viewing. Cells were viewed under WIB fluorescence and DIC. Fluorescent images were recorded at 1.9s exposure, and graded (Protocol 4.3). This assay was repeated according to the above process to investigate FSL A expression in treated RL-95 and HEC-1-A cells after 4 hours and 24 hours post removal (Protocol 4.2).

Results

Endometrial epithelial cells, RL-95 and HEC-1-A, which had been treated with 1 mg/mL FSL A for two hours and stained immediately, had an immune-fluorescent intensity grading of 2-3+ (Table 4). Stained cells showed no signs of background or cross-reactivity, with staining clearly specific to the cell membrane. Two hours after treatment with FSL A, treated cells still stained with an immuno-fluorescent intensity of 2-3+. At twenty-four hours post insertion of FSL A molecule, both RL-95 cells and HEC-1-A cells had reverted

to their pre-treatment levels of A antigen expression, both exhibiting fluorescent intensities of 0-1+. At the intermediate retention time of four hours post insertion, the level of A antigen exhibited fluorescent intensity gradings of 2-3+ with RL-95 cells and 2+ with HEC-1-A cells.

Table 4. FSL A retention in monolayers of endometrial cells. Fluorescent intensity of stained monolayers was graded under WIB fluorescence, with exposure of 1.9s.

Cell Type	Retention Time (hours)			
	0	2	4	24
RL-95	2-3+	2-3+	3+	0-1+
HEC-1-A	2+	-	2+	0-1+

Interpretation

The fluorescent intensity exhibited by RL-95 cells and HEC-1-A cells immediately following 2 hour FSL A insertion had consistent fluorescent gradings of 2-3+. Conversely, non-treated RL-95 and HEC-1-A cells always displayed a fluorescent intensity of 0-1+. Modification of the RL-95 endometrial epithelial cells using FSL A was proven to persist beyond 2 hours post insertion of the molecule, with the fluorescent intensity shown after two hours equivalent to that shown immediately following insertion. Staining of the cells, to detect the inserted molecule 24 hours post insertion, suggested that the inserted molecule was not persistent in the membranes of modified cells after this time. The fluorescent intensity of stained cells was reduced in FSL A treated RL-95 and HEC-1-A cells at four hours post modification compared with observed fluorescence of treated cells immediately following treatment. Together, the data from these assays indicates that FSL A is temporarily retained within the cell membrane of EECs, with FSL A consistently detected two to four hours post insertion. Twenty four hours post insertion, detectable antigen levels had returned to that of non-treated cells. Blanks, containing no primary antibody were consistently invisible under WIB incident light of the fluorescent microscope, confirming that the secondary antibody was not cross-reactive with endometrial epithelial cells. Furthermore, the cells did not exhibit any auto-fluorescence under WIB fluorescent microscope.

2.2 Lavage Design and Development

2.2.1 Development of Lavage Technique

Modification of the uterine epithelia in a murine model using FSL molecules required the reliable administration of molecules directly to the uterine environment. This was achieved, without the necessity for surgery by the development of a trans-vaginal procedure to administer fluid to mouse uterine horns. The development and optimisation of this relatively novel technique was often challenging given the size limitations of the mouse model and the lack of any specialised equipment for this technique. This investigation and development of a new in vivo technique required ethical approval (AEC 10/2003/0183) as discomfort to the mouse could not be precisely predicted. While analogous procedures are performed in women undergoing IVF under sedation, this model required anaesthesia in case of unforeseen discomfort while using this new technique.

Method Overview

Sexually mature female CBA/ C57 mice which were in estrus and later, mice which were pseudo-pregnant (day 4.5) were anaesthetised with Avertin (Protocol 4.17). The lavage apparatus consisted of a 1.5cm long 3mm diameter clear plastic sleeve, and a bluntened 27 gauge butterfly catheter attached to a 1mL syringe. The plastic sleeve was lubricated with water based ultrasound gel, and inserted into the vagina of the mouse. Fluid containing 1% Evans Blue dye was used as a tracker to trace administration of fluid in mouse uterine horns. The cervix was identified in the middle of the sleeve under a dissecting microscope and the bluntened needle of the butterfly catheter, primed with lavage fluid, passed gently through the cervix and held in place. A maximum of 200 µL lavage fluid was gently expelled into the uterine horns. The mouse was then held by the tail in a vertical position for 1 minute to allow complete flow of fluid to the ovary end of the uterine horns, facilitated by gravity. Any back-flow of fluid was aspirated with a 200µL bench pipette, and the process of lavage was repeated for a maximum of three times, depending on the volume of fluid back-flow.

Results

After several investigations using estrus and day 4.5 pseudo-pregnant mice (total of 13 mice), it was ascertained that 100µL was optimal for full inflation of each uterine horn. More than 200µL total lavage fluid into the two horns, risked rupturing the horns, requiring euthanasia of the mouse. Due to the dual horn morphology of the mouse uterus, it was often difficult to insert the needle straight into the uterine junction to allow both horns to be lavaged evenly. When the needle was properly inserted for optimal delivery of fluid, resistance to the needle insertion was felt to lessen as the needle passed through the cervix. Backflow of lavage fluid occurred when the needle was not optimally positioned in the cervix. Complete lavage without backflow occurred in approximately 25% of lavage treatments, with the remainder of treatments requiring at least one repeat procedure. The cervix and uterus appeared to be influenced markedly by the day of estrus, and the state of pseudo-pregnancy. The change in the state of the cervix from a tight, impenetrable sphincter to a relatively loose, feathery opening, influenced the ease of inserting the needle through the cervix, however it also influenced the volume of back-flow which lavage treated horns exhibited. Total surgery time of the uterine lavage procedure was between 10 and 20 minutes.

Interpretation

The optimal volume for sufficient inflation of uterine horns was approximately 150-200µL depending on the size of the mouse/uterus and the state of estrus/pseudo-pregnancy. Pseudo-pregnant horns had a slightly higher capacity for larger volumes than non-pseudo-pregnant horns. Due to the inability to visualise uterine horns during the procedure, caution was warranted with respect to volumes administered. Full inflation of both horns was evident by the blue dye visualised in the horns after the lavage procedure and was obtained with the introduction of 200µL of fluid in a 25g recipient mouse. The use of 1% Evans blue dye for FSL molecules to be administered with was required in order to visually confirm lavage success.

2.2.2 Insertion of FSL A into Dissected Uterine Horns Ex Vivo

Freshly dissected uterine horns were used for ex vivo investigations to determine the modification of uterine epithelia when uterine horns were maximally inflated with fluid containing FSL molecules. Uterine horns were treated for a fixed time period before being embedded in cutting compound and examined for epithelial cell modification

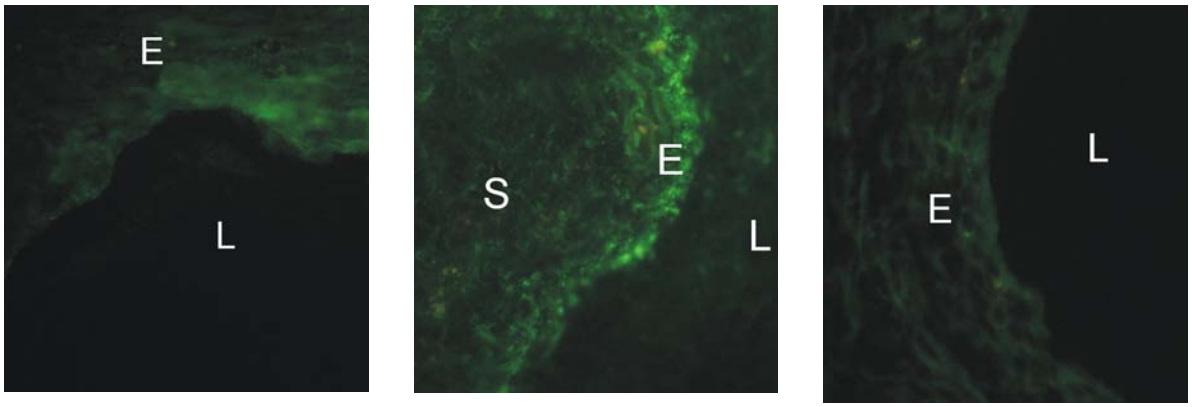
Method Overview

This investigation initially utilised Female CBA/C57 mice at various stages of estrus, and mice at day 4.5 pseudopregnancy. Mice were euthanised by cervical dislocation and uterine horns were immediately removed and placed in a petri dish containing pre-warmed Hanks Balanced Salt Solution (HBSS), where excess fat and connective tissue were removed (Protocol 4.6). Uterine horns were inflated with 0.5 mg/mL FSL A from the cervical junction, allowing each horn to fully inflate. Control lavage horns were inflated with pre-warmed growth media alone. Inflated horns were clamped and placed into microfuge tubes containing Hanks Balanced Salt Solution (HBSS). Horns were incubated in a 37°C incubator for four hours to allow insertion of FSL A into endometrial epithelial cell membranes and monitored to ensure media was not lost due to evaporation. Horns were then removed from HBSS and placed on a clean petri dish where they were separated from the uterine junction, allowing fluid to drain out. Pre-warmed HBSS was then gently passed though the horns using a syringe to remove excess FSL A within the uterine lumens. Individual horns were then embedded into optimal cutting temperature (OCT) compound and rapidly frozen before storage at -85°C. Sections were prepared at 7µm in a cryostat (Protocol 4.7) and mounted onto pre-chilled poly-L-lysine coated microscope slides. Sections were stained for the presence of A antigen (Protocol 4.2), visualised and graded for fluorescent intensity as an indication of insertion of FSL A by the uterine cells.

Results

Stained sections of uterine horns which had been treated with 0.5mg/mL FSL A for four hours ex vivo yielded significant quantities of A antigen present in uterine horns. Fluorescent intensity was graded at 2-3+ around the uterine lumen with a grading of 1-2+ in the stromal cell layer of the cross-section

(Figure 9). Non treated uterine horns elicited a fluorescent intensity of 0-1+, equivalent to blanks observed under WIB fluorescence.



Non-treated uterine horn FSL A at 0.5 mg/mL Blank

Figure 9. Ex vivo uterine horns untreated and treated with FSL A. Images show epithelial cells (E), stromal cells (S) and the lumen (L) of uterine sections. Blank horns which were not treated were stained without primary antibody. Images were taken under WIB fluorescent light at ×200 with exposure time of 1.9 seconds.

Interpretation

Uterine lumen cells were modified by treatment ex vivo with 0.5 mg/mL FSL A, which was detected by immuno-fluorescence. An observable difference in fluorescent intensity was observed between sections of treated uterine horns compared with non-treated horns. The fluorescence seen in the blanks and non-treated uterine horns may be representative of low level auto fluorescence. Alternatively, there may be a low level of cross reactivity of the secondary antibody for the cells of the uterine horn. This would be elucidated by viewing untreated and unstained tissue under fluorescence, which would reveal the degree of auto-fluorescence exhibited by this murine uterine tissue. As the fluorescence observed in non-treated horns was equivalent to that observed in the blanks, the possibility of natural expression of A antigen in the uterine horns causing this low degree of immuno-reactivity was eliminated.

2.2.3 Lavage of Pseudo-pregnant Mice

It has been documented in mice that mechanical stimulation induces the decidual reaction of the endometrium allowing the endometrium to become receptive to an embryo (Yoshioka et al., 2006). When mice are mated to vasectomised males, a state of “pseudo-pregnancy” is elicited in which the endometrium becomes receptive despite the absence of embryos.

It is considered that the uterine state of a female mouse mated with a vasectomised male would be similar to that of a mouse mated with an entire male mouse. Pseudo-pregnant mice, which have been mated with vasectomised male mice, exhibit thickened uterine horns, indicative of decidualisation. As the day of implantation in mice is considered to be 4.5 days subsequent to mating, the initial lavage was carried out at day 3.5 in order to modify cells prior to implantation on day 4.5. Pseudo-pregnant mice at day 3.5 were used together with day 2.5 donor embryos as this was the fertile model used in the laboratory.

Method Overview

Evans Blue dye was prepared at 1% in PBS and used as a tracker for the administration of fluid to the uterine horns. Day 3.5 pseudo-pregnant CBA/C57 adult mice were anaesthetised (Protocol 4.17) and lavage fluid (approximately 200µL) was administered to uterine horns by transvaginal lavage (Protocol 4.12). Any fluid which back-flowed, was aspirated using a 200µL bench pipette, and the lavage procedure was repeated for a maximum of three times subject to the volume of back-flowed fluid. This method was used in order to maximise potential inflation but to avoid over-inflation of uterine horns. Mice euthanized by cervical dislocation and the presence of 1% Evans Blue dye in the uterine horns was evidence of a successful lavage

Results

Evans Blue dye (1%) was clearly seen, evenly distributed in the uterine horns of lavage treated mice (Figure 10). Administration of three aliquots of 200µL each resulted in evenly inflated uterine horns which remained intact with no visible damage seen to uterine horns. Lavage fluid was visibly contained within uterine horns, and dye had not leached to any other tissue or organs within the peritoneal cavity. This procedure was carried out on five different days and was confirmed successful in eight out of 11 mice assayed using 1% Evans Blue dye as a tracker.

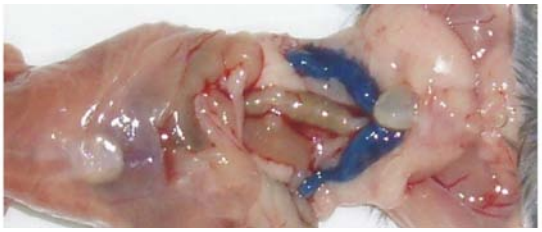


Figure 10. Day 4.5 pseudo-pregnant mouse lavage with Evan's blue dye. Inflation of uterine horns evidenced by blue dye observed in uterine horns. Lavage technique evenly delivered fluid to both uterine horns with no damage to surrounding tissue

Interpretation

Efficient and reliable delivery of fluid to mouse uterine horns was successfully achieved using the developed trans-vaginal lavage technique. Administration of fluid using this novel technique is minimally invasive, only requiring anaesthesia of the mouse to deliver a fluid bolus to the uterine horns in a non-surgical manner. The inclusion of a tracker dye such as Evans Blue dye at 1% provides visual confirmation of a successful lavage when the mouse is dissected.

2.3 Lavage Assay and Analysis

The development of the lavage technique may provide the tools for non-surgical delivery of a liquid bolus directly to the uterine lumen. The transvaginal lavage may be immediately followed by an embryo transfer due to the minimally invasive nature of the procedure. The ultimate goal of the transvaginal lavage technique was to modify the membranes of the uterine epithelial cells in order to manipulate the attachment properties of those cells to an embryo.

2.3.1 Haematoxylin Staining

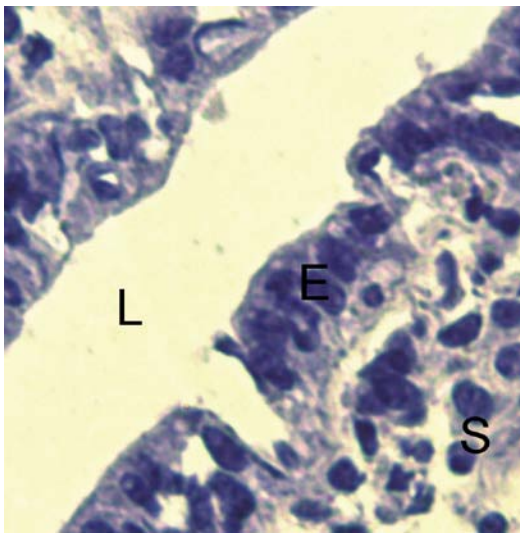
This classic nuclear specific histochemical stain was adopted to visualise cells of the uterus and gauge receptivity of the uterus based on cell organisation at different stages of pseudo-pregnancy in the mouse model. Some researchers have revealed that the plasma membrane transformation of endometrial epithelial cells may be the key to uterine receptivity (Murphy, 2004). Furthermore, the cellular integrity of trans-vaginal lavage treated uteri were investigated by using this stain which enabled the cell structure of sections of the uterine horn to be easily and clearly visualised.

Method Overview

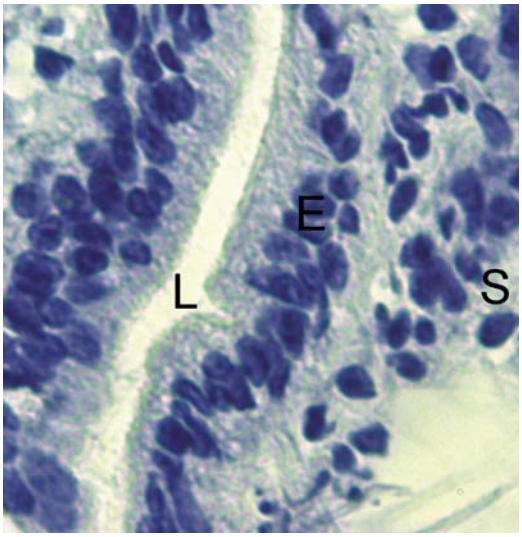
Pseudo-pregnant female mice were prepared (Protocol 4.16) using vasectomised male mice (Protocol 4.20). Mice were euthanased at day 2.5 – 5.5 pseudo-pregnancy (one mouse per day of pseudo-pregnancy), their uterine horns dissected and embedded in cryo-cutting compound (Protocol 4.6). Cryo-sections were prepared at a thickness of 7µm (Protocol 4.7) and mounted onto poly-L-lysine treated slides. Cryo-sections of two Evan's Blue Dye lavage treated horns and two FSL A lavage treated horns were also prepared to determine whether the lavage procedure affected uterine wall either with and without the presence of FSL A. Slides were stained with haematoxylin (Protocol 4.13) for 2 minutes and air dried. Sections were mounted and viewed using an Olympus BX-51 microscope at 100×, 200×, and 400× magnification.

Results

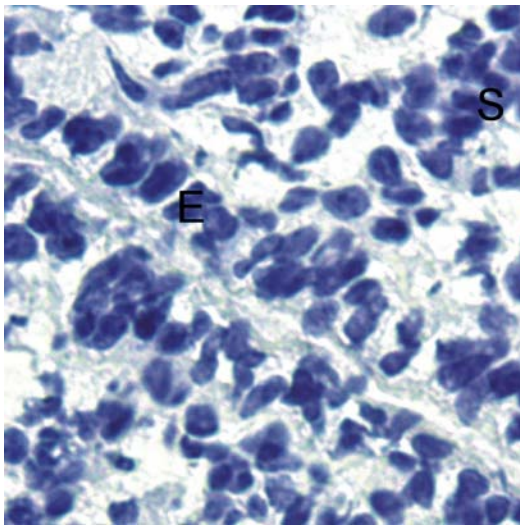
In all sections stained, endometrial epithelial cells layers remained intact and consisted of a single layer of columnar cells lining the lumen of the uterine horn, with stromal cells directly beneath. Nuclei were clearly polarised at the basal edge of the columnar cells distinctly on days 2.5, 3.5 and 5.5 of pseudo-pregnancy (Figure 11). All epithelial cells of the endometrium were tightly packed in images of sections taken at these stages. On day 4.5 however, organisation of epithelial cells was much less uniform, cells did not appear to be forming the tight barrier seen in other sections, and cell nuclei were no longer polarised at the basal edge of the cells. Sections taken from lavage treated horns and stained with haematoxylin showed little or no signs of tissue damage and compared favourably with non lavage treated horns (Figure 12).



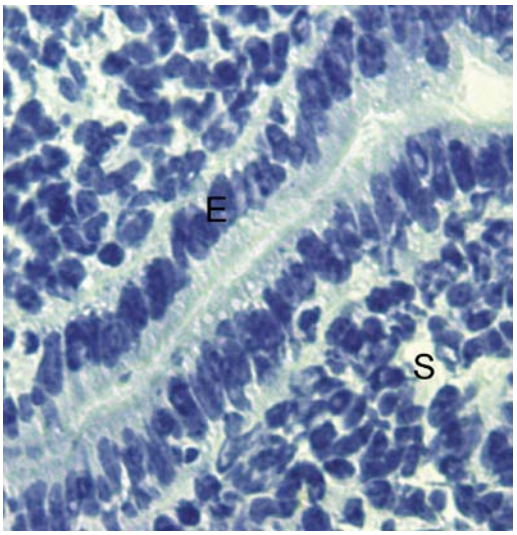
pseudo-pregnant day 2.5



pseudo-pregnant day 3.5

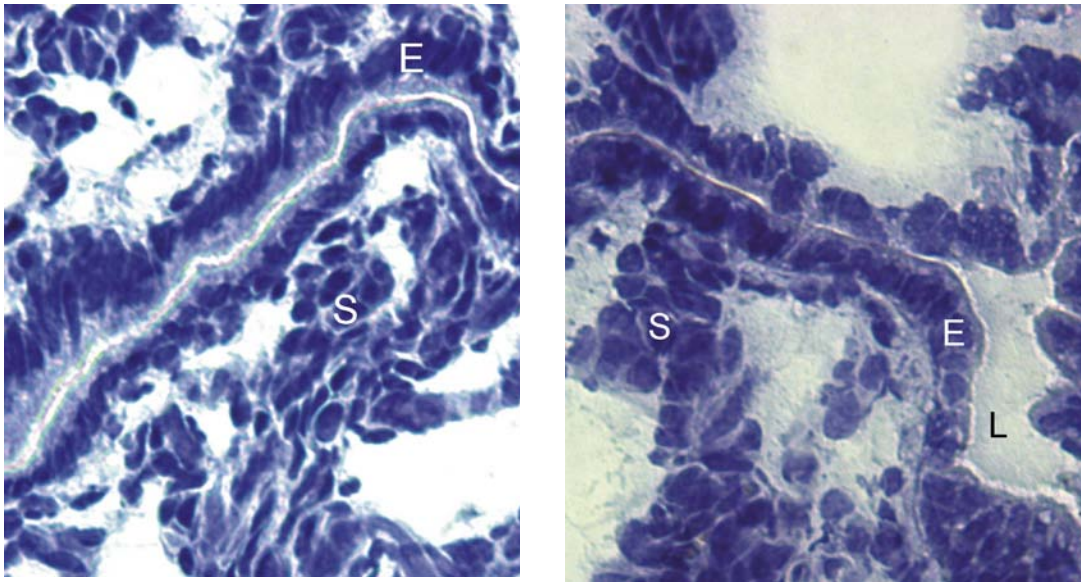


pseudo-pregnant day 4.5



pseudo-pregnant day 5.5

Figure 11. Haematoxylin stain of pseudo-pregnant uterine horns. Images show endometrial epithelial cells (E) and stromal cells (S) of uterine horn sections. Magnification 200×.



No Lavage Lavage with 1% Evans Blue Dye

Figure 12. Haematoxylin stain of lavage treated and non-treated uterine horns. Images show endometrial epithelial cells (E), stromal cells (S) and the lumen (L) of uterine horns. Day 4.5 lavage treated horns included 1% Evan's Blue dye as a tracker dye for successful lavage.

Interpretation

Changes to endometrial epithelial cell morphology and their organisation were visualised at early stages of pseudo-pregnancy. It has been shown that the uterine receptivity is influenced by the organisation of endometrial epithelial cells (Murphy, 2004). This investigation shows that cells are highly organised, and polarised in the non-estrus mouse at days 2.5, 3.5 and 5.5 and correlates with previous reports that uterine receptivity is influenced by cellular organisation of endometrial epithelial cells (Murphy, 2004). At day 4.5 of pseudo-pregnancy however, cell nuclei become visibly non-polarised, and the columnar epithelial cells of the uterine lining appear less tightly bound. This observation may be indicative of a break in the integrity of the uterine epithelial cells as an efficient barrier. While this would require further investigation, this finding would correspond to those of other researchers. Haematoxylin staining also revealed there was minimal disruption to the endometrial epithelial cell layer subsequent to both lavage treatment and FSL A treatment, and whilst also requiring further analyses as confirmation of this, no overt effect on the integrity of the uterine lining was observed. Disruption of the mucous integrity within the uterine lumen may be a limiting factor of this technique and hence care must be taken with the transvaginal lavage administration of a fluid bolus.

