THE UNIVERSITY OF
SYDNEY

MOLECULAR AND HORMONAL STUDIES ON MOLECULES INVOLVED IN THE MAINTENANCE OF UTERINE EPITHELIAL POLARITY IN THE RAT

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A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy (Anatomy and Histology)

The University of Sydney

2014

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DECLARATION

I hereby certify that this thesis does not contain, without the appropriate acknowledgement, any material previously submitted for a degree in any university. I also certify that this thesis does not contain, without the appropriate acknowledgement, any material previously published or written by another person.

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ABSTRACT

Uterine luminal epithelial cells (UECs) undergo dramatic alterations in the apical and basolateral membrane during early pregnancy and these changes are collectively known as the 'plasma membrane transformation'. This study focuses on how these cells maintain a polarised columnar epithelial phenotype despite the loss of a number of junctions and related structures at the time of implantation. Several aspects of cell polarity are investigated in this thesis. Caveolin and cavin proteins PTRF (RNA pol I transcription factor) and SDPR (serum deprivation protein response) were investigated to understand their involvement in membrane curvature as well as membrane lipid and cholesterol polarity. Filamin A, an actin crosslinking protein was studied to understand the role that the actin cytoskeleton plays in the maintenance of cell polarity. Junctional complexes were also studied through the localisation and expression of afadin in UECs during early pregnancy.

The current study investigated caveolin, a major protein of caveolae; as well as PTRF and SDPR which belong to the cavin protein family. These proteins are involved in lipid and cholesterol transport and membrane curvature, this thesis investigated the localisation and expression of these proteins in the uterus during early pregnancy and the hormonal regulation of these proteins. Caveolin 1 and 2 were both found to shift to the basal plasma membrane of UECs at the time of implantation and also under the influence of progesterone. The localisation of caveolin 1 and 2 at the time of implantation corresponded to the increase in the number of morphological caveolae observed at the basal plasma membrane of UECs.

An increase in the protein abundance of caveolin 1 and a decrease in caveolin 2 protein in uterine epithelial cells were observed at the time of implantation and under the influence of progesterone, while the amount of PTRF protein was unchanged during early pregnancy and in response to ovarian hormones. SDPR expression and localisation in UECs was restricted to the time of fertilisation and the influence of oestrogen where it was localised cytoplasmically in UEC. PTRF was found to associate with caveolin-1 at the time of implantation, where these

proteins together serve to increase membrane curvature and also maintain a polarised state of lipid and cholesterol in the plasma membrane of UECs at the time of implantation.

Remodelling of the actin cytoskeleton of UECs and also the underlying stromal cells was also investigated by studying the localisation and expression of filamin A (FLNA) - a protein that cross links actin filaments and also mediates the anchorage of membrane proteins to the actin cytoskeleton. Localisation of FLNA in UECs at the time of fertilisation was cytoplasmic and shifted apically at the time of implantation and under the influence of progesterone. FLNA was also concentrated to the first two to three stromal cell layers below the epithelium at the time of fertilisation and shifts to the primary decidualisation zone at the time of implantation. This shift in localisation was found to be dependent on the decidualisation reaction. Protein abundance of the FLNA 280 KDa monomer, and calpain cleaved fragment (240 KDa) did not change during early pregnancy in UECs. These results suggest that FLNA may be an important regulator of cytoskeletal remodelling of these cells to allow uterine receptivity and decidualisation necessary for successful implantation in the rat.

The role of the actin cytoskeleton and the extracellular matrix was also investigated using the Ishikawa cell line as they have been used extensively as a cell culture model of a receptive endometrium. Due to the important role that focal adhesions and the extracellular matrix ligand fibronectin play in normal uterine receptivity, paxillin and talin were investigated in these cells; with regards to the influence fibronectin has on their localisation and expression. This study also observed the effects of cytochalasin D on the localisation and expression of paxillin, talin and caveolin-1 in these cells when grown on fibronectin. Confocal immunofluorescence microscopy of Ishikawa cells grown on glass coverslips coated with Matrigel™ or fibronectin showed that caveolin-1, talin and paxillin are localised apically in Ishikawa cells grown on a basement membrane. This is in contrast to the localisation of these proteins in UECs *in vivo* where they were localised basally. Actin disruption experiments showed a decrease in apical staining of paxillin and talin in Ishikawa cells grown on either Matrigel™ or fibronectin, however punctate staining of caveolin-1 was observed in cytochalasin D treated Ishikawa cells grown on Matrigel™. Protein abundance of talin and

paxillin was greater in Ishikawa cells treated with cytochalasin D grown on fibronectin, whereas caveolin-1 protein abundance was greater in cytochalasin D treated cells grown on Matrigel™ compared to untreated cells. The results from this study suggested that the presence of fibronectin influences the expression and localisation of these proteins in Ishikawa cells, and hence the adhesive properties that are attributed to this cell line.