2.3.2 Detection of FSL A in Lavage treated horns

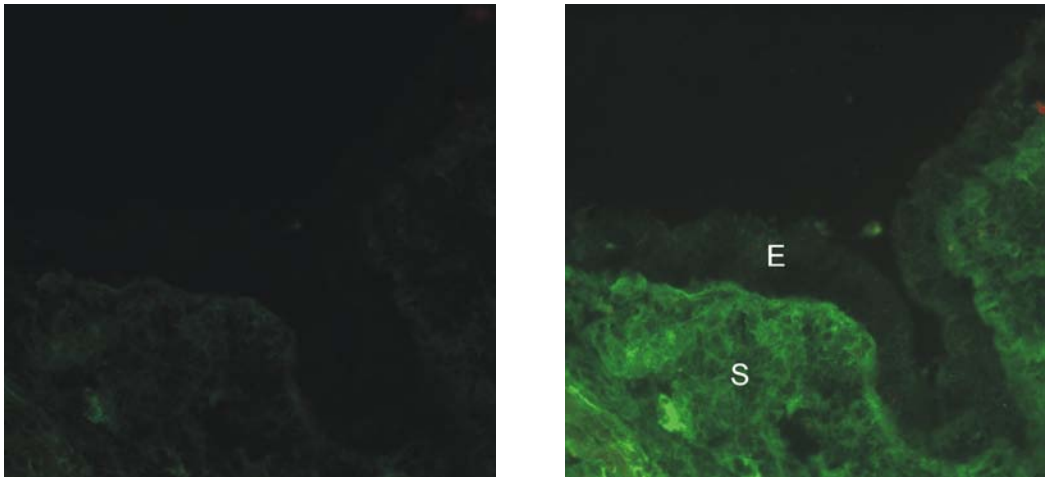
The aim of using the transvaginal lavage procedure to administer a solution containing FSL was to modify uterine endometrial epithelial cells by a non-surgical technique. Due to the relative availability of the molecule, FSL A was chosen to continue in vivo investigations as a proof of principle molecule. Furthermore, the methods developed in previous applications were well established with this molecule (Carter, 2007).

Method Overview

Nine day 4.5 pseudo-pregnant mice (taken on three different days) were anaesthetised using Avertin manufactured in house (Protocol 4.17). Six of the mice were treated with 150-200μL 1mg/mL FSL A in 1% Evan's Blue Dye, administered to uterine horns by the trans-vaginal lavage procedure (Protocol 4.12). Three remaining mice had 1% Evan's Blue Dye only adminstered by trans-vaginal lavage. Mice were allowed to recover for 2 hours to allow for endometrial epithelial cell modification and then euthanased by cervical dislocation. The successful lavage was confirmed by the presence of Evan's Blue tracking dye, in each of the uterine horns. Uterine horns were dissected, embedded in cryo-cutting compound, and stored at - 85°C until use (Protocol 4.6). Sections (7μm) were prepared from embedded uterine horns and mounted onto pre-chilled poly-L-lysine coated slides (Protocol 4.8). An affinity purified human IgG antibody, anti-A affinity purified IgG (50μL) was added to each section and incubated for 1 hour at 37°C on an orbital shaker. Control slides with no primary antibody (blanks) were incubated with 2% BSA (diluent) only. Slides were washed, secondary antibody (50μL) added to each section and then incubated in a humidified dark box for 30 minutes at room temperature. Sections were washed, blotted and covered with cover slips using glycerin containing mountant, before being viewed under WIB fluorescence of Olympus BX51 microscope. Fluorescent images were recorded at 1.9 seconds exposure and DIC images were also recorded. Further fluorescent images were recorded at 5.2 or 6.4 seconds exposure in order to more clearly visualise the intact epithelial lining of the uterine horns.

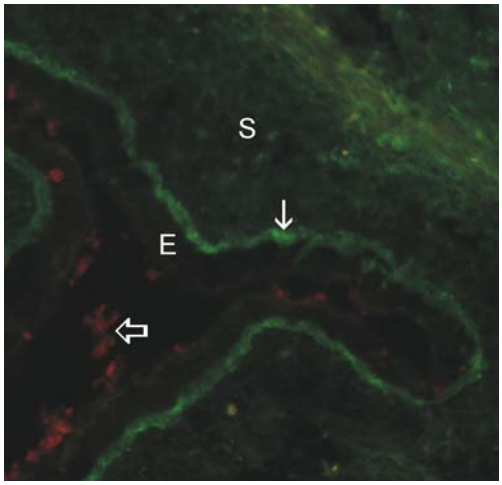
Results

Sections of non-lavage treated horns, which were viewed at 5.2 or 6.4 seconds exposure, clearly indicated intact uterine epithelial cells which were difficult to visualise under fluorescence at 1.9 seconds exposure (Figure 13). Endometrial epithelial cells of uterine horns which had been lavage treated with 1mg/mL FSL A gave a fluorescent intensity of 3+ at an exposure time of 5.2 seconds, compared with 0-1+ observed in non-lavage treated horns (Figure 14). Endometrial epithelial cells elicited significantly lower fluorescence than stromal cells in non-treated uterine horns. In FSL A treated uterine horns however, the fluorescence of EECs is equivalent to that observed in stromal cells. Evan's Blue dye lavage treated horns and blanks also yielded a fluorescent intensity of 0-1+. Evans Blue dye fluoresced red under the WIB fluorescence of the Olympus BX51 microscope, as seen in the lumens of all lavage treated uterine horns containing Evans Blue dye. This red fluorescence was observed in the blanks however was not observed in any sections from non lavage-treated uterine horns (Figure 14).

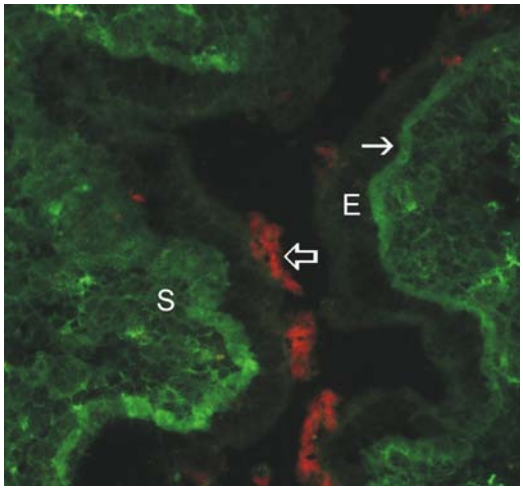


1.9 seconds 6.4 seconds

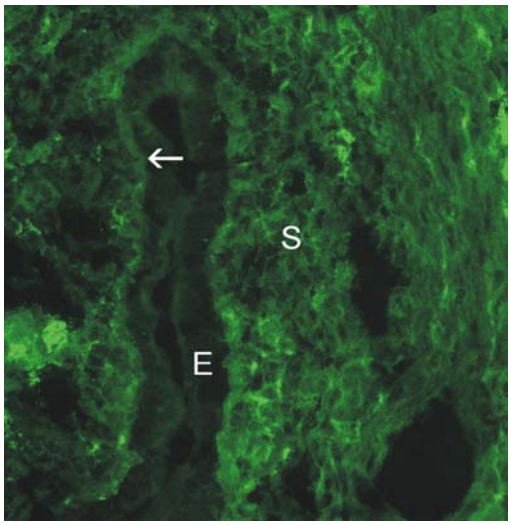
Figure 13. Sections of anti A stained Evan's Blue dye treated uterine horns. The image on the right shows the endometrial epithelial layer (E) and the stromal layer (S) of the sectioned uterine horns. Sections viewed with WIB incident light under fluorescent microscope with exposure times of 1.9 seconds and 6.4 seconds.



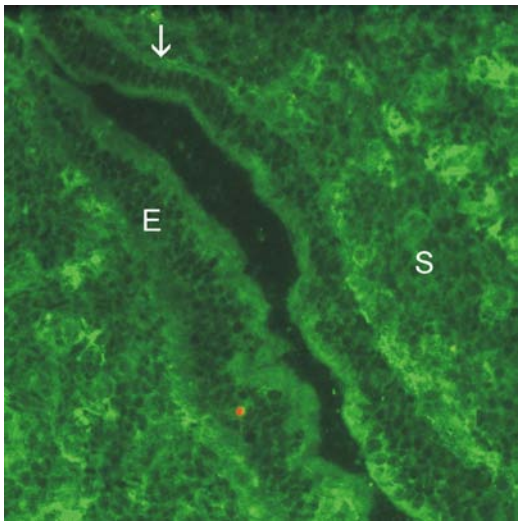
Evans Blue dye lavage, blank



Evan's Blue dye lavage, stained



Non-lavage treated horn, stained



FSL A lavage treated horn, stained

Figure 14. Comparison of lavage treatments of uterine horns. Images show endometrial epithelial cells (E), stromal cells (S), the basal layer of the endometrial epithelial cells (narrow arrow) and cells stained with Evan's Blue dye (wide arrow). Endometrial epithelial cells lining the uterine lumen (E) can be seen in images of non-treated sections, stained with lower fluorescent intensity than background stromal cells (S). The epithelial basal layer (narrow arrow) stains intensely in all sections. The FSL A lavage image shows endometrial epithelial cells with staining of equivalent intensity than the background stromal cells. Images viewed under WIB incident light of fluorescent microscope at an exposure time of 5.2 seconds. Evan's Blue dye (wide arrow) fluoresces red under WIB incident light. Magnification 200×.

Interpretation

The temporary in vivo manipulation of uterine epithelial cells was achieved by the minimally invasive, transvaginal delivery of FSL molecules directly to the uterine lumen. Endometrial epithelial cells lining the uterus were modified to exhibit A antigen via the transvaginal lavage of FSL A molecule. This modification was detected by viewing insertion under WIB fluorescence with an exposure time of 5.2 – 6.4 seconds. The superior sensitivity observed at this

exposure time compared with 1.9 seconds used in previous assays enabled epithelial cells to be more easily visualised to compare FSL A treated with non-treated cells. Due to the higher exposure times, auto-fluorescence may have contributed to increased background fluorescence, observed in blanks. This increased fluorescence of the background was not previously observed with the lesser exposure time of 1.9s. Auto-fluorescence may also be responsible for increased fluorescence observed in the basal layer of EECs, however non-specific binding of the anti-human secondary antibody may also be responsible. Despite this, the difference in the fluorescent signal elicited by EECs from lavage treated uterine horns compared with non-lavage treated horns was evidence of targeted modification of lavage treated endometrial epithelial cells.

2.3.3 In vivo Safety of Lavage Treatment

Any lasting effects on fertility subsequent to lavage, is of particular interest due to the novel nature of this procedure. The aim of this investigation was to determine whether lavage treated mice maintained normal fertility subsequent to recovery from this procedure.

Method Overview

Four pseudo-pregnant mice (day 4.5) were anaesthetised with Avertin (Protocol 4.17), and underwent a transvaginal lavage to deliver either PBS (2 mice) or 1mg/mL FSL A (2 mice) (Protocol 4.12). Female mice were allowed to recover completely from any physical effects of the treatment which may alter reproductive behaviour and after two weeks were placed in housing boxes with male mice as per standard breeding practices. Litters were assessed when delivered and prominent characteristics such as litter size, health of mother, pup weight/size, and pup health were monitored. Both parents and litters were euthanased 2 weeks after birth.

Results

Mice treated by PBS only lavage gave birth to litters of equivalent size (10, 9 and a third litter of 12) when compared with mothers treated by 1mg/mL FSL A lavage, which both delivered 9 pups (Table 5). The litter sizes of all female breeding mice in this investigation were very closely aligned, and correlated with standard average breeding litter sizes of animals bred in this facility over a

2 year period. (Erikson, 2009). While all mice gave birth to a subsequent litter following recovery from lavage and mating with a stud male, one pair produced an initial litter of nine, re-mated and produced a second litter of 12.

	Treatment	
Lavage treatment	1mg/mL FSL A	PBS lavage
Mother 1	9	10
Mother 2	9	9 (+12)

Table 5. Litter sizes of FSL A lavage treated mice. Mice were allowed to recover and naturally mate to assess reproductive ability following the lavage procedure. Litter sizes amongst all mice were equivalent for PBS lavage treated mice or FSL A treated mice. Mother 2 of the PBS lavage treated mice gave birth to a litter subsequent to the first litter post lavage, and this confirmed that subsequent fertility had not been affected by lavage treatment.

Interpretation

Delivery of healthy progeny after natural mating, subsequent to recovery from lavage treatment and exposure to FSL A, was indicative of long term safety of the lavage technique. Exposure of the mouse uterine horns to FSL A also appeared to have no detrimental effect on long term fertility. Furthermore, the lavage procedure appeared to be well tolerated in terms of potential physical stress due to scarring or rupture during the process of the lavage procedure. Limitations of this technique meant that the litter size was not quantified with absolute certainty directly after parturition and further mating was not avoided with the stud male. Whilst the numbers of mice treated in this investigation were low, the normal, healthy litters are indicative that the efficient delivery of modifying solutions to the uterine lumen in vivo is a well tolerated mechanism by which the uterine conditions may be manipulated.

2.3.4 Embryo Transfers in Lavage Treated Recipients

Embryo transfers were performed on recipient female mice that had been previously treated with PBS or 1 mg/mL FSL A by transvaginal lavage. Implantation rates in recipients treated by prior lavage were compared with those of untreated recipient mice. The potential in vivo effects of FSL insertion molecules on the attachment and implantation process in the mouse were investigated using this preliminary assay.

Method Overview

Embryo transfers of blastocyst stage embryos were carried out on two day-3.5 pseudo-pregnant recipients (Protocol 4.18) to provide the baseline implantation rate of non-treated recipients in a fertile model. Further day-3.5 pseudo-pregnant mice were treated by transvaginal lavage with either PBS, or PBS containing 1 mg/mL FSL A to investigate the effects of FSL A lavage on implantation. Three mice had 1 mg/mL FSL A administered via transvaginal lavage, whilst one had PBS only administered. After the lavage treatment, embryos were immediately transferred to recipients, with 5 blastocyst stage embryos transferred to each uterine horn. Mice were placed in separate boxes for recovery and were maintained and monitored for either 7 or 14 days. Mice were then euthanased, uterine horns dissected, and fetuses or implantation sites recorded. Successful implantation was determined by both viable fetuses and re-absorptions, with the frequency of each being recorded.

Results

The implantation rate of mice treated with a trans-vaginal PBS sham lavage displayed an unaltered implantation rate of 100%. Mice which underwent an FSL A lavage treatment prior to embryo transfers showed on average an 83% rate of implantation (Table 6 and Figure 15). Re-absorption of embryos was observed after one day of embryo transfers, and while more frequent in the FSL A treated mice (5/7 implanted), an animal also treated with PBS lavage on that day also exhibited embryo re-absorption (1/11 implanted) (Table 6). Transmigration was observed on one occasion, with an embryo, initially transferred into the left horn migrating into the right horn after the transfer procedure.

Table 6. Implantation rate of recipient mice after lavage treatment. Implantations consisted of both fully formed fetuses and re-absorptions. Numbers are expressed as fractions, with the numerator representing the number of implantations and the denominator representing the total number of embryos transferred, with the corresponding percentage (%) given in brackets.

Treatment (n=recipients)	Number of fetuses (%)	Re-absorbed fetuses (%)	Lost fetuses (%)
No Lavage (n=2)	20/20 (100)	0/20	0/20
PBS Sham (n=1)	10/11 (91)	9/11 (9)	0/11
FSL A Lavage (n=3)	20/30 (67)	5/30 (17)	5/30 (17)

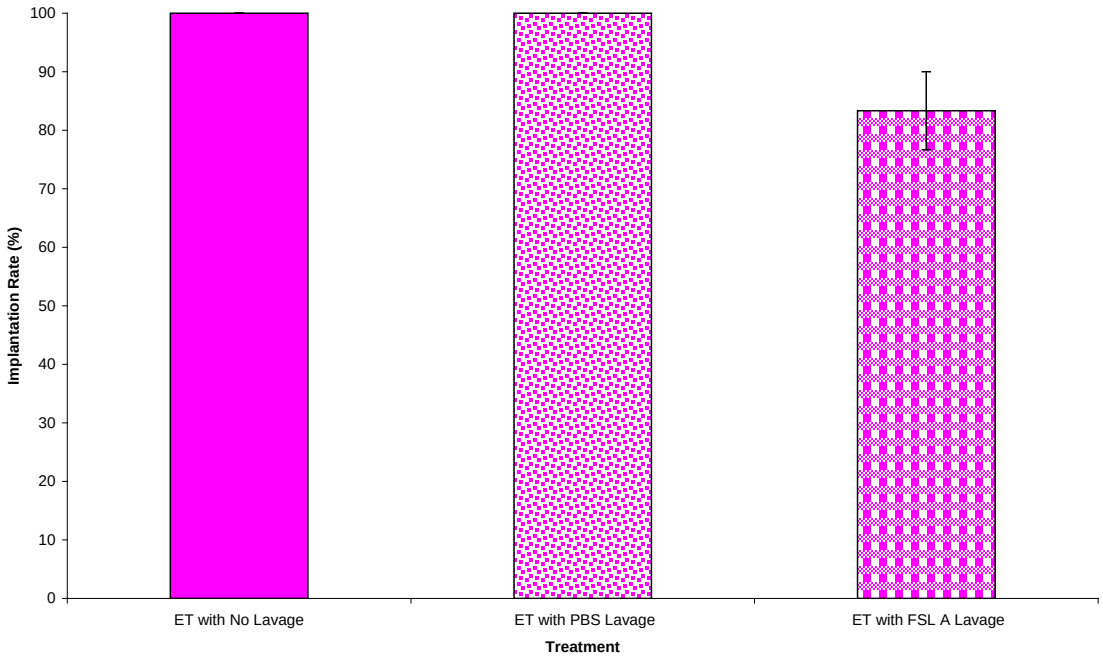


Figure 15. Embryo Implantation rate of lavage treated recipients. FSL A lavage treated subjects (n=3) show slightly lower implantation rate compared with lavage treatment with PBS (n=1). Embryo transfer recipients, without lavage had implantation rates of 100% (n=2). Mean values ±SEM.

Interpretation

Whilst further studies would be needed to confirm, initial data from this investigation are indicative that transvaginal lavage treatment of mice with PBS prior to embryo transfer may not influence implantation success. Higher numbers of recipients and embryos would be required for statistical significance to be confirmed at reliable degrees of confidence. Transmigration may occur infrequently in the mouse, when an embryo which has been transferred to one

uterine horn, migrates out through the uterine junction and implants in the other horn (Erikson, 2009). The potential for trans-migration of embryos, between uterine horns, prior to attachment and implantation may be potentiated by the high degree of inflation of uterine horns observed during this procedure. There is evidence to suggest a lower implantation rate in FSL A treated recipients compared with recipients treated with PBS alone. Since the PBS lavage treatment did not affect implantation rates, the volume of the lavage was not considered to be inhibitory to implantation per se. Initial results of this preliminary assay, suggested that the exposure of the uterine lining to FSL A for an extended period, altered the implantation success of transferred embryos. Furthermore, despite successful implantation in FSL A treated uterine horns, a higher proportion of embryo transfers resulted in re-absorption compared with PBS treated or untreated horns. This phenomenon however occurred on only one day of transfers, and may represent outliers in terms of embryo quality or recipients used, as one re-absorption was also observed in a PBS treated recipient. Due to the small number of subjects used, and the altering of certain parameters during this pilot study, further investigation of FSL A effects on embryo implantation is warranted. Higher numbers will allow statistical analysis to ascertain whether differences observed are significant. Fetal weight and placentas should be precisely measured at a stage of development that is consistent from assay to assay to allow monitoring and inter-assay comparison of embryo development. Day 17 (one day prior to expected parturition) is considered the optimal day to perform euthanasia and subsequent autopsies required for this analysis.

2.4 Screening

Endometrial epithelial cells and sections of uterine horns were screened for the presence of cell surface Hyaluronic Acid (HA) and CD44. These molecules are expressed on the cell surface and are believed to be relevant for embryo attachment to the endometrium. An elevation of expression of these molecules in optimally adherent RL-95-2 epithelial cells compared with sub-optimally adherent HEC-1-A cells may be suggestive of their importance in the attachment of an embryo to endometrial epithelial cells. If there is a reduced expression of either of these molecules in the sub-adherent cells, the use of FSL glycoconjugates may enhance attachment This investigation aims to

elucidate the potential for these molecules to be used as candidate fertility enhancement molecules if they were attached to a lipid tail.

2.4.1 HA Screening of Endometrial Epithelial Cells

Endometrial epithelial cells cultured from Endometrial Carcinoma cell lines RL-95 and HEC-1-A cells were screened for the presence of Hyaluronic Acid (HA) using two different techniques – one using Hyaluronic Acid Binding Protein (HABP) and the other using anti-HA antibody. The two methods were used in order to provide confirmation of the presence of the HA in the cells and observe any staining differences which may be falsely interpreted, in order to enhance the robustness of this assay. Detection was performed on fixed cells to maintain cell integrity during the staining procedure, and also on non-fixed cells in order to elucidate whether fixation had an effect on the detection of these molecules.

Method Overview

Monolayers of RL-95 and HEC-1-A cells were prepared by seeding 20µL of an appropriate concentration of cells into Terasaki plate wells (Protocol 4.9). RL-95 cells were incubated overnight and HEC-1-A cells were incubated for two days at 37°C to produce confluent monolayers over the surface of the wells. Media was gently removed, and some cells were fixed using 3.7% formaldehyde for 10 minutes while some remained un-fixed before being washed gently with PBS. Two methods using different detection molecules were used to detect the presence of HA (Protocol 4.4): i) biotinylated HA Binding Protein (bHABP) or ii) sheep anti-HA antibody. Detection molecule/ primary antibody (20µL) was added to all wells and plates were incubated in a humidified box at 37°C for one hour. Blanks were included in some wells, with 2% BSA added in place of primary antibody. Avidin Alexa Fluor and donkey anti-sheep secondary antibody was required to detect the biotinylated HABP and the anti-HA antibody respectively. After plates were washed, 20µL of reporter molecule/secondary antibody were added to wells and incubated for 30 minutes in a humidified dark box at room temperature for 30 minutes. Wells were washed and 10µL PBS added to all wells for viewing under Olympus BX51 fluorescent microscope. Stained cells were viewed under WIB fluorescence and images were recorded at 1.9 seconds exposure.

Results

A fluorescent intensity of approximately 2-3+ was observed with RL-95 cells stained using anti-HA antibody, compared with a lower fluorescent intensity of approximately 2+ observed in the HEC-1-A cells, (Figure 16). Fluorescent intensities observed using the biotinylated HABP however were just 0-1+ for RL-95 cells and, 2+ for HEC-1-A cells. The staining patterns of these two reporter molecules differed markedly. Biotinylated HA binding protein gave rise to nuclear staining, whilst extra-nuclear staining only was seen with the use of the anti-HA antibody. All blanks having omitted the detection molecule/primary antibody were invisible under WIB fluorescence at 1.9s exposure. Non-fixed cells screened for HA expression using both the anti-HA antibody and the biotinylated HA Binding Protein also gave no fluorescence.

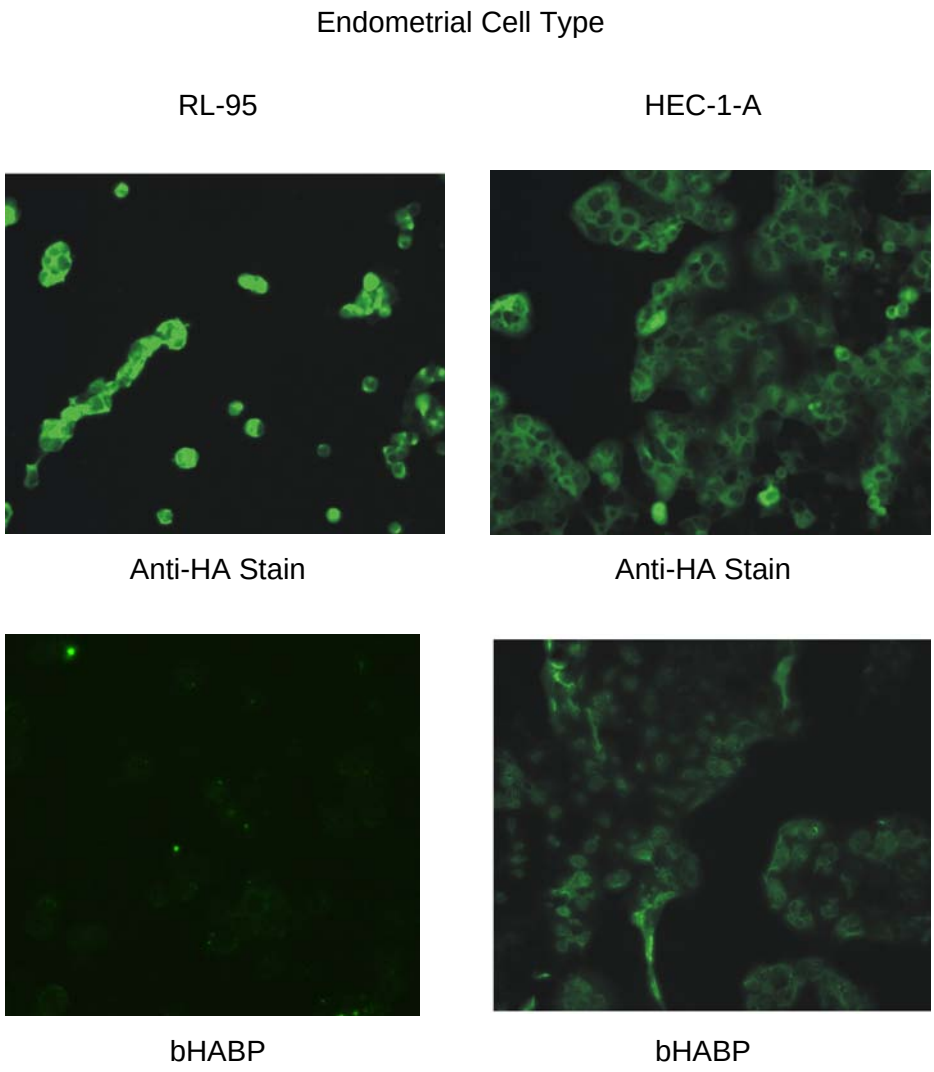


Figure 16. HA screen of fixed endometrial epithelial cells. Anti-HA produced extracellular staining while biotinylated HA binding protein exhibited intracellular staining. Cells viewed under WIB fluorescence at 1.9s exposure, magnification 200×

Interpretation

While HA is clearly present in both RL-95 and HEC-1-A cells, it appears that abundance is higher in the optimally adherent RL-95 cells. Non fixed cells showed no staining at all with either biotinylated HA binding protein (bHABP) or anti-HA antibody. The anti-HA antibody was the preferred detection tool for HA screening, as it was more sensitive than the HA binding protein tested and had been shown previously to detect molecular weights of HA, (Williams, 2008). The extracellular staining exhibited by the anti-HA antibody compared with intracellular staining of HA binding protein also made this the best choice for the detection of HA in the membranes of endometrial epithelial cells. Invisibility of blanks under WIB fluorescence provided evidence that neither the

Avidin Alexa Fluor, nor the donkey anti-sheep secondary antibody reporters bound in a non-specific manner to the epithelial cells.

2.4.2 CD44 Screening of Endometrial Epithelial Cells

CD44 is a trans-membrane protein present in the pre-implantation blastocyst which can recognise glycans such as hyaluronan. Hyaluronan or hyaluronic acid may act as a ligand for CD44 (Afify et al., 2005; Aplin, 1997), which is also expressed in endometrial epithelial cells and in the stroma of the endometrium throughout pregnancy and during the normal menstrual cycle. If CD44 were to be detected in the endometrial epithelial cells, it may represent a potential binding partner for a hyaluronic acid glycoconjugate FSL molecule.

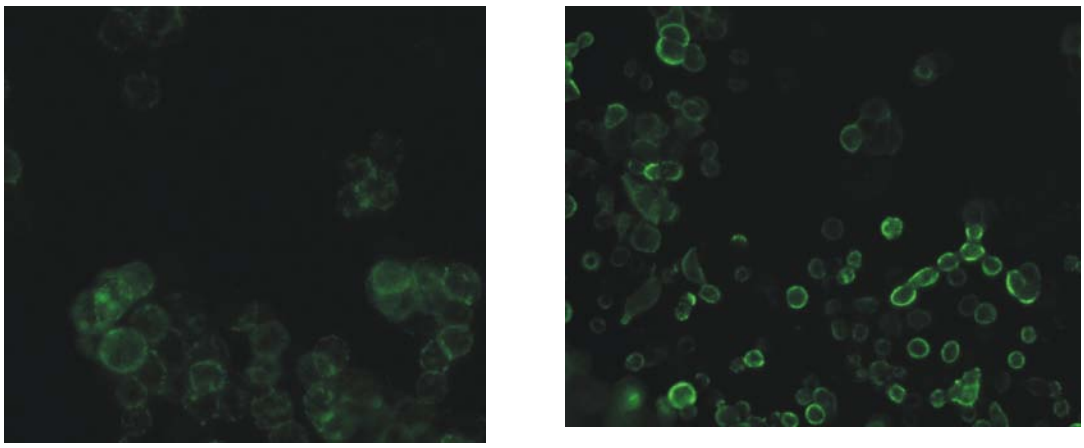
Method Overview

Monolayers of RL-95 and HEC-1-A cells were prepared in Terasaki plates as (Protocol 4.9). In order to obtain appropriate and comparable results to those with RL-95 monolayered cells, HEC-1-A cells were seeded at a higher concentration than in the previous assay, and were incubated overnight along with RL-95 cells (Protocol 4.9). After overnight incubation, cells were either fixed with 37% formaldehyde for 10 minutes at room temperature, or remained unfixed, in growth media (Protocol 4.5). Wells were washed and 25µL rat anti-CD44 was added to wells. Controls, containing no primary antibody, had 2% BSA diluent only added to wells. Plates were incubated at 37°C for 2 hours in a humidified box, washed with PBS and blotted dry. FITC conjugated rabbit anti-rat secondary reporter antibody (1/50) was added to all wells (25µL) and incubated at room temperature for 30 minutes in a humidified dark box. Wells were washed and 10µL PBS added to all wells for viewing purposes. Wells were viewed under WIB fluorescence with Olympus BX-51 microscope, and images recorded at 1.9s exposure time. DIC images were also recorded in order to determine cell localisation.

Results

Monolayered RL-95 exhibited a fluorescent intensity of approximately 2+ fluorescence, with unfixed cells, while HEC-1-A cells gave a fluorescent intensity of approximately 2-3+ (Figure 17). All primary antibody controls, containing no rat anti-CD44, were completely invisible under WIB fluorescence

after being incubated with reporter antibody, rabbit anti-rat. This is indicative that there is no non-specific binding of the secondary antibody to any cellular material. Fixed cells exhibited a fluorescent intensity of 0-1+.



RL-95 HEC-1-A

Figure 17. CD-44 screen of unfixed RL-95 cells and HEC-1-A cells. Adherent RL-95 cells exhibited 2+ fluorescence and sub-adherent HEC-1-A cells exhibited 2-3+ fluorescence when viewed under WIB fluorescence at 1.9 seconds exposure. Magnification 200×.

Interpretation

Both cell types have remained un-fixed and have rounded, producing spherical cells which give rise to optimal staining. RL-95 cells exhibit a slightly less intense fluorescent signal than that of HEC-1-A counterparts. It may therefore be concluded that CD44 is expressed in both adherent RL-95 and sub-adherent HEC-1-A cultured endometrial epithelial cells. These cells may therefore be receptive for embryos expressing HA, either endogenously, or modified with an HA conjugated FSL molecule. Interestingly, detection of CD44 with fixed cells with this antibody/ molecule complex is dramatically reduced compared with unfixed cells. This phenomenon is in contrast with that seen with the HA detection antibody using fixed versus non-fixed cells. The non-fixed cells round up during the staining process, and it is much easier to visualise fluorescence in the resulting spherical cells than in spread out fixed counterparts. Thus, while the quantity of CD44 present in the cell membranes may be consistent, the visible fluorescence of the cultured endometrial epithelial cells appears to be

increased depending on the degree of contraction/ expansion of the plasma membrane surrounding the cells.

2.4.3 Screening of Pseudo-pregnant Uterine Horns for HA

Sectioned uterine horns were screened at various stages of pseudo-pregnancy for hyaluronic acid (HA) using sheep anti-HA primary antibody. This assay was performed to help elucidate the role that HA plays in the receptive endometrium, during the window of implantation in the mouse.

Method Overview

Pseudo-pregnant uterine horns were prepared from CBA/ C57 female mice (Protocol 4.16) and dissected at days 2.5, 4.5 and 6.5 of pseudo-pregnancy. Dissected uterine horns were embedded in OCT cryo-compound immediately following dissection and stored at -85°C prior to sectioning. Three mice per group were used to prepare sections at each of the different days of pseudo-pregnancy (Protocol 4.11). Sections were prepared at 5µm thick using a cryostat, and mounted onto pre-chilled poly-L-lysine coated microscope slides. Sheep anti-HA detection antibody (20µL) was added to uterine sections and incubated on the slides for 2 hours at 37°C in a humidified box. Diluent only blanks, containing no primary antibody were included in the assay. Slides were washed with PBS and dried. Secondary, reporter antibody, donkey anti-sheep was added to all sections and incubated in a humidified dark box at room temperature for 30 minutes. Slides were washed and mounted with cover slips using mountant which contained Citifluor to protect the fluorescent potential.

Results

Hyaluronic acid labelling fluorescence was most intense at the surface of endometrial epithelial cells (Figure 18). Days 2.5, 4.5 and 6.5 elicited fluorescent intensities of 1+, 3+ and 3+ respectively. Blank controls, with primary antibody omitted were not visible under WIB fluorescence, observed only under DIC. This confirmed that there was no cross-reactivity of the secondary antibody for the uterine horns.

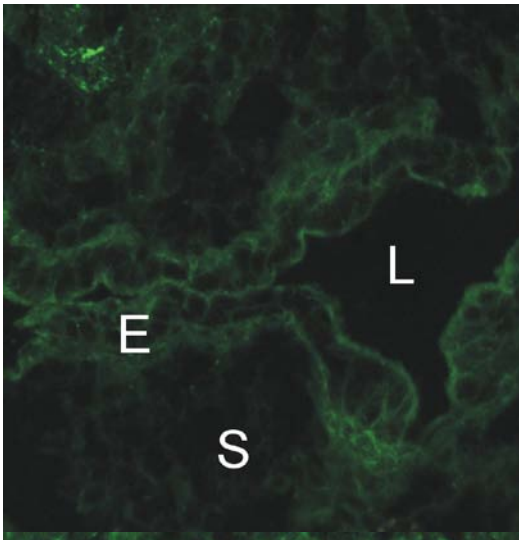
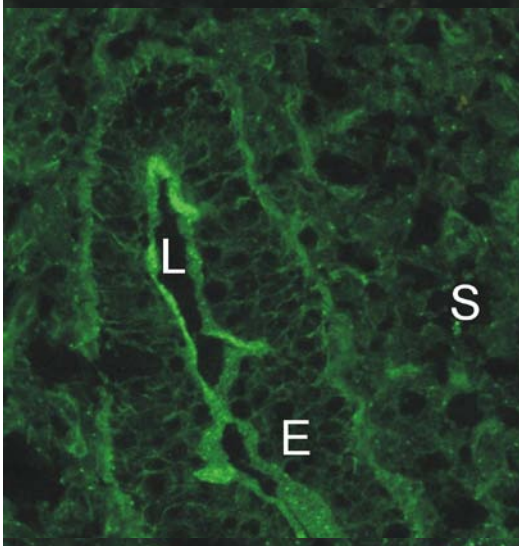
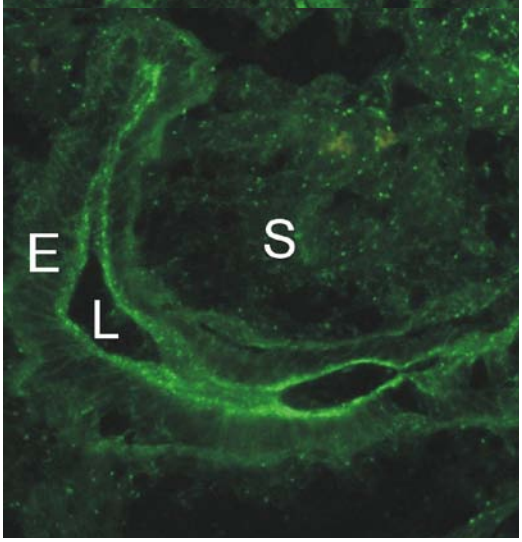
Pseudo-pregnancy day	Image	Score
2.5		1+
4.5		3+
6.5		3+

Figure 18. HA screen of pseudo-pregnant uterine horns. Images show endometrial epithelial cells (E), stromal cells (S) and the lumen (L) of uterine sections. Uterine horns at day 2.5, 4.5 and 6.5 were stained using sheep anti-HA and donkey anti-sheep FITC conjugated secondary antibody. Images were viewed under WIB fluorescence at 1.9 seconds exposure at 200× magnification.

Interpretation

A small amount of HA was detected at early stages of pseudo-pregnancy in the epithelial cells lining the lumen of mouse uterine horns. Dramatically more HA was detected in endometrial epithelial cells (EECs) by day 4.5 and this increase persisted until day 6.5. Consistent with findings in humans (Salamonsen et al., 2001), this increase in fluorescent intensity suggests that there is an up-regulation of HA around the time of implantation in the mouse, that is, day 4.5. The high cell density seen in sections of day 4.5 uterine horns compared with day 2.5 and day 6.5 may provide stable conditions for the implantation of embryos.

2.5 Attachment Assays

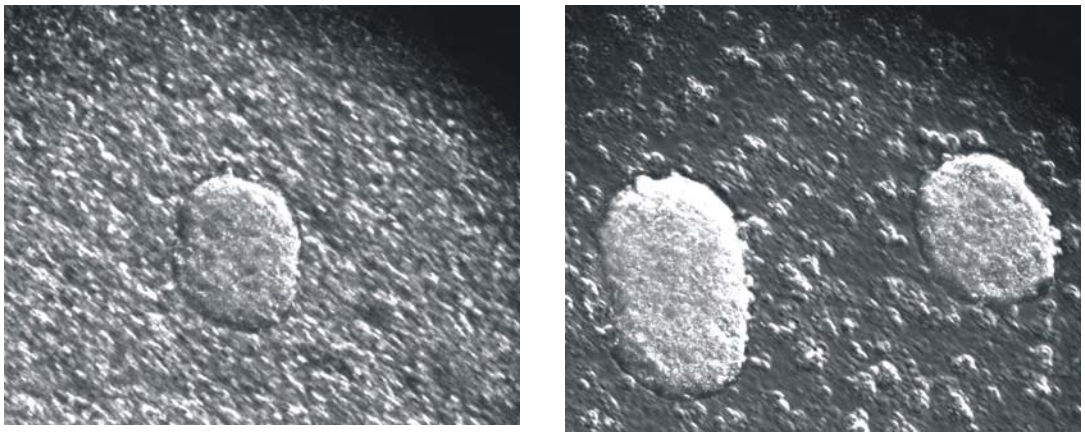
2.5.1 Attachment Assays using Cultured Endometrial Epithelial Cells

Assays were carried out to determine the attachment potential of trophoblastic cells to endometrial epithelial cells known to be optimally adherent and sub-adherent. Trophoblastic BeWo cells were prepared as tiny spheres representative of an embryo using a recognised technique to model an embryo (Protocol 4.10). Attachment assays were performed using these spheroids in co-culture with prepared monolayers of cultured endometrial epithelial cells representative of the endometrial lining of the uterus. Attachment of spheroids to monolayers of two different cell lines, RL-95-2 and HEC-1-A, representing adherent and sub-adherent cells respectively was compared.

Method Overview:

Four replicate experiments were carried out to compare attachment in adherent RL-95 and sub-adherent HEC-1-A endometrial epithelial cells (Protocol 4.9). Monolayers were prepared by seeding RL-95 or HEC-1-A cells into wells of a 96 well culture plate (Figure 19). Cell suspensions of BeWo cells were incubated overnight at 37°C on an orbital shaker to produce tiny spheres of cells approximately 300 μm in diameter (Figure 20). Five of these spheroids were added per well to the prepared monolayers in the 96 well culture plates using a custom made mouth pipette. Spheroids were allowed to attach to the monolayers at for 30 minutes at 37°C (Figure 19). Attachment was challenged

by centrifugation of the inverted plate at 400 rpm (15 ×g) for 5 minutes. Plates were then placed upside down for 5 minutes to allow loosened spheroids to detach and fall off the monolayers due to gravity. Remaining spheroids, attached to monolayers were counted and the attachment potential was expressed as a percentage of the total spheroid number added to the monolayers. Data was analysed using the test of two proportions using Minitab, and p values determined to give a measure of the likelihood of the result if the sets of data were from the same population.



Spheroid on RL-95 Monolayer Spheroids on HEC-1-A Monolayer

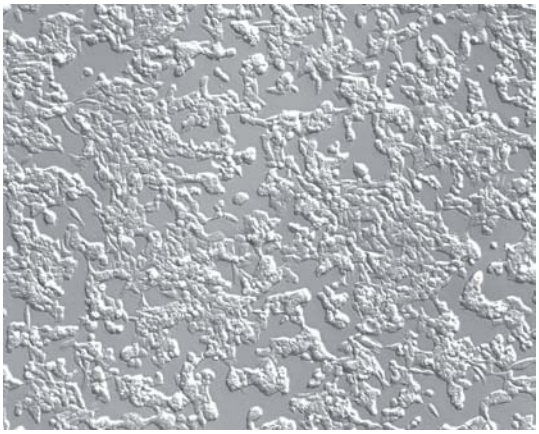
Figure 19. Spheroids attached to RL-95 and HEC-1-A Cell monolayers. Images were taken under an inverted microscope at 200× magnification.

Results:

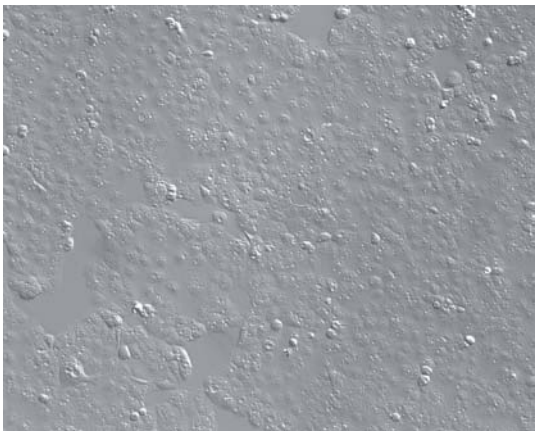
BeWo spheroids attached to RL-95 monolayers with a mean efficiency of 93% ± 2, while the attachment to HEC-1-A cells was significantly reduced with just 18% ± 4 of spheroids remaining attached after centrifugation (Table 7), $p<0.001$. The difference in BeWo spheroid attachment to RL-95 cells versus HEC-1-A cells was shown to be statistically significant, with a p value of less than 0.01 (Figure 21).

Table 7. BeWo spheroid attachment to endometrial epithelial cells. Optimally adherent RL-95 cells exhibited significantly higher attachment efficiencies than the sub-adherent HEC-1-A cells. Attachment efficiencies are expressed as percentages with fractions in bracket representing the number of spheroids remaining attached over total number of spheroids added to monolayers.

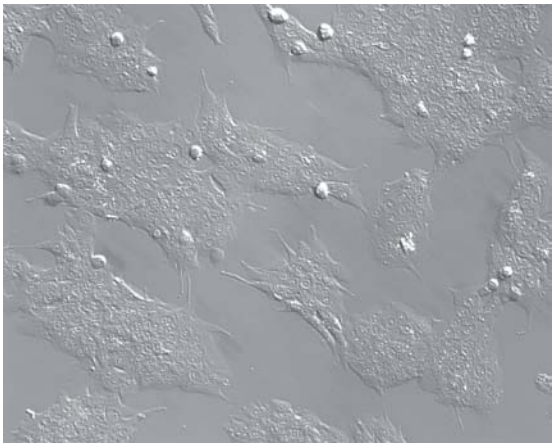
Replicate	Attachment Efficiency (%)	
	RL-95	HEC-1-A
1	92(11/12)	17 (2/12)
2	88 (28/32)	25 (5/20)
3	96 (27/28)	7 (2/28)
4	97 (31/32)	22 (7/32)
Mean	**93**	**18**



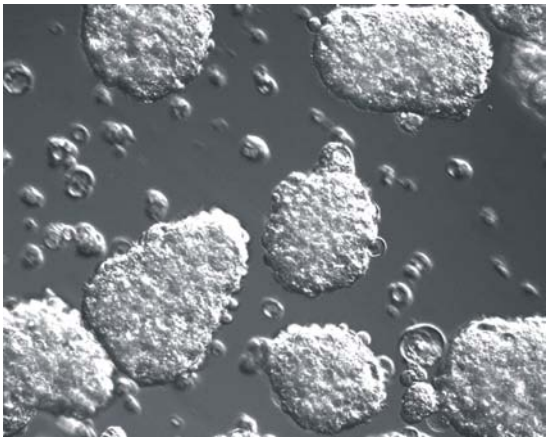
RL-95 monolayer



HEC-1-A monolayer



BeWo monolayers



BeWo spheroids

Figure 20. Cultured Endometrial epithelial cells and trophoblast cells. Magnification ×200. RL -95 cells exhibiting approximately 90% confluence, HEC-1-A monolayer exhibiting 95% confluence, BeWo cells growing in monolayers for culture exhibiting 70% confluence, d = BeWo spheroids produced after culture with gyration at 130 rpm

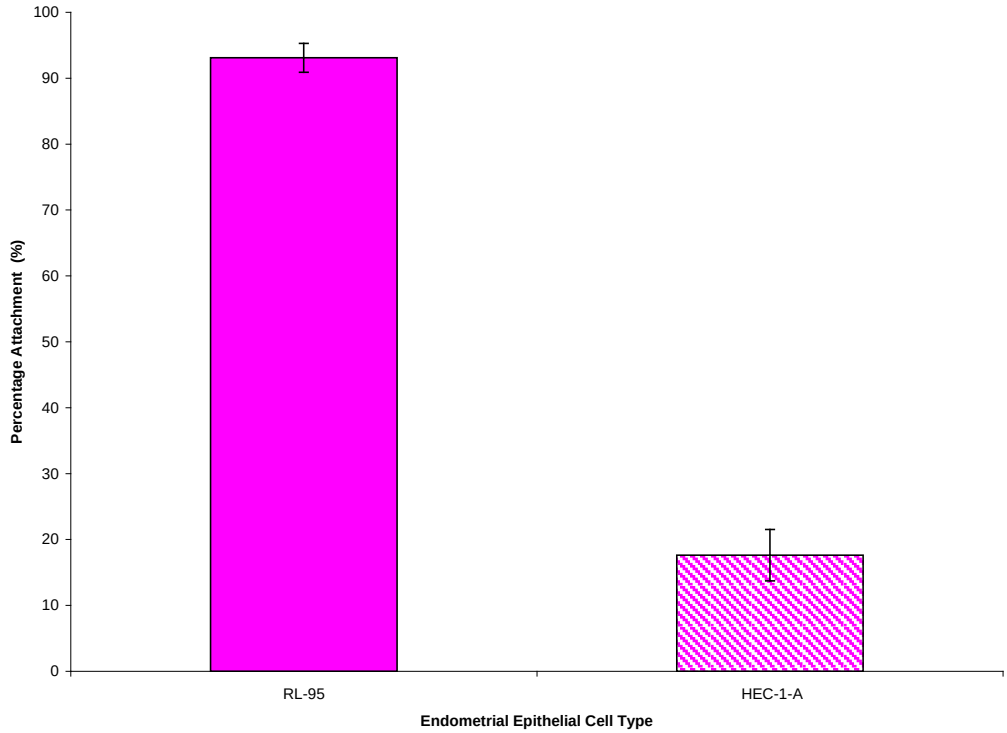


Figure 21. Spheroid attachment to endometrial epithelial cell monolayers. The attachment efficiency (%) adhered to monolayers of adherent RL-95 cells and sub-adherent HEC-1-A cells. Mean ±SEM, $p < 0.001$ analysed with Two Proportions test using Minitab.

Interpretation:

RL-95 monolayered cells demonstrated significantly higher attachment of BeWo spheroids than monolayers of HEC-1-A. The attachment efficiency of BeWo cells to RL-95 monolayers was consistently above 85% with a mean of 93%, while the mean retention of spheroid attachment to HEC-1-A monolayers was just 18% and never above 25%. Monolayers of HEC-1-A cells exhibited a higher variation in attachment relative to RL-95 monolayers. To control for inter-assay variation, validity criteria were established for the attachment assays using RL-95 and HEC-1-A monolayers. A valid assay was deemed to be one in which attachment of BeWo spheroids to RL-95 monolayers was greater than or equal to 85% attachment, while attachment to HEC-1-A cells was less than or equal to 35% attachment. Monolayer culture media was not changed prior to addition of BeWo spheroids. This serves two purposes - not only does the retention of culture media result in reduced exposure of the monolayers to shear forces when changing media, but since no changes or flushing of the uterine environment would occur in the in vivo mouse model, or in the clinical setting, these parameters were followed as closely as possible.

2.5.2 BeWo Spheroid Attachment Assay with FSL A treated monolayers

Adherent RL-95 cells and sub-adherent HEC-1-A cells were exposed to benign FSL molecules which were not expected to exert any functional effect other than to prove the concept of molecular manipulation To test this, potential changes in the attachment characteristics of endometrial epithelial cells towards BeWo spheroids were assessed to determine whether the harmless manipulation of molecules on the surface of the endometrial epithelial cells resulted in any modification of their adherence characteristics.

Method Overview:

In three replicate assays, endometrial epithelial cells were seeded into 96 well plates (Protocol 4.9). After overnight incubation, growth media was carefully removed and replaced with media containing 1mg/mL FSL A. Endometrial epithelial cells were incubated in the presence of FSL A for a period of 30 minutes at 37°C with 5% CO_2. BeWo spheroids were prepared from cell BeWo monolayers as per (Protocol 4.14). Five spheroids per well were then placed on the epithelial cell monolayer using a mouth pipette especially made for the purpose of handling spheroids and incubated for 30 minutes at 37°C. Plates were inverted and taped into centrifuge buckets of Hitachi centrifuge and centrifuged at 400rpm (15 $\times$g) for 5 minutes. Plates were left inverted for 5 minutes following centrifugation to allow loosened spheroids to detach. Spheroids remaining attached to monolayers were counted and expressed as a percentage of the total spheroids added. Data was analysed using the test of two proportions using Minitab, and p values determined to give a measure of the likelihood of the result if the sets of data were from the same population.

Results:

Attachment of BeWo spheroids to non-treated adherent RL-95 monolayers had a mean efficiency of 98% attachment. A mean attachment efficiency of just 20% was observed in FSL A treated RL-95 monolayers (Table 8, Figure 22). Attachment of spheroids to non-treated sub-adherent HEC-1-A monolayers had a mean attachment efficiency of 16.7% however, with just 11.7% attachment observed in FSL A treated HEC-1-A monolayers, the difference in attachment

efficiency compared with non-treated HEC-1-A cells was not statistically significant.

Table 8. Spheroid attachment to FSL A treated endometrial monolayers. Bracketed numbers represent replicates showing the proportion of spheroids attached over the number assayed)

	Endometrial Cell Type	
	RL-95	HEC-1-A
Untreated	98.3 (10/10, 19/20, 20/20)	16.7 (0/20, 3/20, 7/20)
FSL A treated	20.0 (9/20, 0/10, 3/20)	11.7 (5/20, 2/20, 0/20)

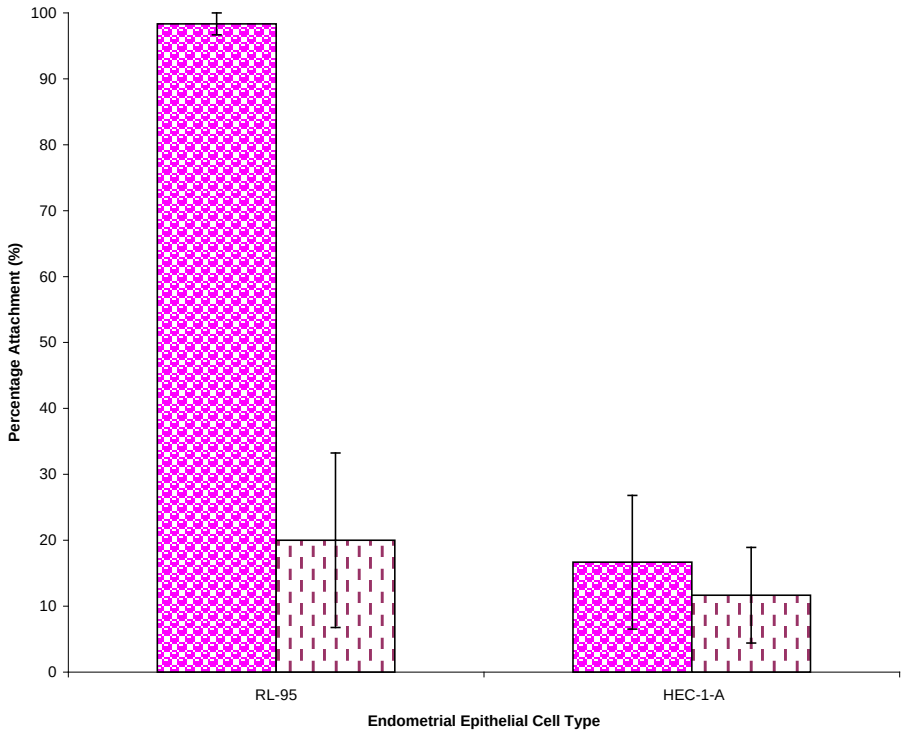


Figure 22. Spheroid attachment to FSL A treated endometrial cells. Dark bars represent non-treated monolayers, while lightly shaded bars represent monolayers treated with 1mg/mL FSL A. Mean ± SEM. The attachment efficiency of RL-95 monolayers is significantly affected by FSL A treatment, $p < 0.001$ analysed with Two Proportions test using Minitab.

Interpretation:

HEC-1-A cells were not significantly affected by treatment with FSL A with respect to attachment efficiency towards BeWo spheroids. However, attachment of BeWo spheroids to optimally adherent RL-95 monolayers was significantly affected by exposure of monolayers to FSL A prior to attachment.

FSL A treatment has altered the cell adherence characteristics of these usually optimally adherent endometrial epithelial cells, rendering them sub-adherent, with an attachment efficiency equivalent to that of HEC-1-A cells. While treatment with FSL A affected the attachment efficiency of adherent RL-95 cells, this result was unexpected due to the benign nature of this FSL molecule with the A antigen head group. The nature of the effect of this molecule on attachment of endometrial epithelial cells remains to be elucidated.

2.5.3 Comparison of FSL A and FSL B Influences on RL-95 Attachment Assay

The influence of the benign FSL A treatment on optimally adherent RL-95 monolayers was compared with RL-95 monolayers treated with the structurally similar FSL B, also a benign molecule. Due to the minor degree of reduction of HEC-1-A adherence efficiency after FSL A treatment, these monolayers were not used for comparing FSL A and FSL B. This comparison was two-fold – firstly since the molecules are structurally similar, differences observed in attachment may be considered to be due to the subtle structural change in the functional head group on the FSL molecules. As these molecules are of similar size, any difference in steric hindrance which may affect the attachment assay would be considered to be minimal. Secondly, this assay will provide a means for comparing the influence of a different FSL molecule with the same lipid tail on the same endometrial epithelial cells under the same conditions. This would provide further data regarding the safety of the insertion molecules/lipid tails with respect to cell monolayer health.

Method Overview

RL-95 cells were seeded into 96 well plates (Protocol 4.9). After overnight incubation, growth media was carefully removed and replaced with media containing 1mg/mL FSL A, or 1mg/mL FSL B. Non-treated wells were included as controls. Spheroids were prepared from BeWo trophoblast cells (Protocol 4.10) and five were added to each well using a mouth pipette. In order to ensure the spheroids did not attach to each other, influencing the assay integrity, spheroids were placed evenly around the well, carefully taking care to avoid disruption of the monolayer. Spheroids were incubated at 37°C for 30 minutes to allow attachment to the monolayers. Plates were inverted and

taped into centrifuge buckets of Hitachi centrifuge and centrifuged at 400rpm (15 ×g) for 5 minutes. Plates were left inverted for 5 minutes following centrifugation and remaining spheroids attached to monolayers were counted and expressed as a percentage of the total spheroids. Data was analysed using the test of two proportions using Minitab, and p values determined to give a measure of the likelihood of the result if the sets of data were from the same population.

Results

Monolayers of RL95 cells treated with FSL A exhibited a mean attachment of 18%. This adherence efficiency for BeWo spheroids is consistent with FSL A treated monolayers observed in the previous assay. Monolayers treated with FSL B exhibited a mean attachment rate of 73% (Figure 23).

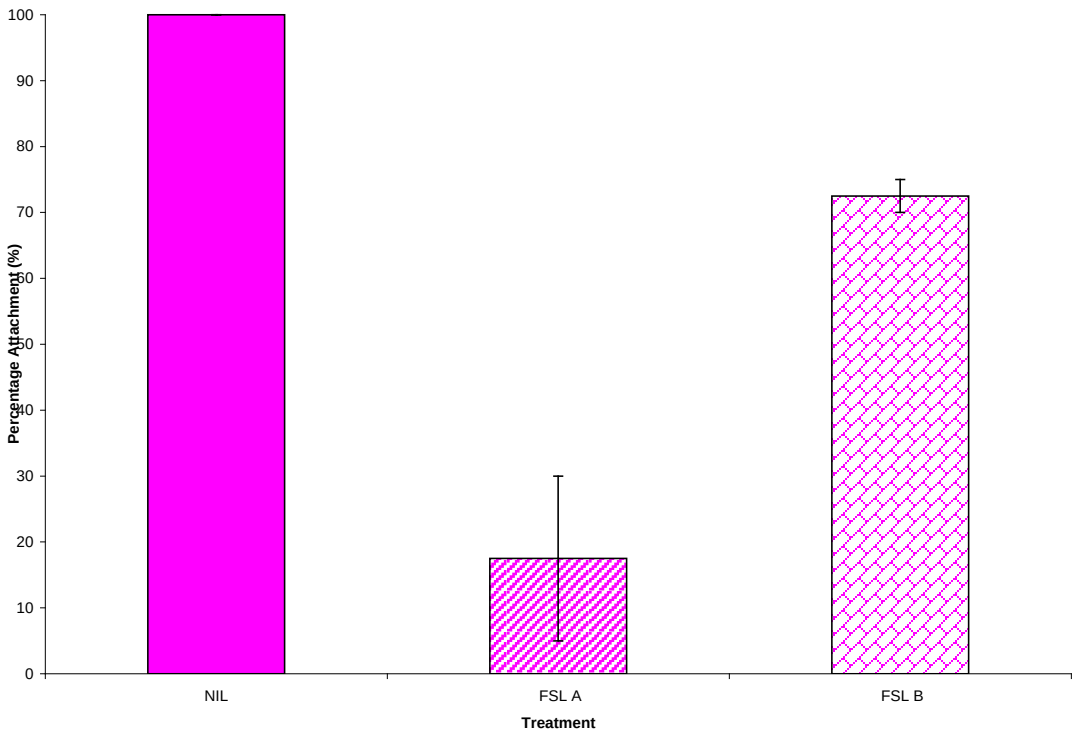


Figure 23. Comparison of FSL A and FSL B on BeWo spheroid attachment. Mean ± SEM. The non treated monolayers, the FSL A treated monolayers and the FSL B treated monolayers all had significantly different attachment efficiencies, $p<0.001$ analysed with Two Proportions test using Minitab.

Interpretation

While the effect of FSL B treatment on monolayers significantly lowered the attachment efficiency of adherent RL-95 endometrial epithelial cells, this

reduction was significantly less than that observed with the FSL A treated monolayers. From a functional saccharide perspective, there is no explanation for the reduced attachment observed between trophoblast spheroids of BeWo cells and the adherent RL-95 endometrial epithelial cells. While it the mechanism for this effect remains to be elucidated, it is interesting to consider that these structurally similar molecules exert significantly different influences.

2.5.4 BeWo Spheroid Attachment Assay with Media Change

The attachment assay was repeated using treated monolayers which then had media aspirated off and fresh media added prior to the introduction of BeWo spheroids to the monolayers. This would help to determine whether the observed decrease in attachment of BeWo spheroids to FSL treated endometrial epithelial monolayers was due to the specific modification of endometrial epithelial cells or due to characteristics of assay media such as steric hindrance and pH changes.

Method Overview:

An attachment assay was carried out using the optimally adherent RL-95 cells (Protocol 4.14). Monolayers of RL-95 were prepared (Protocol 4.9) and treated with either FSL A or FSL B. Subsequent to treatment with FSL molecules, some wells had cell growth media gently removed and replaced with pre-warmed, degassed RL-95 cell growth media, while other wells had FSL molecules remaining in growth media. BeWo spheroids were added to monolayers using a mouth pipette and allowed to attach to monolayers at 37°C for 30 minutes. Adherence efficiency of the monolayers for BeWo spheroids with changed media and unchanged media was assessed in FSL A and FSL B treated cells by centrifugation at 400 rpm (15 $\times$g) according to (Protocol 4.14) and recorded as the percentage of total added spheroids remaining attached after centrifugation challenge. Data was analysed using the test of two proportions using Minitab, and p values determined to give a measure of the likelihood of the result if the sets of data were from the same population.

Results:

RL-95 untreated monolayers yielded an attachment efficiency of 98%, correlating to previous assays where untreated RL-95 cells always attained an

attachment efficiency of BeWo spheroids of greater than 90%. Attachment efficiencies of 23% and 58% for RL-95 monolayers treated with FSL A and FSL B respectively were also in accordance with previous attachment assays performed (3.5.2 and 3.5.3). Monolayers in which FSL insertion media was gently replaced with fresh growth media, an increased attachment potential was exhibited with respect to attachment assays in which media was not changed (Figure 24). FSL B treated RL-95 monolayers with replaced media returned to an attachment potential of 100% compared with an attachment efficiency of 58% exhibited with unchanged media. On the other hand, FSL A treated RL-95 monolayers with replaced media demonstrated an attachment potential of just 70% compared with an attachment efficiency of 23% exhibited with unchanged media.

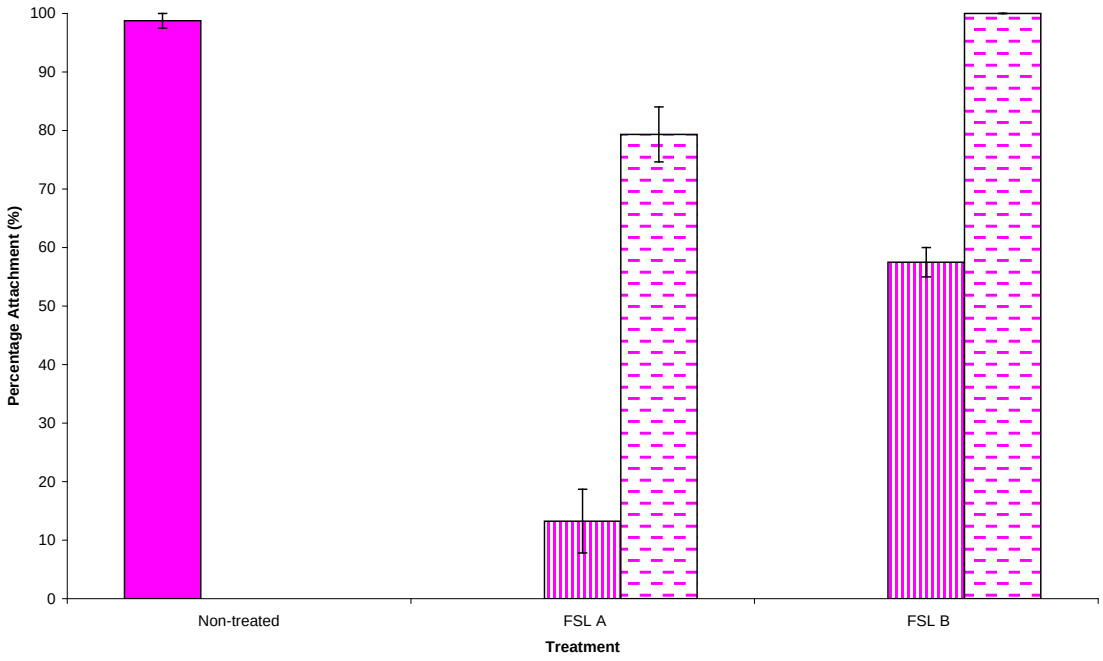


Figure 24. Trophoblast spheroid attachment with changed media. Dark shading represents FSL treatment and attachment assay without a media change, light shading represents FSL treatment and attachment assay with a media change. Untreated optimally adherent RL-95 cells were included as a control (solid shaded bar). Attachment efficiency is expressed as mean ± SEM. Attachment in both FSL A and FSL B treated and unchanged media were significantly different from their counterparts with changed media $p < 0.001$. There was also a significant difference between attachment of FSL A and FSL B treated monolayers $p < 0.001$. Data were analysed by Test of Two Proportions using Minitab.

Interpretation:

Considering the FSL B treated and washed monolayers, the 100% attachment of spheroids is suggestive of two scenarios: either the FSL molecule has

inserted and exerted no effect on spheroid attachment, or the molecule has not inserted, and has been completely removed along with the replaced media. Due to earlier evidence of insertion using immuno-histochemical techniques to visualise endometrial epithelial cell monolayers, it is considered that the former option is most likely – that is, modification of the cell membranes has occurred, but that this insertion has not influenced spheroid attachment. The significant difference seen between FSL A and FSL B treated and washed monolayers, was suggestive that the FSL in solution is responsible for the decreased attachment observed. It can be observed, having excess FSL molecule remaining in attachment media, that is, no change of media prior to attachment, results in inhibition of BeWo spheroid attachment. While this inhibition is exhibited regardless of the specific head group, it is more accentuated with excess FSL A in attachment media compared with FSL B. The excess FSL molecule may be responsible for coating or inserting into the spheroid prior to attachment, thereby causing difficulties with any subsequent attachment of spheroids to endometrial epithelial cells. There has been evidence from previous research that insertion/coating of embryos may occur within minutes of exposure to FSL molecules (Blake, 2003). Another possible indication of these results however, is that non-specific steric hindrance due to the excess of FSL molecules in the attachment media may be responsible for the inhibited attachment of BeWo spheroids to FSL A or FSL B treated monolayers. This steric hindrance may be simply restricting access of BeWo spheroids to monolayered endometrial epithelial cells rather than specifically repelling the spheroid from the monolayer. In the FSL A treated and unwashed monolayers, the inhibitory effect on attachment may be due to both specific repulsion and steric hindrance – hence the dramatic decrease observed in attachment compared with non-treated monolayers.

3 Discussion

3.1 Implication of Results

There are multiple aspects that are required to be fulfilled in order to reproduce successfully, and therefore many reasons why infertility may be a problem for couples. If the reasons for couple's fertility problems are not elucidated, the unfortunately unhelpful diagnosis of "unexplained infertility" is provided. In vitro fertilisation has increasingly become a fix-all and the last ray of hope for couples all over the world, in their quest for bearing their own children. The first successful in vitro fertilised baby was born in the UK almost thirty years ago (Cohen et al., 2005; ESHRE, 2009). Since then, while the IVF arena has advanced with the demand for improved technology, certain limits still remain. Unsuccessful implantation of the embryo into the endometrium lining the uterus is considered to be one of the central causes of "unexplained fertility" (Boomsma et al., 2009). Therefore methods for improving the implantation would have the potential to significantly impact on IVF success.

There are two sides to consider when assessing implantation of a clinical pregnancy: the embryo and the endometrium. Whilst there has been a wealth of knowledge built up about the embryo and endometrium independently, the intricate interactions with each other remain to be fully clarified. Together with the high degree of redundancy which apparently occurs, no single factor has clearly been established as an absolute requirement for implantation. Efforts into improving IVF technology have predominantly been made on improving embryo culture conditions with particular regard to growth and health, in order to optimise embryo quality and enhance implantation. There has been comparatively little investigation into the modification of the uterine environment to enhance attachment. This is perhaps due to the difficulties in establishing definite targets of manipulation and also due to the difficulty in accessing the uterus in vivo. Furthermore, the ability to treat embryos once they are in culture is relatively easy in terms of access to them compared with accessing and treating uterine endometrial cells. The endometrium derives less attention than the intensively cultured embryos, reflective of a gap in the research with regard to optimisation of uterine conditions.

KODE™ technology has previously been employed to successfully modify a range of different cell types, including red blood cells, and blastocysts. A variety of molecules have been investigated by others within this research group, for modification of blastocysts and the influence on implantation (Blake, 2003; Carter, 2007). Exploring the manipulation of the endometrium was a new focus for the group and this project was the first to explore the possibilities of changing the endometrial environment via KODE™ technology. A novel method was developed to administer modifying agents to the mouse uterus in order to allow their influence, tolerability and safety to be ascertained. Both in vitro and in vivo techniques were employed, with modification parameters initially determined using modified cells in vitro.

The major challenge for modification of uterine cells in vivo is simply a matter of access. The in vivo lavage technique was accordingly designed and optimised prior to use with KODE™ molecules, which were administered directly to the mouse uterus to modify uterine cells in vivo. The successful treatment of EECs of the uterus with KODE™ molecules in order to engineer and tailor cell surface molecular expression is a significant advancement. This achievement has potential for overcoming implantation difficulties and exploring novel approaches. Further in vitro techniques were employed to determine the expression of two attachment molecules regarded as candidates for development of novel KODE™ molecule constructs. Finally, attachment of embryonic cells to endometrial epithelial cells of human origin was observed in vitro and this assay was used to observe and measure the effects of modification with benign KODE™ molecules. The successful development of a murine trans-vaginal lavage technique to administer modifying fluid to the lumen of the uterus has also potentiated research of any agent delivered to the uterus to be investigated in the mouse model.

3.1.1 Insertion and Retention

This study is the first to demonstrate that endometrial epithelial cells have been modified to exhibit an exogenous antigen on their surface using KODE™ technology. Endometrial epithelial cells were transformed within two hours of treatment with FSL molecules and this modification was stable for a further two hours after removal of the modification molecule. Cultured endometrial

epithelial cells were modified to display the blood group antigen A by treatment with the synthetic construct, FSL A. This molecule was the proof of concept molecule chosen for its availability, and more importantly, the previous experience and knowledge base within the research facility. This molecule was not expected to exert any biological effect, and was selected to act purely as a proof of principle molecule. Modification molecules were not detected in modified cell membranes after 24 hours, lost probably through metabolic endocytosis and expulsion (Frame et al., 2007; Kovalenko et al., 2004; Schwarzmann, 2001). The implication of this for the clinical setting is that lavage fluid may have to be irrigated over the target cells (uterine endometrial epithelial cells) and retained in the uterus for at least 2 hours in order to achieve reliable modification. Due to the retention time constraint, this procedure would need to be undertaken immediately prior to embryo transfer in the clinical setting in order to insert into cells and be maximally presented on the cells' surfaces, to have maximal effect on embryo attachment

In vitro cell culture of specific, human derived cells requires specialised cell culture conditions and components. Optimal sustenance of cells for their metabolism ensures that they behave in a similar manner to their analogous cells in vivo. A common component of cell culture is serum, most commonly fetal bovine serum (FBS), and cells that don't have this component added to their media usually demonstrate marked differences in cell growth and morphology. The insertion of KODE™ molecules is affected by the presence of lipids due to the bilipid tail component that inserts into the membranes of cells. The FBS was considered likely to contain factors which would interfere with KODE™ molecule insertion. This component was therefore omitted from cell media during modification with KODE™ molecules under this assumption. This limited the length of time to which cultured endometrial epithelial cells could be treated with KODE™ constructs which may in turn influence the retention ability of modification molecules in these cells. If insertion was to occur over an extended period of time however, this would be best performed with FBS present as although efficiency of insertion may not be optimal, cell nutrition prevails as cell health is crucial in any modification. In vitro investigations may therefore under-estimate the retention ability of cells. Cells treated in vivo do not exhibit the same constraints associated with cell culture requirements.

With methods established for modification of cultured endometrial epithelial cells in vitro, modification of their in vivo counterparts was subsequently targeted. To achieve this, an effective delivery method was required with the requisite for being non-surgical in its nature.

3.1.2 Lavage Design and Development

The delivery of modification molecules directly to the uterus via a catheter passed trans-cervically into the uterus is a relatively novel procedure. The success of the lavage technique was confirmed using a tracker dye. This achievement has facilitated the use of the mouse model for treatment and investigation of KODE™ molecules administered to the uterus in vivo. The novel aspect of the trans-cervical lavage was that the cervix of the mouse was able to be viewed under the dissection microscope and could then be penetrated with a catheter in order to administer a large enough volume to the uterus to inflate the uterine horns. This technique in the mouse is analogous to the cervico-vaginal procedure used to transfer embryos in the human. This procedure may therefore be employed in the clinical setting prior to embryo transfer for the administration of lavage fluid. The mouse model provides an indication of suitability of modification molecules which, after certain preliminary in vitro investigations, may be considered for clinical trials.

The success of the lavage technique was not without its hurdles. The lavage was performed with greatest ease in mice in estrus or those exhibiting pseudo-pregnancy. This may not be surprising since an easily penetrable cervix would facilitate passage of sperm immediately after mating into the uterus. Considerable mouse to mouse variability demonstrated that some mice were unable to be lavage treated as catheterisation of the cervix was sometimes technically challenging. Lavage fluid in excess of 100-200μL risked rupturing of the uterine horns and consequent internal injuries. Due to the lack of visibility of the uterus during the lavage, over-inflation was considered to be the biggest risk of this technique. For this reason, a smaller volume of fluid was preferable to use if there were doubts regarding the capacity of the mouse uterine horns. Fluid occasionally flowed back through the vagina leading to loss of inflation of uterine horns, a further challenge for the lavage procedure. To overcome this, mice were kept in a supine position with back ends elevated for

a period of time before anaesthetic recovery in order to discourage liquid flowing out by gravity.

As this technique is relatively novel, it is interesting to consider similar techniques employed by others, to study the uterus or its surrounding environment after manipulation. At the time this project was undertaken, only one other researcher had reported a lavage of the murine reproductive tract with parameters approximate to those included in the current investigation. In 2005, Johnson et al performed a mouse vaginal lavage of lipiodol, an iodised poppy seed oil traditionally used as an oil soluble contrast media (OSCM) for hystosalpingograms (Johnson et al., 2005). Their methodology involved a vaginal instillation using a blunt needle and delivery of 0.5 mL into the vagina, holding the vaginal opening closed to prevent backflow. Another method they adopted to deliver lipiodol directly to the uterine horns of the mouse was via a surgical laparotomy involving a small incision made into the lower abdomen and injection of 0.1 mL to the exposed uterine horn via a 27 gauge needle (Johnson et al., 2005). From their methods, it appears that, delivery into the uterus by lavage relied on full inflation of the vaginal canal with 0.5 mL of fluid. The associated pressure within the vaginal canal caused by holding the vagina closed forced fluid to be pushed up through the cervix to enter the uterus (Johnson et al., 2005).

Another, more recent development of a cervico-vaginal lavage, was used to administer pseudo-virions to investigate the establishment of papilloma-virus infection (Roberts, 2007; Roberts et al., 2007). In the mouse model, small volumes of fluid up to 15μL containing pseudo-virions were administered directly to the cervico-vaginal opening using a paediatric nasal speculum to visualise the cervix. A gel loading tip was then used to cannulate the cervical opening and administer fluid directly to the cervical canal. Their method does not deliver the larger volumes of up to 200μL delivered to the mouse uterus as this current investigation does. Also, rather than delivery of fluid directly inside the uterus, fluid is administered just outside the uterus, at the point of cervical infection. This technique does not require complete penetration of the cervix. It is interesting to note however, that Roberts et al report that due to the anatomical variability of the mouse, it was not possible to cannulate the cervix

of every individual mouse (Roberts, 2007). The findings of these researchers correlate with the current investigation as it was observed that the stage of estrus or pseudo-pregnancy appeared to influence the ease of cannulation for a successful lavage.

The methods of Roberts et al do not appear to differentiate subjects on the basis of estrus. The use of relatively large volumes of 150μL to 200μL may increase the potential for loss due to a capillary action whereby once fluid starts to flow out of the uterus, the complete bolus dose flows out in its entirety. Furthermore, not only is the chance of over-inflation increased, risking rupture of the uterus, but the larger volumes delivered by transvaginal lavage into uterine horns prior to an embryo transfer, may increase the transmigration phenomenon of embryos between uterine horns. With the use of instruments such as the nasal speculum employed by Roberts et al, together with mice optimally prepared, and smaller volumes used, the transvaginal technique developed in this investigation may be further enhanced.

3.1.3 Lavage Assay and Analysis

The results of this investigation revealed the surprising phenomenon that mice treated with benign and harmless FSL A by transvaginal lavage exhibited a reduced implantation rate of transferred embryos. These results are confirmation that the trans-vaginal uterine lavage successfully delivered fluid to the uterus and are suggestive that this led to modification of the endometrial cell surface expression. Sections of lavage treated uteri stained for detection of the A antigen, showed an increase in A antigen expression of the endometrial epithelial lining of the uterine horns. This modification elicited a change in the receptive character of uteri of the optimally fertile recipient mice. As the proof of principle modification molecule, FSL A, is considered to be benign, the mechanism for this effect on cell attachment remains to be elucidated.

For the first time, KODE™ technology has been utilised to target and successfully modify a specific organ in a live animal. While KODE™ modification has been used extensively with marked success, in situ modification of a targeted organ in an animal model has not been attempted previously. Both red blood cells and embryos which have been transformed

with KODE™ molecules have been treated by in vitro culture and therefore conditions of insertion may be tightly defined. Taking these molecules into the live animal model and attempting to modify target cells in vivo, exposes the molecules to conditions which are under the control of the complete and complex biological system and beyond the control of the technician. This is a potential challenge for in vivo modification and must therefore be considered when applying this technology.

In vitro exposure of the lipid-containing KODE™ constructs to solvents is known to strip synthetic molecules from modified cells. It was therefore necessary to consider this when employing sensitive detection methods of molecule insertion. KODE™ modified uterine horns were frozen in freeze media (OCT) and sectioned with a cryostat. Cryostat sectioning and subsequent staining of sections, whilst being the preferred technique for antigen preservation, is widely considered to be suboptimal in terms of conservation of cell morphology. However, due to the solvents used in dehydration steps required for staining paraffin embedded sections (usually acetone mixtures or alcohol gradients), these methods were unsuitable for KODE™ modified tissue. Detection of modified tissue was therefore confined to cryostat sectioned tissue which did not conserve tissue morphology as efficiently. This made it more technically challenging to get complete, clean sections with no artefacts, and suitable freezing conditions. Due to the importance of FSL antigen preservation, overcoming this challenge was a priority.

Preliminary investigations indicated that modification by lavage treatment of the mouse EECs was shown to be harmless to the recipient, both in terms of the safety of the lavage procedure and the toxicity of KODE™ molecules. Sectioned uterine tissue from lavage treated mice compared with controls, showed that no major disruption had been sustained by the uterine tissue subsequent to lavage treatment. Furthermore, when subsequent fertility was investigated in lavage treated mice, the ability of treated mice to successfully mate and give birth naturally, indicated that short term exposure of uterine endometrial epithelial cells to FSL A did not have long term consequences. No lasting repercussions on fertility were observed. This also confirmed that the transvaginal lavage procedure was gentle enough to ensure that subsequent

fertility was not affected due to physical damage such as scar tissue. Due to both the temporary nature of the modification previously described, and the fact that uterine epithelial cells are continuously sloughed off and shed from the uterine lumen (Aplin & Kimber, 2004; Diedrich et al., 2007), FSL A was expected to be relatively quickly eliminated from the reproductive system with no effects on implantation lasting beyond the next estrus cycle.

Not only were endometrial epithelial cells of mice successfully transformed with the proof of principle molecule, FSL A via transvaginal lavage, but this modification was unexpectedly shown to alter the receptivity of the endometrium. A decrease in the implantation rate of transferred embryos was observed in mice which underwent transfers immediately subsequent to lavage treatment with FSL A. It is interesting to note that in another study, using KODE™ molecules which targeted the embryo, FSL A treated embryos which were transferred into recipient mice showed no difference in their implantation and live birth rates compared with non-treated controls (Erikson, 2009). This investigation confirmed that lavage with FSL A resulted in modified endometrial epithelial cells which disrupted the attachment of a transferred embryo. This disruption may have been due to introduced steric hindrance within the mouse uterus. In other words, while the inserted molecules may not have caused a specific repulsion of the embryos, their presence within in the EEC membrane may simply have blocked the access of the embryo for implantation

Further investigations with other novel FSL constructs, mimicking proteins or carbohydrates such as MUC-1 or LIF may provide further evidence of fundamentally important attachment molecules of the uterus. Attachment molecules recognised in the literature, such as LIF or cell surface integrins such as L-selectin, or $\alpha v\beta 3$ components may represent useful candidates of target molecules in the preparation of a new generation of synthetic molecule constructs. As LIF is soluble, and secreted from the uterine epithelium into the uterine cavity (L. Salamonsen, personal communication, 2009), LIFR may be a more appropriate molecule to be used in conjunction with KODE molecules to transform cell membranes of the uterine epithelium. Cell signalling molecules such as cytokines may be considered to be useful candidates in order to manipulate the uterine environment and steer tolerance/intolerance to an

embryo. Investigation of a specific sub-fertile mouse model treated with new molecules may help to confirm the importance of such molecules for implantation. While the trans-vaginal technique may be disruptive to the uterine mucous, also called the glycocalyx, this may in practice prove useful for IVF application. Large molecular components of uterine mucous, such as MUC-1 are thought to hinder access of the embryo to the endometrium leading to a consequential loss of receptivity (Aplin, 1997; Aplin & Kimber, 2004). The transvaginal uterine lavage may help to clear this mucous. This mucous, whilst normal at certain phases of the menstrual/estrus cycle, may be considered inhibitory to receptivity of the endometrium to an embryo during the window of receptivity (Aplin, 1997; Aplin & Kimber, 2004).

3.1.4 Screening

Screening of endometrial epithelial cells for two potential attachment molecules was carried out to determine the presence of these molecules on sub-adherent versus adherent cells. This information would provide an indication of the importance of the molecules for attachment, and determine whether their binding partner would be useful when attached to an FSL glycolipid construct for increasing attachment efficiency of sub-adherent cells. Screening results indicated that the surface cell expression of hyaluronic acid (HA) was heightened on day 4.5 of pseudo-pregnancy, the established window of receptivity for embryo implantation in the mouse (Afify et al., 2005). This heightened expression, consistent with reports in literature regarding expression of HA in the human endometrium (Afify et al., 2005) was not observed at day 2.5. Elevated expression was still observed by day 6.5 of pseudo-pregnancy. Due to the presence of different isotypes of hyaluronic acid observed, complications in the measurement of hyaluronic acid may be encountered especially with respect to antibody recognition using immuno-histochemical techniques. In these assays, the choice of antibody was based on an isotype of HA particular to the uterus and associated with embryos of the mouse, (Williams, 2008). Hyaluronic Acid (HA) and CD44 are influential in the attachment of embryos to endometrial epithelial cells (Afify et al., 2005). These molecules may represent potential candidates to be conjugated to a synthetic FSL construct to increase attachment and implantation of embryos to EECs.

Indications from screening assays using immuno-histochemistry are that HA appears to be expressed at a higher level in adherent cells compared with non-adherent cells. This supports the hypothesis that the increased presentation of these molecules at the cell surface by modification with KODE™ molecules may enhance embryo attachment.

Challenges encountered in the detection of KODE™ modification of cultured EECs were due to the phenomenon of cultured cells exhibiting varied morphologies when in grown in monolayers. In suspension, cultured cells tend to round up, and the cell membrane may becomes condensed, which may then lead to an interpretation of heightened molecular expression compared with that seen in situ. As the cells in situ are organised into functional areas and exhibit a range of morphologies, it is considered that monolayers are a closer approximation to the in vivo setting than the rounded cell morphology seen in suspensions. Staining monolayers, whilst relatively easy and relevant to the in vivo setting, may introduce an element of variability to the staining procedure. Since different cell types exhibit significant variation in cellular morphology and size, consistent and comparable staining may be most effectively achieved by using cell suspensions. Since all cultured cells round up in suspension irrespective of their size, staining cells when they have this morphology may circumvent certain artefacts introduced into the staining/detection technique using monolayered cells.

The detection of KODE™ modified, in vivo lavage treated uterine horns of the mouse involved immuno-histochemical techniques. Many of the detection antibodies used in immuno-histochemistry utilise monoclonal antibodies which are raised in mice. This posed a challenge for the detection of successful molecule insertion in vivo. Secondary antibodies contain a reporter dye, in this case, Fluoroscein isothiocyanate (FITC), so that molecular expression may be visually measured. Secondary antibodies must be raised in a species other than that of the primary antibody. If a primary antibody raised in a mouse was utilised, an anti-mouse secondary antibody would have to be used to recognise the first antibody. Clearly this poses a problem as any anti-mouse antibody will bind to all murine tissue. Binding of the secondary antibody to the sectioned uterine horns will obscure any specific binding to the primary antibody.

The use of the in vivo mouse model therefore required a primary antibody which was raised in a non-murine species. Such antibodies are not only much harder to source, but due to not being monoclonal in nature, may not be as specific. Furthermore, while FSL A was a useful proof of principle molecule, FSL B could not be used to compare insertion as it was in the in vitro assays. This was due to the galili antigen which is ubiquitously expressed by non-primate species, and is closely related to the human blood antigen, group B (Macher & Galili, 2008). Detection antibodies directed towards B antigens for detection of FSL B modification are cross reactive to the galili naturally expressed in murine tissue, also masking detection of FSL B modification in the mouse model.

Immuno-histochemistry staining of uterine tissue from biopsies ex vivo, or in vitro cultured uterine cells, is an important tool for the screening of tissue for natural molecular expression. It is also an efficient method for confirming molecular insertion of synthetic KODE™ molecules. The use of detection and reporter antibodies is not without its limitations. Detection would be enhanced by the use of other techniques such as electron microscopy to visualise insertion. A measure for the modification of cell attachment characteristics in vitro is required to demonstrate whether molecular insertion causes a change to cell behaviour.

3.1.5 Attachment Assays

Trophoblast cells of an embryo are responsible for making the initial attachment to epithelial cells of the endometrium which leads to successful implantation of the embryo. Commercially available trophoblast cells of human origin were specifically cultured to produce small clusters of cells in spherical balls which would model the embryo. Attachment assays were established to measure the attachment of these trophoblast spheroids to monolayers of cultured endometrial epithelial cells of human origin. This assay represented an in vitro model of embryo attachment to the endometrium of the uterus in vivo.

Correlating with published data of adherent endometrial RL-95 cell attachment characteristics towards trophoblast cells (Hannan et al., 2009; Heneweer et al., 2002; Hohn & Denker, 2002; Hohn et al., 2000; Thie et al., 1998), these cells consistently exhibited attachment potentials of 90-100%. The sub-adherent

HEC-1-A cells on the other hand demonstrated attachment potentials of not more than 40%, however these cells also exhibited a much higher variation in attachment potentials. Initially, treatment of the two types of endometrial epithelial cells with the proof of principle molecule FSL A prior to the attachment assay sought to determine whether there was any negative effect of the molecules on attachment, prior to considering potential molecules which may be useful for a positive effect. Two approaches were taken when measuring the influence of the KODE™ molecules. First, the practice of co-culturing the monolayers with trophoblast spheroids, was undertaken without the removal of the KODE™ molecule from the media. This ensured that conditions remained as close as possible to that experienced by an embryo in a uterus which had previously been treated by lavage with these constructs. It is considered likely that the KODE™ treated uterus would retain residual molecules, exposing the embryo to modifying molecules in utero. Secondly, attachment assays with the co-culture of trophoblast BeWo spheroids were performed in treated monolayers in which the KODE™ molecule was removed and replaced with fresh media. This approach ensured that any effect of the KODE™ molecule was due to the modification of the endometrial monolayers alone, and not confounded with potential influences on the spheroid or purely an influence of excess molecule in the media.

Endometrial epithelial cells inserted with FSL A molecules exhibited a decrease in attachment potential towards trophoblast spheroids. This effect, observed in treated monolayers that were washed, was amplified when excess KODE™ molecules remained in attachment assay media. This pattern was reflected with FSL B treated EEC monolayers, albeit to a lesser degree. In this investigation, there is not enough data to prove the effect on attachment of endometrial epithelial cells was due specifically to the functional head. To resolve this question, un-related FSL molecules with different functional head groups may be assayed to determine whether there is an effect on attachment efficiency caused by other head groups. Also, a lipid tail with a spacer may be assayed, however due to problems with charge and solubility, this may not prove viable. The FSL B molecule carries the blood group B antigen as the functional head group in place of the blood group A antigen of FSL A, conjugated to the lipid tail and spacer. The mechanism for the decrease in

attachment potential seen in modified cells treated with FSL A and FSL B remains to be elucidated. The difference of these molecules is essentially due to one residue of the molecule being an N-acetyl-galactosamine in blood group B in the place of the galactose residue of blood group A. Conformational differences between the two molecules may influence how they pack together and some may simply be bulky as a result which may cause steric hindrance in the uterine setting, hampering access of the embryo to the endometrium. Subtle changes to the head group of the modifying molecule may allow tailoring of cell characteristics accordingly.

Interestingly, both FSL A and FSL B treated monolayers which had media changed prior to spheroid attachment, exhibited increased attachment compared with monolayers in which KODE™ molecule was maintained. Intriguingly, the media changed, FSL A treated monolayers, did not return to the levels exhibited by non treated cells. This difference may be due to the presence of the molecules free within the media in the unchanged media assays. The excess molecules within the media could potentially influence the attaching spheroid. The resulting modification of the spheroid or embryo within the attachment environment may mask their own surface attachment molecules and interfere with attachment. Excess molecules within the attachment media may cause crowding on the cell surface causing some molecules to inappropriately associate with the plasma membrane and not properly insert. Antigen presentation will thus be altered on the cell surface and may render them unable to be recognised by their receptor/ligand.

The difference exhibited in FSL A treated cells with changed versus unchanged media is considered to be due to insertion of the FSL A molecule into the endometrial epithelial cell membrane which affected attachment efficiency in these cells by an unknown mechanism. It remains to be explained why the FSL B molecule appeared to be easily removed from the target cells when changing the attachment media, whilst FSL A continued to exert an effect on attachment efficiency. It may be that the configuration of the FSL B antigen attached to the bilipid tail does not allow easy access for appropriate insertion to occur. Electron microscopy may help to unravel the insertion characteristics at

the molecular level in order to unequivocally determine plasma membrane insertion as opposed to mere adherence of molecules to the cell membrane.

These assays are designed to be a simplified, in vitro representation of the attachment and implantation of an embryo into the endometrium. Trophoblastic cells were grown as spheroids, a collection of cells in round balls, in order to mimic embryos. These balls were typically up to 400µm diameter while an implanting blastocyst is around 80µm. The larger size provided a larger surface area together with increased weight, which may increase the attachment potential of spheroids compared to embryos. However, spheroids were still considered a good in vitro model of embryos for several reasons: This larger size, a function of incubation time, gyration and growth conditions, made the in vitro handling of spheroids simpler compared with embryos. Spheroids were also easily visualised under the dissection microscope. Furthermore, the organisation of the trophoblast cells into a spheroid configuration conserves the polarised morphology of the cells (Heneweer et al., 2002), observed in embryonic trophoblast cells. An embryo exhibiting polarised trophoblast cells on its surface must undergo apposition/orientation prior to the initial attachment and this apposition is influenced by the cell's polarity.

The attachment potential of monolayers of EECs could be considered to be semi-quantitative as it is a measurement of the combined force of many discrete points of attachment of many cells of the attaching spheroid. The age of the cells making up the spheroid may influence the expression of attachment molecules, which in turn influences attachment of that single spheroid. Variability may occur between spheroids due to surface cells of the spheroid and their molecular expression. Parameters such as the age and passage number of the EECs, or BeWo spheroids may affect this assay, and this should be kept in mind when measuring and drawing conclusions from the effects of KODE™ molecule treatment on cell attachment. These assays always utilised monolayers seeded with consistent cell numbers, grown for a consistent length of time. The attachment time of spheroids was also strictly adhered to for all assays. Monolayer confluence, which was dependant on seeding concentration, incubation and media conditions, was critical. Gaps in the monolayers expose the surface of plastic culture vessels, which attracts cells

and may lead to a falsely high interpretation of attachment. A consistent rate of gyration of trophoblast cells also had a real impact on regular spheroid size and morphology, important for consistency of contacts between trophoblast cells and the EEC monolayer. Media pH, temperature, atmosphere, particularly CO_2 concentration and osmolarity all affect the optimal growth of cultured cells and levels of all these parameters were monitored to prevent fluctuations influencing the cell behaviour.

3.2 Future Applications

For the purposes of strengthening evidence for the potency of KODE™, a continuation of this investigation would be beneficial to answer unsolved questions and to realise its potential with recognised cell attachment molecules. This study investigated only benign FSL molecules in the context of proving the principle of the technology for application in the uterine environment. Further investigation into safety in vitro by studying cell growth characteristics closely may provide evidence of safety not just at the gross level, but at the cellular level, and may provide clues into the cellular metabolism of these molecules, and the kinetics of such metabolism. To begin, these studies could simply involve evaluation of cell counts after exposure to the synthetic constructs and observation of the morphology of cells. It would be relevant to observe the incorporation of different bilipid tails with respect to retention in endometrial epithelial cells both in vitro and in vivo. Different cell types exhibit different modification characteristics and the high turn-over of these epithelial cells may mean they would benefit from having optimised retention of modifying molecules. The customisation of KODE™ constructs with well recognised and documented molecules important in embryo-endometrium dialogue, cell signalling and attachment and the use of these in a sub-fertile molecule would confirm the benefits of modification using KODE™ with respect to enhancing implantation. Furthermore, increased exhibition of specific, targeted molecules at precise concentrations may be examined to ascertain the importance of such molecules to implantation, without affecting other molecular attachment influencing attachment in vivo. An example of an interesting molecule to investigate would be an FSL-LIFR molecule which may enhance attachment of an embryo to the endometrium. LIFR is part of the signalling receptor which binds LIF ligand when dimered with gp130 and transduces the signalling

receptor resulting in changes to gene expression, (L Salamonsen, personal communication, 2009). The use of the delivery of custom engineered KODE™ molecules via the in vivo transvaginal lavage may be useful with sub-fertile gene knockout mice. An investigation of treatment of both the embryo and the endometrium with corresponding receptor-ligand molecules would specifically target attachment, ensuring both entities exhibit recognition molecules. Alternatively, treatment and modification of both embryo and endometrium with the same modification molecule may drive attachment via an introduced bridging molecule which would recognise the modifying molecule present on both the embryo and the endometrium. Clues for potential molecules which may be beneficial to add to sub-fertile mice and sub-adherent cells may be obtained from more extensive screening assays of fertile tissue and optimally adherent cultured cells.

The ability to deliver a liquid to the endometrium in order to modify conditions of the uterus in vivo may have a number of potential applications. Whether the fluid is soluble or an emulsion and whether it targets cell membrane modification or is simply becomes a beneficial player in the general uterine milieu, delivery of fluid directly to the uterine lumen of the mouse by the relatively non-invasive technique of transvaginal lavage has the potential to be very useful with respect to in vivo research into the value of putative fertility enhancement molecules. With respect to lavage treatment with KODE™ technology, and the development of new synthetic molecules allowing peptides to be conjugated to bilipid tails, many attachment and recognition molecules may be added to the surface of cells to specifically engineer an array of cell surface characteristics.

3.2.1 Research Models

The power of KODE™ technology for the application of research is threefold: i) customised modification FSL molecules can be synthesised with head groups of complete or functional portions of carbohydrate or protein recognition molecules, ii) the ability to modify tissue in vivo through the administration of KODE™ molecules to the appropriate tissue, iii) tailoring of specific levels of expression of target molecules to appropriate levels by variation of treatment molecule concentration. Specific attachment molecules may be used to

synthesise novel constructs, designed to modify endometrial epithelial cells in a controlled manner in vivo. Using the transvaginal lavage procedure, non-receptive cells may be manipulated to become more receptive to an embryo, or alternatively, cells with an undesirable adherence potential, may be manipulated to become less adherent.

Using a transvaginal or other lavage-like procedure for molecule delivery, the interaction between any two cell types which may be easily accessed may be manipulated in vivo. There are two approaches to making such specific changes to cell surface expression: biological and chemical. Biological methods include such techniques as genetically modified organisms which are engineered to produce specific cell characteristics. These methods require the cell to express the desired modification of its own. This process can be inefficient and labour intensive and often requires complex ethical and regulatory approval. By chemically synthesising customised molecules for cell modification, the process is far more targeted, more efficient and does not involve the high degree of regulation involved with genetically modified organisms. Revolutionary bio-engineering of cells may be achieved with KODE™ technology without the constraints of efficiency and ethics associated with alternative methods for in vivo manipulation at the cellular level. While this technology is time bound, in that modification is temporary in nature, another advantage of using technology such as KODE™ is that, as an alternative to genetic modification, this technology may be much more widely accepted, as it does not affect the germ line. Modification may be highly specific and precisely targeted, without introducing novel genetic entities, which receives widespread scepticism in the general population.

The introduction of molecules capable of modifying the endometrial epithelial cells via a non-invasive delivery of liquid directly to the uterine environment in vivo provides a strategic weapon in the arsenal to combat infertility. Not only may a wide variety of molecules be isolated, purified and engineered to be expressed on EEC surfaces with the use of KODE™ technology, but the developed lavage technique may also be employed to administer any number of agents directly, efficiently and non-surgically to the uterine lumen in the mouse. Valuable in vivo information may be obtained with respect to any uterine aspect,

which may pave the way for further investigations of potentially useful molecules in clinical trials.

3.2.2 Improvement to Current IVF Practice

A diagnosis of unexplained infertility accounts for around 10% of all couples presenting to clinicians with fertility problems. This sub-set includes those with problems associated with implantation. In many of these cases the problem may be with the endometrium being refractory to embryonic attachment (Boomsma et al., 2009; OBGYN., 2009). In these instances the embryo transferred in an IVF setting, may be completely healthy but not able to adhere effectively for implantation to occur. Obviously two entities are involved in embryo-endometrium attachment and subsequent implantation. Few researchers have targeted the endometrium and attempted to modify it to optimise conditions suitable for embryo attachment. It has taken a long while to start to finally identify critical players with respect to molecules and cell types essential for implantation. One of the difficulties is that there exists a lot of redundancy in terms of functional properties, which makes it difficult to categorically specify essential molecules which can not be substituted. This makes it challenging for selection of a candidate molecule to be incorporated into a novel KODE™ molecule. It may be possible in the future to modify cells with a combination of potentially useful molecules for enhanced attachment.

Historically it was common for multiple embryos to be transferred in any attempt at embryo transfer during IVF (ESHRE, 2009; Illingworth, 2008; OBGYN, 2009). Each extra embryo was treated as an insurance policy in case something went wrong with the first transferred embryo, such as an inexplicable failure to implant. There is a growing concern regarding the practice of multiple embryo transfer due to the heightened risks of pregnancy complications seen with multiple pregnancies especially when numbers above 2 or 3 (ESHRE, 2009; Illingworth, 2008) are transferred. In many parts of the world it is now widely regarded as a rather irresponsible way to achieve pregnancy due to risks introduced to both embryos and mother (Illingworth, 2008). By targeting the potential causes of unexplained infertility, such as implantation, the temptation to transfer multiple embryos may diminish.

As a more complete understanding of the essential components that are requisite for attachment and implantation emerges, specific molecules which have been unequivocally identified as necessary attachment molecules may also be identified as candidate functional heads for novel KODE™ constructs. It is well recognised that high estrogen exposure, typical during hyper-stimulation of IVF cycles, leads to an advanced endometrium which is not conducive to optimal attachment of an embryo (Delvigne, 2009; Kramer et al., 1993). With the common IVF practice of transferring blastocysts stage embryos to the uterus, the endometrium may be delayed even longer. KODE™ lavage may be a novel way to address the potential problems of disrupted synchrony of the endometrium and the embryo. Treatment with modification molecules may ensure that the endometrium receives feedback that would naturally be embryo derived. A delivery of KODE™ molecules, communicating to the uterus that an embryo is present, could be repeatedly administered in a booster like fashion prior to embryo transfer. In this manner, the embryo-endometrium dialogue may be mimicked, with the message articulated to the endometrium that an embryo would soon be ready for attachment.

It is conceivable that in the future a customised approach may be taken to assist reproduction for individuals at the IVF clinic. Uterine conditions may be sampled and monitored by performing a cervico-vaginal lavage and measuring components of the flushed out lavage fluid. A cocktail of KODE™ modifying agents may then be tailored and administered prior to or along with an embryo transfer. In this manner, specific hindrances to implantation may be estimated, targeted and specifically remedied. Used in conjunction with pre-implantation genetic screening or metabolomics, inadequacies of both the embryo and the endometrium may be addressed (Botros et al., 2008; Jones et al., 2008)

3.2.3 Potential for the Clinical Setting

This investigation set out to prove the principle of KODE™ modification in endometrial epithelial cells, using both human derived cells with in vitro assays, and using the in vivo mouse model to modify cells lining the uterus in situ. The results obtained demonstrated an unexpected alteration to the cells' adhesive character. Cells treated with FSL A and FSL B proof of principle molecules, containing the benign head-groups of A and B human blood group antigens,

showed a reduced adherent capability. This alteration suggests that not only may endometrial epithelial cells be targeted using KODE™ technology in order to enhance attachment potential for fertility purposes, but that these cells may also be targeted specifically in order to bring about a reduced attachment potential.

KODE™ molecules were administered directly to the uterus by transvaginal lavage in the mouse model, in order to treat endometrial epithelial cells with the modifying molecules. If the analogous method was adopted in the human, by administering molecules directly to the uterus using standardised equipment and techniques usually employed for IVF purposes, human endometrial epithelial cells may also be targeted to reduce uterine receptivity of an embryo. Indications of KODE™ modification of endometrial epithelial cells in situ have shown that this technique, and the molecules themselves are well tolerated, safe and modification is temporary. Such contraceptive purposes in the clinical setting may be useful for patients in which imminent pregnancy would be undesirable. Achieving a contraceptive effect without the use of exogenously administered hormones may be desirable in certain clinical situations. For example, after dilation and curettage treatment of women for biopsy purposes the treatment of endometrial cells with KODE™ molecules to bring about a contraceptive effect may be useful. Such non-hormonal treatment may discourage embryo implantation, while allowing the monitoring of naturally expressed hormones and other uterine components. Inhospitable conditions for sperm survival may also be targeted to hinder sperm access to the fallopian tubes for fertilisation of the oocyte.

Another clinical scenario which would benefit from a reduction in adhesive potential of endometrial epithelial cells would be the treatment of endometriosis. Endometriosis is associated with infertility complications, and affects about 10% of women of reproductive age (Ilad et al., 2004). One theory regarding the cause of endometriosis is retrograde menstruation whereby endometrial cells which are sloughed off and usually expelled with menstrual blood, flow backwards up the fallopian tubes and into the peritoneal cavity (Ilad et al., 2004). These cells then attach to tissue in other abdominal areas such as the outside of the uterus, the ovaries, fallopian tubes and many other surfaces

inappropriately. As with stromal cells of the endometrium, the inappropriately attached cells are subject to the hormonal influence of the regular menstrual cycle, causing these sites to bleed with the menstrual cycle. If it is possible to alter the adherent potential of sloughed endometrial epithelial cells temporarily at specific points of the menstrual cycle, there may be potential to use KODE™ technology as a therapeutic agent to treat endometriosis. Treatment could be administered after routine operations to remove endometrial plaques inside the peritoneum to discourage further attachment.

Further investigation of the use of FSL technology targeting a reduction in adhesive potential of endometrial epithelial cells relating to both the contraception context and disease therapy is certainly warranted.

This investigation set out to determine the applicability of KODE™ technology to the uterine environment. The aim was to explore the possibility of modifying the antigenic presentation of the cells lining the uterus using KODE™ technology. The proof of principle molecule, FSL A was chosen for reasons of availability and the extensive experience of others within the research facility with this molecule. Results showed that endometrial epithelial cells were easily modified both in vitro and in situ in the mouse model. This modification lasted approximately 2 hours after cells no longer had contact with the solution containing the modifying molecules. Ultimately, if modification of endometrial epithelial cells (EECs) could be achieved, specific attachment molecules would then be investigated for the potential synthesis of constructs which may be useful to enhance the attraction of the endometrium for an embryo. Unexpectedly, this proof of principle molecule also altered adherence characteristics of treated cells, rendering usually highly adherent cells, sub-adherent. This change in cell character was observed not only with in vitro cell treatment, but even after administration in vivo. Modification of EECs with a molecule carrying a structurally similar functional group, FSL B, indicated that the change in cell characteristics observed was specific to the dissolved FSL construct in solution. The variation of effect caused by the FSL A and FSL B molecules was not explained, as both molecules are considered to be benign. With continued development of KODE™ technology, future constructs may be engineered using a wide variety of functional groups, including proteins.

The in situ manipulation of the uterine environment may lead to cell signalling, immuno-tolerance and attachment molecules to be synthesised for use in an environment where glycolipids are commonplace and insertion into cells is easily achieved. Cell characteristics were easily modified in situ using a proof of principle molecule administered to the murine uterus via a novel transvaginal lavage procedure. The introduction of custom made constructs, carrying powerful recognition, signalling or attachment molecules into the endometrial epithelial cells in situ, has the potential to truly impact the field of IVF and beyond.

4 Appendix: Experimental Protocols

4.1 Inserting Functional Spacer Lipid (FSL) A into Endometrial Cells

OBJECTIVE

Treat cultured endometrial epithelial cells with a basic Functional Spacer Lipid (FSL) in order to modify cell membrane expression.

REAGENTS AND EQUIPMENT

Reagents

- Monolayers of Endometrial Epithelial Cells RL-95, HEC-1-A or AN3-CA (Protocol 4.9)
- Endometrial Epithelial Cell Maintenance Media (Protocol 4.9)
- FSL A molecule at 1mg/mL
- Formaldehyde fixative at 3.7% (BDH Cat # 101136C)
- Anti A Bioclone monoclonal IgM DEV 01102 (Ortho-Clinical Diagnostics # 606-20-954-5)
- PBS
- Secondary Antibody anti-mouse Ig-FITC (Chemicon # 985031020 AP 326F) at 1/50 dilution

Equipment

- Poly-L lysine coated cover slips (Protocol 4.8)
- Sterile 24 Well culture plates (Greiner BioOne # 662160)/ Micro test (Terasaki) plates (Greiner BioOne 60 #GR 659180)
- Olympus BX 51 Fluorescent Microscope with WIB fluorescent filter
- Microscope camera (Optronics Magnafire fitted with UPM-TVC/ USPT lens)
- 37°C Incubator with 5%CO_2 atmosphere (Binder E045.06.984)
- Bench top pipettes 1000μL and 200 μL (Eppendorf)

PROCEDURE

Insertion

- Place pre-prepared Poly-L-lysine coated cover slips into all wells of a sterile 24 well tissue culture plate to be used for cell growth.
- Seed cell suspensions of endometrial epithelial cells to be treated into wells of the 24 well plate containing cover slips at an appropriate seeding concentration. RL-95 cells should be seeded to give an initial density of approx 1.6 x 10^5 cell/cm_2 and cultured for 1-2 days at 37°C with 5%CO_2.
- Check that cells are healthy, pH is neutral – indicated by a medium which is orange-pink, media is clear, not turbid and that confluence is appropriate for the required assay.
- Gently remove cell culture media by aspirating off gently using a 1 mL bench top pipette.
- Replace media on cells to be inserted with an appropriate concentration of FSL A prepared in the growth media appropriate for the cell type being inserted. Insertion media should contain no FBS, as this may interfere with the insertion process of the FSL A molecule.
- Incubate the plate with insertion media at 37°C with 5%CO_2 atmosphere for at least 2 hours.

- Remove plates from the incubator, and wash the monolayers gently using a sterile 1mL pipette charged with sterile PBS. If the cells are to be cultured on for a retention investigation, the PBS should be pre-warmed.
- Monolayers must either be fixed prior to staining for A antigen detection, or placed in pre-warmed culture media for any subsequent retention investigations. If retention is being investigated, monolayers are returned to culture conditions without delay.

VARIATIONS

Variation 1

Insertion into monolayered cells on cover slips in 24 well culture plate

- Seed RL-95 cells into 24 well culture plates containing poly-L-lysine coated cover slips to give a density of 1.6×10^5 cell/ cm_2. This requires 1 mL of cell suspension at $1.8x10^5$ cell/ mL. Grow overnight at 37°C with a 5% CO_2 atmosphere.
- Add FSL A at a concentration of 0.5 mg/ mL and incubate at 37°C with 5% CO_2 for approximately 18 hours.
- A screen of remaining antigen is carried out 2 hours after removal of FSL A.

Variation 2

Insertion into two day monolayered cells in Terasaki plates

- Seed RL-95, and AN3-CA cells into Terasaki plates to a density of $7.7X10^5$ cell/ cm_2 and $3.8x10^5$ cell/ cm_2 respectively, in a volume of 20μL. Incubate cells for two days at 37°C with an atmosphere of 5% CO_2. Seed HEC-1-A to give a density of $5.2x10^5$ cell/mL in a volume of 20μL. Incubate HEC-1-A cells for approximately 18 hours under the same conditions as the previous cells.
- Add FSL A or FSL B to wells containing monolayered endometrial cells at concentrations prepared at 0.1 mg/ mL, 0.5 mg/ mL, 1.0 mg/ mL and 2.0 mg/ mL.
- Incubate Terasaki plates containing cells for 4 hours at 37°C with 5%CO_2.
- Add 20 μL primary antibody, Bioclone A or Bioclone B undiluted. Incubate with cell monolayers for 60 minutes at room temperature. Blank wells should be included with 2% BSA diluent used as controls.
- Keep cells away from light exposure. Add 20 μL secondary antibody, anti-mouse IgFITC at 1/50 dilution in 2% BSA Incubate plates for 45 minutes at room temperature in a dark box.
- Wash cells gently three times using a transfer pipette containing PBS. Place 10 μL PBS into wells prior to viewing.
- Remove the Terasaki lid and invert Terasaki plate. View wells under WIB fluorescence of Olympus BX51 fluorescent microscope

Variation 3

Insertion into one day monolayered cells in Terasaki plates

- Seed RL-95 cells into Terasaki plates to a density or $1.3x10^6$ cell/ cm_2. Incubate overnight to ensure cells adhere to the plastic culture tray.
- Add 20μL FSL A at a concentration of 1 mg/ mL prepared with RL-95 growth media as the diluent to wells to be treated. Ensure diluent does not contain the FBS used as a growth supplement.
- Incubate cells with FSL A for 2 hours at 37°C with a 5%CO_2 atmosphere.
- Add 20μL primary antibody, Bioclone A, undiluted to wells for staining. Diluent only (2% BSA) blank wells should be included as controls. Incubate wells for 1 hour at 37°C for 1hour
- Add 20μL secondary antibody, anti-mouse IgFITC at 1/50 dilution to all wells and incubate at room temperature in a humidified dark box for 30 minutes.
- Prepare and view wells as per variation 2.

Variation 4

Retention Investigation

- Carry out insertion assay as per variation 3 in Terasaki plate.
- Remove insertion media gently, and wash three times with pre-warmed sterile PBS.
- Replace insertion media with appropriate pre-warmed cell culture media

- Replace Terasaki trays into incubator at 37°C with a 5% CO_2 atmosphere for the length of time required to test retention.
- Carry out detection step as per 4.2

4.2 Screening Endometrial Cells for Antigen A and B

OBJECTIVE

To detect expression of endogenous and synthetic, FSL-modified A and B blood group antigens at the cell surface of endometrial epithelial cells

REAGENTS AND EQUIPMENT

Reagents

- Monolayers of Endometrial Epithelial cells RL-95, HEC-1-A or AN3-CA (Protocol 4.9)
- Endometrial Epithelial Cell maintenance media (Protocol 4.9)
- Formaldehyde fixative at 3.7% (BDH Cat # 101136C)
- Anti A Bioclone monoclonal IgM DEV 01102 (Ortho-Clinical Diagnostics
- # 606-20-954-5)
- Anti b Bioclone monoclonal IgM DEV 01103 (Ortho-Clinical Diagnostics
- # 606-20-956-5)
- PBS
- Secondary Antibody anti-mouse Ig-FITC (Chemicon # 985031020 AP 326F) at 1/50 dilution with 2% BSA in PBS
- BSA at 2% concentration in PBS (Gibco # 30063-573)

Equipment

- Olympus BX 51 Fluorescent Microscope with WIB Fluorescent filter
- Microscope camera (Optronics Magnafire fitted with UPM-TVC/ USPT lens)
- 37°C Incubator with 5%CO_2 atmosphere (Binder E045.06.984)
- Bench tip pipettes1000μL and 200μL (Eppendorf)
- Forceps

PROCEDURE

- Gently remove media from wells of 24 well plate containing endometrial epithelial cells on cover slips using a sterile 1000μL bench-top pipette.
- Add 200μL 3.7% Formaldehyde solution to each well using 200μL pipette and fix for 10 minutes at room temperature.
- Remove fixative using 1000μL bench-top pipette, and wash cells, three times with PBS, using a 1000μL bench-top pipette loaded with 1mL PBS. Transfer cover slips into dry wells using forceps.
- Add 50μL undiluted primary antibody, anti-A Bioclone to surface of cover slips. Incubate cover slips in wells at room temperature for 30 minutes.
- Wash cells as previously, three times using PBS. Transfer cover slips to new dry wells.
- Turn on mercury burner of Olympus fluorescent microscope BX-51 to allow the lamp to warm up before viewing.
- Add 50μL secondary antibody, anti-mouse Ig-FITC at 1/50 to the surface of cover slips, and incubate in a humidified dark box at room temperature for 30 minutes. Variations may be incorporated to both temperature and time of this incubation.
- Taking care to minimise light exposure, wash cells three times with PBS as previously performed.
- Invert cover slips, and mount onto clean dry microscope slides. Label slides with treatment of cells and staining procedure.
- View cells with Olympus BX-51 fluorescent microscope under WIB filter (emission wavelength of 550nm) at 200 x magnification. Record images at an exposure time of approx 1.9s exposure.

- Grade fluorescent intensity at 1.9s exposure using a scale of 0-3 (Protocol 4.3). A grade of 0 representative of no fluorescence, with cells invisible under WIB fluorescent filter, and 3+ representative of very high fluorescence.

VARIATIONS

Variation 1

Detection of A antigen and B antigen using anti-A or anti-B Bioclone in Terasaki plates

- Add 20µL fixative to cells layered in Terasaki plates and fix cells at room temperature for 20 minutes.
- Remove fixative using a 200µL bench-top pipette. Wash cells by gently adding a drop of PBS per well with a transfer pipette, and remove this liquid carefully with 200µL pipette. Repeat this process three times. Blot plate with paper towels to remove excess liquid from wells.
- Add 20µL primary antibody, anti-A Bioclone, or anti-B Bioclone undiluted to cells to be stained. Add 2% BSA in PBS as a block buffer for non-primary antibody controls. Incubate plates at room temperature for 60 minutes.
- Remove primary antibody carefully with a 200µL bench-top pipette, and wash wells three times with PBS as above.
- Add 20µL secondary antibody, anti-mouse IgFITC at 1/50 dilution to all wells, taking care to minimise light exposure. Incubate plate in a humidified dark box at room temperature for 45 minutes.
- Remove secondary antibody, and wash wells three times with PBS as above.
- Place 10µL PBS into all wells for viewing wells under fluorescent microscope. Remove lid and invert Terasaki plate. View plate with fluorescent Olympus BX-51 microscope under WIB filter (emission wavelength 550nm) at 1.9s exposure.
- Grade fluorescent intensity as above.

Variation 2

Detection of A antigen using anti-B Bioclone

- Add 20 µL 3.7% Formaldehyde fixative to cells monolayered in Terasaki plates. Fix cells at room temperature for 10 minutes.
- Remove fixative gently using a 200µL bench-top pipette and wash wells three times with PBS as above. Blot plates dry with paper towel to remove excess liquid.
- Add 20µL undiluted primary antibody, anti-A Bioclone to wells to be stained. Add 2% BSA to wells for non-primary antibody controls. Incubate plates at 37°C for 1 hour on an orbital shaker at 130 rpm.
- Remove primary antibody carefully using a 200µL bench-top pipette and wash wells three times with PBS as above, and blot dry.
- Add 20 µL secondary antibody, anti-mouse Ig-FITC at 1/50 dilution to all wells, taking care to minimise light exposure. Incubate plates in a humidified dark box at room temperature for 30 minutes.
- Remove secondary antibody carefully with a 200µL bench-top pipette, and wash wells as above.
- Place 20µL PBS into all wells for viewing under fluorescent microscope. Remove Terasaki plate lid and invert plate. View wells with fluorescent Olympus BX-51 microscope under WIB filter (emission wavelength 550nm) at 1.9 s exposure.
- Grade fluorescent intensity as above.

4.3 Grading of Fluorescent Intensity

OBJECTIVE

To grade the intensity of the fluorescent signal of an image under WIB filter of Olympus BX-51 microscope. This grading will provide a semi-quantitative measurement of the molecular expression at the surface of cells using immuno-fluorescence assays.

REAGENTS AND EQUIPMENT

Equipment

- Olympus BX-51 Microscope with WIB filter (emission wavelength 550nm) fitted with DIC lens
- Microscope camera (Optronics Magnafire fitted with UPM-TVC/ USPT lens)
- Sections or cells stained with FITC or Alexa Fluor conjugated reporter molecule or antibody
- Dark box to store slides

PROCEDURE

- Turn on mercury burner, which provides UV light for fluorescence microscopy, at least 30 minutes prior to viewing cells or sections. The mercury lamp is not stable if turned off and on for periods of less than 30 minutes. Ensure the hours accumulated for the running of the mercury lamp total less than 200 hours.
- View stained cells or sections at 200x magnification using Olympus BX-51 microscope under DIC.
- Focus the image until a clear image of cells or sections is seen under DIC.
- Turn down the incident light, change the filter WIB and turn on the UV fluorescing light from the mercury burner.
- View images under the microscope, and adjust focus if necessary.
- Turn on the computer with camera software, and ensure the image is displayed on the screen correctly. Adjust the exposure time on the computer software to 1.903 seconds. This allows for inter-assay comparison of fluorescence grading.
- Take an picture at this exposure time and record the computer's external drive. Take a corresponding image under DIC of the fluorescent image.
- If further detail can be derived from a different exposure time than 1.903, take an image at this exposure time also, carefully noting the exposure time used.
- Grade the images by categorising the fluorescent signal from 0 to 3. A section or cells which are completely invisible under WIB fluorescence at 1.9 seconds exposure, is giving a grading of 0. A very bright green fluorescence observed in or on cells or sections at 1.9 seconds exposure is given a grading of 3+. Refer to table below for examples.
- To maintain inter-assay comparability, this grading must be carried out by the same technician due to the subjectivity of this semi-quantitative technique.

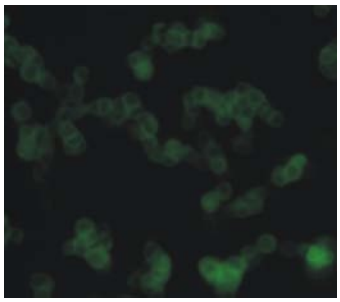
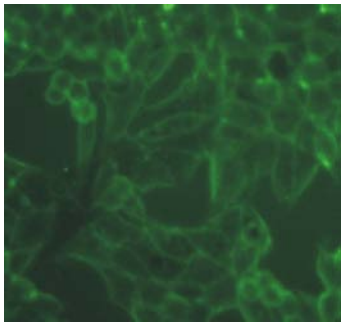
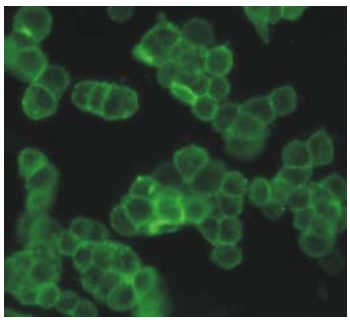
Grading	Photo Example
0	
1+	
2+	
3+	

Table 1: Fluorescent intensity gradings of cultured endometrial epithelial cells. Cells were stained with a FITC conjugated reporter antibody and viewed with Olympus BX51 microscope under WIB filter at 200× magnification. Images taken at 1.9 seconds exposure.

4.4 Detection of Hyaluronic Acid in Endometrial Epithelial Cells

OBJECTIVE

To detect and measure the endogenous expression of hyaluronic acid (HA) in three endometrial epithelial cell lines of varying adherence potential: RL-95, HEC-1-A and AN3-CA.

REAGENTS AND EQUIPMENT

Reagents

- Monolayers of endometrial epithelial cells, RL-95, HEC-1-A or AN3-CA (Protocol 4.9)
- Endometrial epithelial cell maintenance media (Protocol 4.9)
- Formaldehyde fixative at 3.7% (BDH Cat # 101136C)
- Phosphate buffered saline (PBS)
- BSA at 2% in PBS (Gibco 30063-573)
- Biotinylated Bovine HA binding protein (bHABP) at 2.5µg/ mL (Sigma H9910 0.05MG)
- Primary antibody, sheep anti-HA (Biogenesis 5029-9990) at 1/100 in 2% BSA
- Avidin Alexa Fluor (Molecular Probes A-21370, Lot 84C1-1) at 1/200 in 2% BSA
- Secondary antibody, Alexa Fluor conjugated donkey anti-sheep (Invitrogen A-11015) at 1/200 in 2% BSA

Equipment

- Olympus BX 51 Fluorescent Microscope with WIB Fluorescent filter
- 37°C Incubator with 5%CO_2 atmosphere (Binder E045.06.984)
- Bench top pipettes 1000µL and 200µl (Eppendorf)
- Forceps

PROCEDURE

- Gently remove media from wells of Terasaki plates containing monolayered RL-95 or HEC-1-A cells using 200µL bench-top pipette.
- Add 20µl 3.7% formaldehyde fixative to wells and fix cells at room temperature for 10 minutes.
- Gently remove fixative using a 200µL bench-top pipette. Wash wells gently three times with PBS using a transfer pipette, removing fluid from wells with 200µL bench-top pipette. Blot Terasaki plate using paper towels to remove excess liquid.
- Add 20µL primary antibody, sheep anti-HA at 1/100 dilution, to wells to be stained. Add diluent, 2% BSA to non-primary antibody control wells. Incubate plates in a humidified box at 37°C for one hour.
- Remove primary antibody using a 200µL pipette, and wash wells with PBS using a transfer pipette as above.
- Taking care to minimise light exposure, add 20µL secondary antibody, donkey anti-sheep at 1/200 dilution to all wells. Incubate plate in a humidified dark box at room temperature for 30 minutes.
- Remove secondary antibody and wash wells with PBS using a transfer pipette as above.
- Remove lid of Terasaki Plate and place 10µL PBS into all wells for viewing under fluorescent microscope. Invert Terasaki plate and view wells with Olympus BX-51 microscope under WIB filter (emission wavelength 550nm) at 1.9s exposure.

VARIATIONS

Variation 1

Detection of Hyaluronic Acid using biotinylated HA Binding Protein

- Remove media and fix cells as per above procedure
- Add 20µL biotinylated HA Binding Protein at 2.5µg/ mL to all wells to be stained. Add diluent, 2% BSA only to non-bHABP wells as blanks. Incubate plate in a humidified box at 37°C for one hour.
- Remove bHABP from wells gently using a 200µL bench-top pipette. Wash wells with PBS using a transfer pipette as per above procedure.
- Taking care to minimise light exposure, add 20µL Avidin Alexa Fluor at 1/200 dilution to all wells. Incubate plates in a humidified dark box at room temperature for 30 minutes.
- Remove secondary antibody carefully using a 200µL pipette. Wash wells with PBS using a transfer pipette as above.
- View wells of Terasaki plate with Olympus BX-51 microscope under WIB filter (emission wavelength 550nm) at exposure time of 1.9 seconds.

4.5 Detection of CD44 in Endometrial Epithelial Cells

OBJECTIVE

Detect endogenous expression of CD44 in three endometrial epithelial cell lines, of varying adherence potentials, RL-95, HEC-1-A and AN3-CA.

REAGENTS AND EQUIPMENT

Reagents

- Monolayers of endometrial epithelial cells, RL-95, HEC-1-A or AN3-CA (Protocol 4.9)
- Endometrial epithelial cell maintenance media (Protocol 4.9)
- Formaldehyde fixative at 3.7% (BDH Cat # 101136C)
- PBS
- BSA at 2% concentration in PBS (Gibco # 30063-573)
- Primary antibody, rat anti-CD44 at dilution of 1/20 in 2% BSA (BD Pharminogen Pgp-1 Ly-24 553131)
- Secondary antibody, Affinity purified FITC conjugated rabbit anti-rat at dilution of 1/50 in 2% BSA (Zymed Laboratories 61-9511)

Equipment

- Olympus BX 51 fluorescent microscope with WIB fluorescent filter
- 37°C Incubator with 5%CO_2 atmosphere (Binder E045.06.984)
- Bench top pipettes 1000μL and 200μL (Eppendorf)
- Forceps

PROCEDURE

- Gently remove media from wells of Terasaki plates containing mono-layered RL-95 or HEC-1-A cells using a 200μL bench-top pipette.
- Add 20μl 3.7% formaldehyde fixative to wells and fix at room temperature for 10 minutes.
- Gently remove fixative using a 200μL bench-top pipette. Wash wells three times with PBS using a transfer pipette and remove liquid gently with a 200μL bench-top pipette. Blot plate using paper towels to remove excess liquid.
- Add 20μL primary antibody, rat anti-CD44 at 1/20 dilution to wells to be stained, and 20μL 2% BSA to non-primary antibody control wells. Incubate plate in humidified box at 37°C for 2 hours.
- Gently remove primary antibody using a 200μL pipette. Wash wells with PBS using a transfer pipette as above.
- Taking care to minimise light exposure, add 20μL secondary antibody, FITC conjugated rabbit anti-rat at 1/50 dilution to all wells. Incubate plate in a humidified dark box at room temperature for 30 minutes.
- Gently remove secondary antibody using a 200μL bench-top pipette and wash wells with PBS using a transfer pipette as above.
- Remove lid of Terasaki plate a place 10μL PBS into all wells for viewing with fluorescent microscope. Invert the Terasaki plate and view using Olympus BX-51 microscope under WIB filter (emission wavelength 550nm) at 1.9s exposure.

VARIATIONS

Variation 1

Detection of CD44 without fixation step

- Carry out the above procedure omitting the fixation using 20μL of 3.7% formaldehyde fixative. After removing growth media, add primary antibody directly to wells, without fixing first.

4.6 Dissection and Embedding of Uterine Tissue for Storage

OBJECTIVE

Preserve freshly dissected uterine tissue by embedding in Optimal Cutting Temperature (OCT) compound, for storage and later sectioning.

REAGENTS AND EQUIPMENT

Reagents

- Optimal Cutting Temperature embedding medium (Tissue-Tek OCT Compound 4583 # 64678)
- Phosphate Buffered Saline
- 95% Ethanol (Ajax finechem A5077-20L)

Equipment

- Scalpel and blade
- Forceps
- Surgery scissors
- Petri dishes (Technoplas # S9014-07)
- Aluminium foil
- -85°C Freezer
- Stainless steel blocks (heat sink)

PROCEDURE

- Prepare aluminium mould for holding embedding medium with uterine horns and half fill with OCT.
- Remove stainless steel block, to be used as a heat sink, from -85°C freezer, and place mould containing OCT on top. Hold flat to ensure efficient cooling and freezing of the OCT.
- When OCT has become white and opaque, place the mould in the cryostat at -20°C ready for dissected horns.
- Euthanase mouse by cervical dislocation. Swab abdomen with 70% ethanol and dissect uterine horns, making a lateral incision along mouse midline using blunt forceps and scissors. Make a lateral incision along mouse peritoneum using sharp, sterile scissors and forceps. Cut body wall (peritoneum) from the midline incision towards the hip joint of both hind legs.
- Locate uterus, and hold gently with forceps. Remove connective tissue from beneath uterine horns using sharp scissors. Locate the ovaries, and cut these free of the connective tissue. Locate the bladder, and holding this with forceps, cut through the uterus below the uterine junction. Remove the entire including the bladder and ovaries. Place the uterine horns on a clean petri dish and using a scalpel, trim off excess fat, connective tissue and bladder.
- Remove OCT in cryo-mould from the cryostat and carefully place dissected horns along the length of the frozen OCT. Wait briefly while the tissue freezes, and overlay tissue with more OCT taking special care to avoid air bubbles forming around the tissue.
- To determine tissue orientation after freezing, push pins through liquid OCT and into the solid bottom layer.
- Place embedded uterine horns within the cryo-mould back into the cryostat at -20°C, and leave until completely solid and opaque.
- Remove embedded tissue in blocks from cryo-moulds, and wrap in aluminium foil and into a zip-lock bag.
- Label and store embedded tissue at -85°C until use.

4.7 Cryostat Sectioning of Embedded Uterine Tissue

OBJECTIVE

Preparation of uterine sections from embedded uterine horns for tissue staining using immuno-histochemical techniques or dyes.

REAGENTS AND EQUIPMENT

Reagents

- 95% Ethanol (Ajax finechem A5077-20L)
- Embedded uterine horns (Protocol 4.6)

Equipment

- Poly–L–lysine coated microscope slides (Protocol 4.8), pre-chilled at -20°C
- Cryostat (Leica CM 1850)
- Fine paint-brushes, pre-chilled at -20°C
- Microscope boxes
- Soft lead Pencil

PROCEDURE

- Remove required blocks of embedded uterine horns from storage at -85°C and place storage bags in cryostat set at -20°C.
- Leave tissue at -20°C for 30 minutes to allow OCT media and horns to equilibrate to -20°C.
- Cut OCT block containing the embedded uterine horn in half using a clean, dry scalpel, and mount the embedded tissue onto a pre-chilled cryostat mounting stand using extra OCT.
- Section tissue, initially preparing sections of approx 20µm thick, until the entire sectioned face the OCT block creates a complete section of tissue. Reduce the thickness of sections by degree, until smooth sections are obtained.
- Prepare uterine sections at 5-7µm thick, no more than 4 sections at a time.
- Place pre-chilled poly-l-lysine coated microscope slides beneath the cryostat cutting stage, and carefully brush sections onto slides with pre-chilled fine paintbrushes.
- Orientate the sections on the slide using the paintbrush, whilst still holding the slide within the cryostat.
- Set the section on the slide, by melting the OCT onto the slide using heat from a finger.
- Label the slide, and store in microscope slide storage boxes at -85°C until use.

4.8 Preparation of Poly-L-Lysine Coated Slides and Cover slips

OBJECTIVE

To prepare cover slips and micro-scope slides pre-coated with Poly-L-lysine in order to make glass surfaces more adherent for prepared sections and cultured cells.

REAGENTS AND EQUIPMENT

Reagents

- Poly-l-lysine (Sigma Aldrich # P 8920)
- Sterile Milli Q Water (from Biolab Milli Q Simplicity system)
- Detergent
- 70% Ethanol (Ajax finechem A5077-20L)

Equipment

- Glass microscope slides (Sailbrand # LOMSL710501)
- 1cm diameter round glass cover slips (Lomb # LOMCSC121GP)
- Plastic trays
- 50mL screw top conical plastic tubes (Greiner BioOne Cellstar)
- Aluminium foil
- Refrigerator
- Drying oven set at 60°C
- Drying oven set at 140°C
- 0.22 µm syringe filters (Millipore, Millex # MILSLGVO33RS)
- Sterile forceps
- Glass petri dishes

PROCEDURE

- Prepare a 1/10 dilution of poly-l-lysine solution by adding 5mL undiluted solution to 45 mL Milli Q water.
- Store diluted poly-L-lysine solution at 4°C until use. The diluted solution should be given an expiry date of 3 months.
- Thoroughly wash glass slides. Initially scrub slides with hot soapy water. Rinse slides with water and then finally rinse with 70% ethanol to remove all traces of detergent. Wipe surfaces dry using paper towels
- Bring poly-L-lysine to room temperature, and immerse clean, dry slides into solution. Ensure all slides are covered with solution.
- Leave slides soaking in poly-L-lysine solution for at least 10 minutes at room temperature.
- Remove slides from solution, and blot excess liquid from the edges on paper towels.
- Place slides in a plastic microscope staining rack and place into drying oven at 60°C for one hour.
- Remove slides from drying oven, ensuring all liquid is dry. Wrap packets of slides in aluminium foil.
- Store slides in fridge at 4°C and give slides an expiry date of 3 months.

VARIATIONS

Variation 2

Sterile Poly-L-lysine coated Cover slips

- The basic technique is the same as for variation 1. As the cover slips are to be used for cell culture, it is essential to maintain sterile technique for the preparation of these.
- Prepare 1/10 dilution of poly-L-lysine in a 50mL conical tube and bring the solution to room temperature.
- Sterilise 50 mL by passing solution through a 0.22 µm filter into a second sterile conical plastic centrifuge tube.
- Sterilise cover slips by placing them into glass vials, and treated with dry heat in an oven at 140°C for 3 hours. Allow to cool.
- Place sterilised cover slips directly into the filtered poly-L-lysine in the 50 mL conical tube. Gently shake the tube, observing cover slips flowing freely within the tube.
- After at least 10 minutes, remove individual cover slips using sterile forceps. Flame forceps as required to sterilise tips using a Bunsen burner.
- Place cover slips onto the surface of a pre-sterilised glass petri dish, ensuring all cover slips are laid flat with no overlapping of edges.
- Dry poly-l-lysine on cover slips by placing the glass petri dish containing into drying oven at 60°C and leaving for one hour.
- Remove cover slips from the petri dish using sterile forceps, and place coated into a sterile glass vial.
- Label coated cover slips with an expiry date of 3 months and store at 4°C.

4.9 Maintenance of Epithelial and Trophoblast Cell Lines

OBJECTIVE

To provide healthy cultured cells of endometrial epithelial carcinoma cells lines, and trophoblastic cell lines.

REAGENTS AND EQUIPMENT

Reagents

- RL-95-2 Cells ATCC CRL-1671
- HEC-1-A Cells ATCC HTB-112
- AN3-CA Cells ATCC HTB-111
- BeWo Cells ATCC CCL-98
- RL-95 Media: DMEM-F12 (Gibco 11320-033, Invitrogen) containing 10% Fetal Bovine Serum (ICP Biologicals FBS-500), 5µg/ mL insulin (Gibco 12585-014, Invitrogen), Penicillin/ Streptomycin at 1 U/ mL and 1ug/ mL respectively (Gibco 15140-122, Invitrogen).
- HEC-1-A Media: McCoy's 5A Medium (Gibco 16600-082, Invitrogen) containing 10% Fetal Bovine Serum (ICP Biologicals FBS-500), Penicillin/ Streptomycin at 1 U/ mL/ 1µg/ mL, (Gibco 15140-122, Invitrogen).
- AN3-CA Media: Eagle's Minimal Essential Medium (Invitrogen, # 12561056) containing 10% Fetal Bovine Serum (ICP Biologicals FBS-500), Penicillin/ Streptomycin at 1 U/ mL/ 1µg/mL (Gibco 15140-122, Invitrogen).
- BeWo Media: 12K (Invitrogen # 21127-022) containing 10% Fetal Bovine Serum (ICP Biologicals FBS-500), Penicillin/ Streptomycin at 1U/ mL/ 1µg/ mL, (Gibco 15140-122, Invitrogen).
- Trypsin: EDTA (Gibco 25200-072, Invitrogen)
- DMSO, Dimethyl Sulphoxide (Sigma #34H06231))

Equipment

- 37°C Incubator with 5% CO_2 atmosphere (Binder E045.06.984)
- 37°C Water bath/ thermometer
- Biohazard Hood (ESCO)
- 25cm^2 Culture Flasks, (Becton Dickinson # 353108)
- 75cm^2 Culture Flasks (Becton Dickinson # 353136)
- 24 Well Culture Plates (Greiner BioOne)
- 96 Well Culture Plates (Greiner Bio One Cellstar #655180)
- 60 well micro-test (Terasaki) Plates (Greiner BioOne #GR 659180)
- Sterile serological pipettes – 5mL, 10mL (Falcon)
- Bench top pipettes 1000µL, 200µL, 20µL (Eppendorf)
- Filter tips for bench top pipettes (Labcon Sterile Aerosol 1057-965-018)
- Haemocytometer (Hawkesley Neubauer Improved BS 748)

PROCEDURE

Thawing

- Prepare a 37°C water bath in a beaker using hot tap water. Monitor water temperature using a thermometer.
- Remove a vial of frozen cells from storage in liquid nitrogen, and place into a pre-chilled container containing isopropanol at -85°C for transport.
- When water-bath reaches 37°C, remove vial from the transport container, and gently agitate stir within the water-bath, taking care to avoid splashes of water above the level of the screw top.

- When the cells are approx 95% thawed, remove from the water-bath, and wipe the exterior of the tube with 70% ethanol on a clean tissue and dry.
- Remove cap carefully with one hand, and maintaining aseptic technique, transfer contents from the tube into a pre-warmed 25cm^2 culture flask containing growth media appropriate for cell type.
- Label the flask with the cell type, passage number, and date, and place into incubator at 37°C with 5% CO_2 atmosphere, without delay.

Culture

- Maintain cells, in sterile conditions, in a 37°C incubator with 5%CO_2 atmosphere. Monitor growth conditions daily, including pH – by observing the colour of media which contains a pH indicator, cell growth and turbidity.
- If media appears yellow, indicative of acidic conditions caused by metabolism of cells, gently pipette off the spent media using a sterile 10mL volumetric pipette, and discard.
- Replace media (7mL) with fresh, pre-warmed media appropriate for the specific cell type using a sterile 10mL volumetric pipette. Replace cells into 37°C incubator.

Splitting

- Split cells 2-3 times a week, depending on growth and the desired confluence of monolayers.
- Thaw Trypsin:EDTA and pre-warm in 37°C incubator. Do not leave Trypsin:EDTA in the incubator for extended periods of time however, enzyme activity may deteriorate.
- Remove growth media from cells using a 10mL volumetric pipette, maintaining aseptic technique, and discard used media.
- Rinse cell monolayer with 1mL pre-warmed trypsin: EDTA, to remove traces of serum from the media which inhibits enzyme action of trypsin.
- Add 1.5mL trypsin: EDTA to the monolayer, and incubate at 37°C with 5%CO_2 for approximately 5 minutes. When cells appear to be lifting from the surface of the flask, remove the flask from the incubator and resuspend cells with 3.5 mL pre-warmed media. Make sure cells are well suspended within media, by aspirating with volumetric pipette.
- Remove 20µL from cell suspension aseptically, and place 10µL on each side of a clean haemocytometer. Count cells under haemocytometer which holds 0.1 mm^3, or 0.0001cm^3. Average the cell count of the two sides of the haemocytometer and multiply the average by 10^4 to calculate the cell concentration of the suspension in cells/ mL.
- Seed cells at an appropriate density into sterile 25cm^2 flasks containing pre-warmed media to give a total of 5mL per flask.
- RL-95 cells should be seeded into new flasks with at least $4x10^6$ cells per 25cm^2 flask. HEC-1-A cells and AN3-CA cells should be seeded into new flasks with at least $1.2x10^6$ cells per 25cm^2 flask and BeWo cells should be seeded into new flasks with at least $7.7x10^5$ cells per 25 cm^2 flask.
- Label flask with new passage number and place into incubator at 37°C with 5%CO_2 atmosphere.

Freezing

- Prepare growth media with 5% DMSO cryo-protectant and usual growth supplements. Omit antibiotics from freeze media.
- Pre-chill freeze media in fridge at 4°C.
- Treat cells with trypsin as above and count cells as per splitting protocol above. Determine the volume of cell suspension to give minimal cell numbers per 25cm^2 flask (above), and place this volume into sterile polystyrene tubes.
- Centrifuge suspensions in Immunofuge, bench top centrifuge on low for 1 minute. Aseptically remove supernatant using filter tips on 1000µL bench-top pipette.
- Resuspend the cell pellet with 1.5mL pre-chilled Freeze media and allow cells to equilibrate at room temperature for 10 minutes.
- Label vials with cell type, passage number, and date.
- Place vials into freeze container containing pre-chilled isopropanol and place in -85°C freezer.
- When vials have frozen, place in liquid nitrogen vapour phase for storage.

VARIATIONS

Variation 1

Seeding RL-95 and HEC-1-A cells into 96 well plates for Attachment Assays

- Prepare RL-95 cells at a concentration of $1.5x10^6$ cell/ mL and add 300µL of this concentration to wells of 96 well plate to give $4.5x10^5$ cells per well, or $1.2x10^6$ cell/ cm^2. NB Wells of 96 well plates have a diameter of 0.7cm giving a surface area of $0.38cm^2$.
- Prepare HEC-1-A cells at a concentration of $4x10^5$ cell/ mL and add 300µL of this concentration to wells of 96 well plate to give $1.2x10^5$ cells per well, or $3.2x10^5$ cell/ cm^2.

Variation 2

Seeding RL-95 cells into 24 well plate with cover slips for Insertion and Screening

- Prepare RL-95 cells at a concentration of $1.8x10^5$ cell/ mL and add 1mL of this concentration to wells of 24 well plate containing poly-L-lysine coated cover slips to give$1.6x10^5$cell/ cm^2. NB Wells of 24 well plates have a diameter of 1.5 cm, giving a surface area of $1.7cm^2$.

Variation 3

Seeding RL-95, HEC-1-A and AN3-CA cells into Terasaki Plates for Insertion and Screening

- Prepare concentration of RL-95 cells to 3.0 – $5.0x10^5$ cell/ mL. Add 20 µL of this suspension to wells of sterile Terasaki plates (surface area = $0.78mm^2$) to give $1x10^4$ cells per well, or $1.3x10^6$ cell/ cm^2.
- Dilute HEC-1-A cell suspension to give 2.0-$2.5x10^5$ cell/ mL. Add 20µL per well to give a density of $6.4x10^5$ cell/ cm^2.
- Dilute AN3-CA cell suspension to give $1.5x10^5$ cell/ mL, or $3.8x10^5$cell/ cm^2. Add 20µL per well to give a density of $3.8x10^5$ cell/ cm^2.

4.10 Preparation of BeWo Spheroids

OBJECTIVE

To prepare BeWo cells in spherical balls, known as spheroids for use in attachment assays with endometrial epithelial cell monolayers.

REAGENTS AND EQUIPMENT

Reagents

- BeWo Cells ATCC – CCL-98
- BeWo Media: F12K (Invitrogen # 21127-022) containing 10% Fetal Bovine Serum (ICP Biologicals FBS-500), Penicillin/ Streptomycin at 1U/ mL/ 1µg/ mL (Gibco 15140-122, Invitrogen).
- Sterile 1% Poly Vinyl Pyrrolidone (PVP), (Sigma, P0930-50g).

Equipment

- Serological pipettes 5mL and 10 mL (Falcon)
- Bench top pipettes 1000µL, 200µL and 20µL (Eppendorf)
- Filtered pipette tips (Labcon, Sterile Aerosol 1057-965-018)
- 8mL Ehrlenmeyer flasks (Kimble # 26500-25)
- Gyrator (Grant-bio POS-300)
- 37°C Incubator with 5%CO_2 atmosphere (Binder E045-06-984)
- Mouth pipette with custom made glass tips (prepared in house)
- Sterile 35 mm petri dish (Greiner BioOne CellStar # 627160)
- Haemocytometer (Hawkesley Neubauer Improved BS 748)
- Biohazard cabinet (ESCO)

PROCEDURE

- Split cells as per Maintenance of Epithelial and Trophoblast Cell Lines (Protocol 4.9) and estimate the cell concentration using a haemocytometer.
- Coat sterile Ehrlenmyer flasks with 1% PVP in biohazard cabinet and dry in 37°C incubator.
- Add 5.5 mL pre-warmed media to the PVP coated flask.
- Calculate the volume of cells given the concentration from the cell count, to give $4.8x10^5$ cells per flask and adjust the volume in the flask to give 6mL. This will give a final concentration of BeWo cells in the flask of $8x10^4$ cells/ mL in 6mL.
- Incubate flasks at 37°C with an atmosphere of 5%CO_2 on top of a gyrator set at 130 rpm for 16-18 hours until small spheroids are visible in the flask.

COMMENTS

Spheroids prepared after 16-18 hours incubation are estimated to be approximately 400µm to 500µm based on investigations other members of this research facility (Stockman, 2007) 16-20 hr incubation yields spheroids of 400-500µm.

4.11 Immuno-staining of Uterine Horns

OBJECTIVE

To detect modification of uterine endometrial epithelial cells subsequent to treatment with FSL molecules in vivo or ex vivo.

REAGENTS AND EQUIPMENT

Reagents

- Cryostat sectioned uterine horns (Protocol 4.7)
- 3.7% Formaldehyde fixative (BDH Cat # 101136C)
- Anti A Bioclone monoclonal IgM DEV 01102 (Ortho-Clinical Diagnostics
- # 606-20-954-5)
- Secondary antibody anti-mouse Ig-FITC (Chemicon # 985031020 AP 326F) at 1/200 in 2% BSA
- PBS
- 2% BSA in PBS (Gibco 30063-573)
- Citifluor anti-fade glycerol mountant (Citifluor Cat # AF1)

Equipment

- Olympus BX-51 fluorescent microscope with WIB filter (emission wavelength 550nm)
- Wax pencil
- Bench top pipettes 200μL and 1000μL (Eppendorf)
- Transfer pipettes
- Paper towels

PROCEDURE

- Remove sections from storage at -85°C, and encircle sections to be stained with wax pencil.
- Add 50μL 3.7% formaldehyde to each section and fix at room temperature for 10 minutes.
- Rinse sections three times with PBS using a transfer pipette and blot edge of slide dry with paper towel.
- Add 50μL pre-diluted primary antibody using a 200μL bench-top pipette to one section per slide. To the other, add 50μL 2% BSA diluent. Incubate slides for 1 hour at room temperature.
- Rinse sections three times with PBS using a transfer pipette, and shake off excess liquid.
- Add 50μL secondary antibody to all sections using a 200μL bench-top pipette and incubate at room temperature in a humidified dark box for 30 minutes.
- Rinse sections three times with PBS using a transfer pipette. Blot edges of slides dry with paper towels and wipe off excess liquid surrounding sections.
- Mount sections with Citifluor containing mountant and cover with clean glass cover slips.
- View sections with Olympus BX-51 microscope using DIC and WIB filter of fluorescence (emission wavelength 550nm).
- Record DIC images and fluorescent images under WIB filter 1.9s exposure.

VARIATIONS

Variation 1

Staining of FSL A Lavage Horns with anti-A Affinity purified IgG

- Prepare 1.75 dilution primary antibody, affinity purified anti-A IgG with 2% BSA diluent
- Prepare 1/50 dilution of secondary antibody, anti-human Ig-FITC with 2% BSA diluent.
- Thaw sections on slides, and omitting fixation step, add 50µL prepared primary antibody affinity purified anti-A IgG to each section. Incubate at 37°C in a humidified box for 1 hour, on top of orbital shaker
- Rinse slides three times with PBS using a transfer pipette, and blot excess liquid from side of slides using clean paper towels.
- Add 50 µL secondary antibody, anti-human Ig FITC to all sections, and incubate in a humidified dark box at room temperature for 30 minutes.
- Rinse slides three times with PBS in a transfer pipette, and blot excess fluid using paper towels.
- Mount slides using Citifluor containing mountant and cover with cover slips.
- View images with WIB filter (emission wavelength 550nm) of Olympus BX-51 microscope, and record images of fluorescence under WIB filter at 5.2s exposure.

Variation 2

Screening of Uterine Horns for HA

- Prepare primary antibody, sheep anti-HA at 1/100 with 2% BSA diluent.
- Prepare secondary antibody, donkey anti-sheep at 1/200 with 2% BSA diluent.
- Thaw sections to be screened, and omitting fixation step, add 50µL prepared primary antibody, sheep anti-HA to each section. For non-primary antibody controls, add 2% BSA only to sections. Incubate at 37°C for 2 hours in a humidified box.
- Wash slides three times with PBS using a transfer pipette. Blot excess fluid from the side of slides using clean paper towels.
- Add 50µL secondary antibody donkey anti-sheep to all sections and incubate in a humidified dark box at room temperature for 30 minutes
- Rinse sections three times with PBS, and mount with Citifluor containing mountant. Cover sections with cover slips.
- View sections with Olympus BX-51 microscope under WIB filter (emission wavelength 550nm) and record images at 1.9s exposure. Record images under DIC.

4.12 Lavage of Pseudo-pregnant Mice

OBJECTIVE

Administration of a fluid bolus directly to the uterine lumen of mice using a non-surgical transvaginal lavage procedure.

REAGENTS AND EQUIPMENT

Reagents

- Sterile PBS
- 1% Evan's Blue Dye in PBS
- Avertin anaesthetic (Protocol 4.17)
- Day 4.5 pseudo-pregnant CBA/C57 female mice
- Lubricating Jelly (PDI T00128)

Equipment

- Lavage apparatus: 23 Winged Needle infusion set (Venisystems P304 A05)
  1.5cm x 3mm diameter plastic tubing speculum
  1mL syringe (Becton Dickinson # 639461)

- Dissecting microscope (Zeiss Stemi SV6)
- Fibre Optic light source (Zeiss KL 200)
- 1mL tuberculin syringes with needles (BD # 302100)

PROCEDURE

- Prepare day 4.5 pseudo-pregnant CBA/ C57 (Protocol 4.16)
- Anaesthetise mouse with a bolus dose of Avertin anaesthetic of approximately 26 µL/mg bodyweight.
- Wait for 5 minutes, placing mouse in a quiet area with minimal light exposure. Test anaesthesia by tail or foot pinch.
- If there is no reflex on tail or foot pinch, begin the procedure.
- Lubricate plastic speculum with ultrasound gel.
- Insert the plastic speculum into the vagina of the anaesthetised mouse.
- Using the fibre optic light source, locate the cervix under the dissecting microscope, with the aid of the fibre optic light source.
- Gently push the bluntened needle end of the catheter through speculum and into the cervix of the mouse – taking care not to damage the cervix or disrupt the vaginal or uterine lining.
- Holding the catheter in place, gently administer 100µL of fluid from the syringe attached to the catheter lead.
- Hold mouse upright vertically with posterior end upwards to allow gravity to encourage lavage fluid down into uterine horns. Hold in this position for 1-2 minutes.
- If there is back-flow of lavage fluid, blot dry with a clean tissue, and repeat the process.
- Administer a maximum of three boluses of fluid containing 100µL per bolus.
- Gently remove lavage apparatus.
- Identify mouse by an ear notch using sharp surgical scissors, creating a very small notch of approximately 3mm long.
- Wrap mouse in tissue and place in recovery box.
- Label box with lavage type, and mouse identification details.

4.13 Haematoxylin Stain of Uterine Horns

OBJECTIVE

Monitoring of changes in the endometrium of pseudo-pregnant mice around the window of implantation (day 4.5), observation of characteristics of the endometrium. Additionally, this technique is used to monitor any damage to uterine tissue resulting from lavage treatment.

REAGENTS AND EQUIPMENT

Reagents

- OCT embedded uterine horns dissected from pseudo-pregnant mice (Protocol 4.6)
- 95% Ethanol (Ajax finechem A5077-20L)
- Tap Water
- Undiluted haematoxylin (Sigma Aldrich Accustain Hematoxylin Gill II # GHS216)
- Scott's Tap Water (Sigma Aldrich S5134)
- Glycerin Mountant (G5150-1L)

Equipment

- Poly-L-lysine coated microscope slides (Protocol 4.8) pre-chilled at -20°C
- Cryostat (Leica CM1850)
- Olympus BX-51 microscope with 100x, 200x and 400x objective lenses
- Wax pencil

PROCEDURE

- Prepare 7µm sections from uterine horns embedded in OCT which have been stored at -85°C – (Protocol 4.7) and mount onto pre-chilled poly-L-lysine coated slides (Protocol 4.8)
- Thaw sections of uterine horns and circle with a wax pencil.
- Fix sections by placing into a coplin jar containing 95% ethanol, for 2 minutes.
- Remove sections and rinse slides in a jar of running tap water.
- Remove excess water from slides by shaking and lightly blotting the edge against paper towels.
- Stain slides by flooding with undiluted haematoxylin, using a transfer pipette. Allow sections to stain for 2 minutes.
- Wash stain off slides with excess running tap water, until water runs clear. If using a jar under a running tap, change the water in the jar during washing procedure.
- Remove excess water as previously described by shaking and blotting with paper towels.
- Dip slides into a 50mL tube containing Scott's Tap Water for a total of 10 dips per slide.
- Mount all sections with 1 drop per section of glycerine mountant and cover with cover slips.
- View sections with Olympus BX-51 microscope using DIC filter with 100x, 200x and 400x objective lenses.

4.14 Attachment of BeWo Spheroids to Endometrial Cell Monolayers

OBJECTIVE

An in vitro assay to model the attachment of an embryo to the endometrium. Cultured, human derived endometrial epithelial cells from commercially available cell lines will be used to model the endometrium surface cell. Spheroids prepared from commercially available human trophoblast cells are used to mimic an embryo.

REAGENTS AND EQUIPMENT

Regents

- BeWo Spheroids (Protocol 4.10)
- Confluent monolayers of RL-95 cells and/ or HEC-1-A cells in 96 well culture (Protocol 4.9)
- RL-95 media (Protocol 4.9)
- HEC-1-A media (Protocol 4.9)

Equipment

- 96 Well Culture plates (Greiner BioOne Cellstar # 655180)
- Mouth pipette (prepared in house)
- Bench top pipettes 1000μL, 200μL, 20μL (Eppendorf)
- Filter Tips (Labcon Sterile Aerosol 1057-965-018)
- 35 mm petri dish (Greiner BioOne Cellstar # 627160)
- Hitachi 05PR-22 centrifuge
- Biohazard cabinet (ESCO)
- Incubator at 37°C with 5% CO_2 atmosphere (Binder E045.06.984)
- Dissecting microscope (Olympus SZ61)
- Transfer pipettes
- Self adhesive plate sealer (Greiner BioOne M207-1 67605)

PROCEDURE

- Prepare spheroids (Protocol 4.10) and remove spheroids from Ehrlenmeyer flasks on orbital shaker.
- Prepare monolayers of RL-95 and HEC-1-A cells in 96 well plates (4.9).
- Place spheroids into a sterile 35mm^2petri dish using a 1000μL bench-top pipette fitted with a filtered tip.
- Remove 96 well plate with monolayered endometrial epithelial cells, RL-95 or HEC-1-A from incubator, and place onto a heat plate under the dissecting microscope.
- Using a clean, dry mouth pipette, carefully select spheroids of small, uniform size, and dispense 5 spheroids per well monolayered with endometrial epithelial cells, taking care not to disrupt the monolayer.
- Incubate spheroids with monolayers for attachment, at 37°C with a 5%CO_2 atmosphere, for 30 minutes.
- Carefully remove 96 well plate with spheroids from the incubator.
- Using a transfer pipette, place pre-warmed media appropriate to the monolayer type into each well containing spheroids until a bubble forms by surface tension above the well.
- Cover wells with adhesive film, to form an airtight seal with no air bubbles using a centrifuge tube to squeeze out excess fluid and air bubbles.
- Centrifuge the entire 96 well plate, using adhesive tape to hold in buckets of Hitachi centrifuge. Place an appropriately balanced plate in the opposite bucket of the centrifuge.

- Centrifuge plate at 400 rpm (15×g) for 5 minutes.
- Invert plate and allow spheroids to settle for 5 minutes.
- Count spheroids remaining attached to monolayers under the dissecting microscope.

VARIATIONS

Variation 1

Attachment assay of FSL treated monolayers

- Dilute FSL A or FSL B stock to 1mg/ mL with media appropriate for the cells to be treated. Prepare cell maintenance media to be used as diluent for FSL molecules, omitting Fetal Bovine Serum from media
- Carefully remove growth media from epithelial cell monolayers using 1000μL and 200μL bench-top pipettes with filtered tips.
- Replace media with 100μL FSL A or FSL B prepared in maintenance media, to wells being treated. For non-treated wells, remove 200μL media from these wells, to ensure all wells have same volume.
- Incubate monolayers with FSL molecules for 2 hours at 37°C with 5% CO_2 atmosphere.
- Remove plate from incubator and carry out attachment assay as per above protocol.

Variation 2

Washed versus non-washed monolayers

- Carry out protocol as per Variation 1, with the following added step:
- After treatment of monolayers with FSL A or FSL B molecules, carefully remove insertion media containing the molecules using a 200μL bench-top pipette with filtered tip.
- Replace media with 100μL fresh, pre-warmed insertion media containing no FSL molecules.
- Carry out attachment assay as per above protocol.

4.15 Insertion of FSL Molecules into Murine Uterine Horns Ex Vivo

OBJECTIVE

A preliminary investigation to give an indication of the modification potential of FSL A molecules in the uterine lining in vivo. The modification potential of FSL molecules using precise volumes delivered to the uterine horns may be investigated.

REAGENTS AND EQUIPMENT

Reagents

- Freshly dissected uterine horns (Protocol 4.6)
- Hanks Balanced Salt Solution (Gibco # 14170-112)
- DMEM-F12 (Gibco 11320-033, Invitrogen) containing 0.4% Bovine Albumin
- FSL A at 0.5mg/ mL diluted in RL-95 insertion media
- Optimal Cutting Temperature embedding medium (Tissue-Tek OCT Compound 4583)

Equipment

- Dissection microscope (Olympus SZ61)
- Blunt forceps
- Scalpel blade
- Blunt needle
- 1mL syringes (Becton Dickinson # 639461))
- Serrafine clamps (Incus, # G052-020)
- 1.5 mL microcentrifuge tubes
- Petri dish (Techno Plas # 59014-07)
- Aluminium foil
- Pre-chilled stainless steel heat-sinks at -85°C

PROCEDURE

- Dissect uterine horns from mouse, using clean, dry surgical scissors and forceps, immediately after euthanasing the animal by cervical dislocation (Protocol 4.6).
- Aspirate insertion media, with or without FSL A, into a 1mL syringe.
- Attach a blunt need to the syringe and insert through the cervix of the uterine horns.
- Hold the cervix closed using forceps and deliver 200µL of fluid into the uterine horns.
- Clamp the cervical end of the horns shut with a Serrafin clamp and place into a 1.5mL microfuge tube containing pre-warmed HBSS.
- Incubate microfuge tubes containing inflated uterine horns at 37°C for 4 hours.
- Remove horns from HBSS solution and place onto a clean, dry petri dish. Remove cervical junction below Serrafin clamp using a scalpel.
- Embed uterine horns into prepared cryo-mould (Protocol 4.6) and store at -85°C until use.

4.16 Preparation of Pseudo-pregnant Mice

OBJECTIVE

Preparation of pseudo-pregnant recipient mice. A pseudo-pregnant state is a requirement in female mice for their endometrial lining to be receptive to an embryo. Cervical stimulation, usually by copulation, is required to induce a change in the pattern of hormones required for maintenance of the corpus luteum which in turn produces progesterone.

REAGENTS AND EQUIPMENT

Reagents

- Recipient female (day 40-120) CBA/ C57 mice in estrus
- Vasectomised male mice (Protocol 20)

Equipment

- Housing cages

PROCEDURE

- Monitor the overt appearance of the vaginal opening of day 40-120 CBA/ C57 mice and identify candidate recipient female mice in estrus. Mice in estrus exhibit a red, swollen opening to their vaginas, often with a slight excretion of vaginal fluid. A group of females housed together in a single cage, often have aligned estrus cycles.
- Choose the most in estrus recipient candidates, and place 1 or two females in a cage with proven vasectomised male mice.
- Allow mice to mate ad libitum over-night.
- The following morning, check female recipient mice for the presence of a seminal plug at the external opening of the vagina indicative of successful mating. These females become recipient mice and this stage of pseudo-pregnancy is counted as 0.5.
- Identify and house recipient females together in cages and label with day of seminal plug observation. Keep these females together until use in embryo transfers, lavage procedures, or other investigations.

4.17 Avertin Preparation and Anaesthesia of Mice

OBJECTIVE

The preparation of Avertin anaesthetic to be used for the anaesthesia of mice prior to and during surgery or any invasive techniques.

REAGENTS AND EQUIPMENT

Reagents

- 2,2,2 Tribromoethanol (Sigma Aldrich # T48402-5G)
- Tertiary amyl alcohol (Aldrich Chemical Company Inc # 15,246-3)
- 18mΩ Milli Q water (from BioLab Milli Q Simplicity System)

Equipment

- Sterile 15 mL conical centrifuge tubes (Greiner BioOne Cellstar)
- 58°C water bath from tap water
- 0.22µm filter (Millipore, Millex # MILSLGVO33RS)
- Sterile 10mL syringe (Henk Sass Walf 10mL Norm Ject Luer)
- Aluminium foil
- Sterile 27 gauge tuberculin syringes (BD # 302100)
- Bench top pipettes 1000µL, 200µL (Eppendorf)
- Sterile filter tips (Labcon Sterile Aerosol 1057-965-018)
- Balance (Sartorius CP 2245)

PROCEDURE

Preparation of Avertin

- Weigh 0.1563g tribromoethanol into a sterile conical centrifuge tube, taking care to minimise static electricity within balance.
- Add 315µL tertiary amyl alcohol and 12.5mL 18mΩ Milli Q water and place lid on tube.
- Dissolve tribromoethanol to obtain a homogenous mixture by placing into water bath at 58°C, and swirling to dissolve. Monitor the temperature of water bath with a thermometer to ensure the water bath stays close to this temperature. Add further hot tap water if necessary.
- Dissolve persistent lumps of tribromoethanol and/ or droplets of amyl alcohol by aspirating with a 1000µL bench-top pipette, and expelling several times. Ensure contents are fully dissolved by holding up to light and checking clarity of the solution.
- Sterilise this solution by passing through a syringe fitted with a 0.22µm filter at the end. Pour contents of the centrifuge tube into the syringe, and firmly expel liquid through the filter into a new, sterile 12mL centrifuge tube, taking care to eliminate back pressure which may damage the filter.
- Wrap the centrifuge tube with aluminium foil and label the tube. Give the Avertin an expiry date of 14 days.
- Store Avertin at 4°C prior to use.

Anaesthesia of Mouse

- Dispense Avertin into a sterile 1mL tuberculin syringe, and pre-warm at 37°C under aluminium foil to protect from light.
- Weigh mouse to determine bolus dose of Avertin to administer. Dosage is based on .015 mL/ g mouse body weight.
- Administer Avertin by intra-peritoneal injection, and allow anaesthesia to occur by placing mouse in housing box, and minimising light and noise exposure.
- Prior to surgery, ensure anaesthesia is complete, by testing mouse with a light tail pinch, or toe pinch. Anaesthesia is sufficient when no reflex is elicited from this stimulus. If there is a twitching reflex, administer a small amount more Avertin, and wait a further 5 minutes before testing again.

4.18 Transfer of Embryos into Pseudo-pregnant Recipient Mice

OBJECTIVE

Transfer of blastocyst stage embryos into pseudo-pregnant recipient female mice.

REAGENTS AND EQUIPMENT

Reagents

- Recipient female CBA/ C57 F1 mice (Protocol 4.16)
- Day 2.5 flushed embryos in micro-drops of BAV1/ BAV2 (Protocol 4.19)
- Avertin anaesthetic (Protocol 17)
- Sterile mineral oil (Sigma M5310)
- Sterile PBS
- 70% ethanol (Ajax finechem A5077-20L)

Equipment

- 1mL tuberculin syringes (BD #302100)
- Mouth pipette (prepared in house)
- Sterile surgical equipment (Sharp scissors, forceps, Serrafin clamp)
- Surgical Suture (Cooper Medical Ltd # CM 41413)
- Petri dishes (Techno Plas # 59014-07)
- 35mm petri dish (Greiner BioOne Cellstar #627160) with blue-tak
- Moustache clippers
- Heat pad (Techne Circulator C400)
- Dissection microscope (Zeiss Stemi SV6)

PROCEDURE

- Collect embryos from donor female CBA/ C57 mice which have been super-ovulated and mated with stud males as per (Protocol 4.19). Maintain embryos after harvesting in appropriate culture media in a 37°C incubator with a 5% CO_2 atmosphere.
- Anaesthetise a recipient mouse (Protocol 4.17) and shave flanks with moustache clippers between the ribs and the upper hind legs.
- Swab shaved area with 70% ethanol and place mouse into a clean petri dish lid with a smaller 35 mm^2 petri dish containing blu-tak within the larger petri dish.
- Make a small transverse incision through the skin using surgical scissors, and repeat this for the body wall. The incision should be roughly 1cm below the ribs. Care should be taken to avoid cutting any blood vessels of the body wall.
- Locate the ovary by lifting the body wall up and manipulating fat layers with forceps. Grasp the fat pad behind the ovary with the Serrafin clamp, and adhere into the blue-tak contained in the 35mm^2 petri dish. Take care to minimise any twisting of the uterine horn during this step.
- Targeting the area of the uterine horn immediately below the oviduct. Make a puncture hole with a sterile 25 gauge needle attached to a syringe containing sterile PBS, whilst holding the uterine horn with forceps.
- Fit the mouth pipette with a sterile glass transfer tip and load it with 5 late morula or blastocyst stage embryos. Ensure there is a series of air bubbles surrounding media in which embryos are contained.
- Line up the transfer tip with the opening in the uterine horn made previously.
- Carefully expel air bubble of tip of mouth pipette, until section with embryos is reached. Leave a minimal air bubble of no more than 2mm, at the end of the tip.
- Insert pipette tip into the horn of the uterus through the opening made previously to a depth of approximately 5mm.

- Expel the media containing the embryos into the uterine horn by gently blowing on mouth pipette. Observe air bubbles in the tip moving in response to the pressure.
- Gently remove the tip from the uterine horn with a careful sliding action.
- Release the uterine horn from the Serrafin clamp, and lifting the body wall, allow the uterine horn to retract into the peritoneal cavity.
- Suture the body wall closed using surgical suture, and repeat with the skin layer.
- Wash the stitches with sterile PBS and wipe dry with a clean dry tissue.
- Repeat embryo transfer process on the opposite side of the mouse.
- Identify the mouse by a small ear notch using clean, sharp surgical scissors.
- Wrap the mouse in a clean dry tissue and place in housing box with clean bedding for recovery.
- Observe the mouse 30 minutes post surgery to ensure recovery is underway. The mouse should have resumed consciousness within this time.
- Post surgical observations should be continued 2 hours later, and then daily for the following 7 days.

Autopsies

- Perform an autopsy of all embryo transferred recipients 14-17 days post surgery in order to ascertain the implantation efficiency and any overt abnormalities of the fetuses or placenta.
- Place recipient mouse into a container with CO_2 flowing to displace oxygen, and euthanize the mouse by exposing the mouse to 1.5L/ minute CO_2 in the closed atmosphere.
- After 5 minutes exposure, ensure the mouse is dead, by performing cervical dislocation on the mouse.
- Dissect uterine horns containing implantations/ fetuses and examine for possible re-absorptions. Record these.
- Working from the top of each uterine horn, peel the horn away from the fetus, slide the scissors under the uterine wall cutting the tissue while holding the placenta near the fetus.
- Carefully dissect out placenta of each fetus with the amniotic sac attached. Detach the placenta from the amniotic sac very carefully using clean sharp surgical scissors, and weigh on balance. Remove the amniotic sac from around the fetus and weigh the fetus. Record placental and fetal weights for each fetus.
- Record any further comments regarding the autopsy or any unusual features or abnormalities.
- Repeat the above procedure for all fetuses. Discard the mouse

4.19 Preparation and Maintenance of Embryos in Culture

OBJECTIVE

To harvest embryos from donor female mice and maintain these embryos in culture until they are required for use in the laboratory.

REAGENTS AND EQUIPMENT

Reagents

- 8-10 Pre-pubescent CBA/ C57 F1 female mice (21-28 days)
- CBA male stud mice
- FSH (Folligon at 1000 IU – Intervet NZ)
- hCG (Chorulon at 1500 IU – Pregnyl Organon NZ)
- Sterile PBS
- 70% ethanol (Ajax finechem A5077-20L)
- KSOM Embryo handling media buffered with 0.3% BSA (made in house)
- Embryo culture media One (Embryo Assist # 12130016, Medicult)
- Embryo culture media Two (Blast Assist, #12160010, Medicult)
- Sterile Mineral oil (Sigma M5310)

Equipment

- 1mL tubulin syringes (BD # 302100)
- Sterile surgical equipment (sharp scissors, rat tooth forceps, mosquito forceps)
- Dissection microscope (Zeiss Stemi SV6)
- Embryo 4 well culture dish with lid (Nunclon, # NUN144444)
- Micro-drops containing 50µL culture media overlaid with oil and pre-gassed for 2 hours
- Sterile petri dishes (Techno Plas # 59014-07)
- Mouth pipette (prepared in house)
- Sterile glass handling pipettes for embryos (prepared in house)

PROCEDURE

- Prepare Folligon at a concentration of 50 IU and sterile filter using 0.22µm filter. Aliquot into 1mL tuberculin syringes and store at -20°C until required.
- Prepare Chorulon to a concentration of 50 IU, sterile filter and store as above.
- Super-ovulate 8-10 day 21-28 CBA/ C57 female mice by administering 100µL Folligon by intra-peritoneal injection on day one of super-ovulation.
- Administer 100µL Chorulon by intra-peritoneal injection to these subjects 48 hours after the Folligon injection.
- Place super-ovulated female mice immediately with CBA stud male mice in housing boxes and allow mating ad libitum over-night.
- Check females for the presence of a seminal plug the following morning, indicative of a successful mating.
- Retrieve embryos from the donor mice between days 1-3 following plugging. Euthanase the female donor mouse by cervical dislocation and dissect out the uterine horns.
- Depending on the day of fertilisation, target the appropriate area for retrieving embryos. Day 1.5 embryos will be present in the oviduct located just above the uterine horns.
- Place dissected uterine horns onto a sterile petri dish. Flush embryos by inserting a sterile tuberculin syringe containing sterile handling media into the bottom of each horn, and flush embryos up through the oviduct with approx 0.3-0.4mL of media. Tear the oviduct open with the needle

- Visualise the uterine horns under the dissection microscope and tear the region where embryos are expected with a 1mL tuberculin syringe, holding the uterine horn with sterile forceps.
- Locate embryos expelled into the handling media and collect these by aspirating into mouth pipette tip, and placing in holding well of a 4 well embryo culture plate containing handling media, pre-warmed to 37°C.
- After completing embryo retrieval of all donor mice, pooling embryos into handling media, place embryos in groups of 20 into micro-drops prepared with 50µL culture media, overlaid with sterile mineral oil. Micro-drops must be pre-warmed and degassed for at least 2 hours at 37°C with an atmosphere of 5% CO_2. It is expected that on average 25 embryos will be harvested from each super-ovulated mouse. Twelve micro-drops should be prepared in advance for an embryo flush of 10 donor mice

Culture

- Culture embryos in micro-drops in groups of 20 or less. Incubate at 37°C with an atmosphere of 5%CO_2 until required. Any embryos which are not required for use are discarded immediately.

4.20 Preparation of Vasectomised Male Mice

OBJECTIVE

Preparation of sterile male mice. Vasectomised mice are used to produce pseudo-pregnant female recipient mice for embryo transfer procedures.

REAGENTS AND EQUIPMENT

Reagents

- Avertin Anaesthetic (Protocol 4.17)
- Sexually mature male mouse (at least day 50)
- 70% Ethanol (Ajax finechem A5077-20L)
- Sterile Saline

Equipment

- Dissecting microscope (Zeiss Stemi SV 6)
- 1mL insulin syringes (BD # 302100)
- Surgical Suture (Cooper Medical Ltd # CM 41413)
- Gloves
- Clippers
- Bunsen burner
- 37°C Heat pad (Techne Circulator C400)
- Petri dishes (Techno Plas # 59014-07)
- Tissues
- Sterile surgical equipment (fine, sharp scissors, fine mosquito forceps)

PROCEDURE

- Prepare microscope, heat pad and area surrounding microscope for surgery. Turn heat pad on and ensure heating achieves 37°C. Swab area surrounding microscope with 70% ethanol, and allow it to dry.
- Aspirate 1mL pre-warmed Avertin anaesthetic at 37°C into a 1mL syringe, and administer by intra-peritoneal injection into a male mouse. Administer approx 0.015 mL/g of body weight. Place mouse into a covered box in a quiet area, to limit stimulation to mouse. Wait 5 minutes for anaesthetic to take effect. Ensure anaesthesia is complete by pinching tail or foot lightly and watching for a reflex. If there is no reflex, anaesthesia is deep enough to begin surgery.
- Shave an area just above the penis with moustache clippers, and wipe shaved area with 70% ethanol.
- Transfer mouse to heat pad positioned under dissection microscope.
- Make a transverse incision with sharp surgical scissors, through the skin, and then the peritoneum, approximately 1cm wide.
- Separately locate each testicle, exposing the vas deferens, identified by the prominent blood vessel running along its length.
- Heat surgical forceps under Bunsen burner with a hot, blue flame, until the tips glow red.
- Quickly, without letting tips of forceps cool, use the red hot forceps to cauterise a section of the vas deferens approximately 8-10mm long. Ensure the two ends of the vas deferens are well separated and well cauterised before returning the body cavity. Repeat the procedure on the other vas deferens.
- Close the incision with sutures through the peritoneum, and then the skin, and clean the wound with saline.
- Wrap the animal in a clean, soft, dry tissue, and place into housing box for recovery.
- Ten days after surgery, test the vasectomy success by housing male with a female mouse in estrus. Mating of the female mouse is determined by the presence of a

seminal plug in the vagina of the female up to 6 hours post mating. If no pregnancy results in the female mouse within 30 days, the vasectomy is considered successful.

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Refer to: Festing et al – The Design of Animal Experiments

Hogen et al – Manipulating the Mouse Embryo

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