Alterations in cellular junctions during early pregnancy were also investigated in this thesis with particular focus on the adherens junction. This study investigated the morphological changes of the adherens junction and the localisation and expression of afadin a protein that is part of the nectin-afadin-ponsin protein complex found at cell-cell adherens junctions during early pregnancy in the rat to explore its role in the maintenance of epithelial polarity. This study showed a loss of the morphological adherens junction at the time of implantation, and afadin was found to co-localise to apical cell-cell junctions between UECs at both the time of fertilisation and implantation, and co-localise with ZO-1 at both these times. Afadin protein is increased at the time of implantation in uterine epithelial cells. The co-localisation of afadin with ZO-1 and increase in afadin protein in UECs at the time of implantation suggests that it is associated not only with the adherens junction at the time of fertilisation but also the tight junction at both the time of fertilisation and implantation.

In conclusion this thesis shows the involvement of the molecules in the maintenance of uterine polarity and their contribution to uterine receptivity with regards to the influence they have on the composition of apical and basolateral plasma membranes throughout early pregnancy. At the time of implantation in particular when a number of structures such as the tight and adherens junctions are altered or absent, the apical plasma membrane is rigid due to the elevated membrane cholesterol and FLNA associated actin networks, while the basolateral plasma membrane is less rigid, enabling caveolae formation and membrane curvature at this time. Afadin at this time is also associated with the tight junction, where it may serve to maintain distinct apical and basolateral domains. The collective findings presented in this thesis contribute to our understanding of the mechanisms that these cells employ to provide a receptive state during early pregnancy as well as cell polarity.

ACKNOWLEDGEMENTS

The experience of a PhD is unique, in which I have had the opportunity to not only meet and know number of people; but also grow and develop both as a scientist and a person. For this, I would firstly like to thank my supervisor Professor Chris Murphy for the opportunity of doing a PhD in your lab. Thankyou for always having time to talk, your advice and support has been greatly appreciated.

I would also like to thank my associate supervisor Dr. Laura Lindsay for your advice and support, as well as your amazing proof reading abilities.

Thankyou to my other associate supervisor Dr Margot Day, for letting me invade your lab, as well as your advice, and expertise in all things cell culture and blastocyst related.

To my fellow lab members Dr Laura Lecce, Dr Yui Kaneko, Connie Poon, Sam Dowland, Dr. Joanna Biazik Jacquie Herbert, Dr. Bridget Murphy, Melanie Laird, Dr. James Van Dyke Sadaf Kay, Leigh Nicholson, Kevin Danastas, Vie Ngyugen, and Chad Moore thankyou for all the chats, chocolate, fun and keeping me sane throughout, it has been a privilege of meeting and working with you.

Thankyou Dr. Donna Lai and Dr. Louise Cole for their expertise and help on all scientific techniques.

Thankyou Suzanne Ollerenshaw and Robin Arnold for the histology teaching experience that I have gained. I have enjoyed the experience immensely, and hope to continue teaching in the future.

An acknowledgement wouldn't be one if there wasn't a big hearty thankyou to my family and friends. Mum, Dad, Dilan, and Ranesha thankyou so much for your love and support. There is not a lot that I could have done if you weren't behind me.

Publications arising from this thesis

Madawala R.J, Dowland S.N, Lindsay L.A, Murphy C.R. Caveolins redistribute in uterine epithelial cells during early pregnancy in the rat: *An epithelial polarisation strategy?* Histochemistry and Cell Biology; 2014

Madawala R.J, Poon C.E, Dowland S.N, Murphy C.R. Actin Crosslinking Protein Filamin A During Early Pregnancy In The Rat Uterus. Reproduction Fertility and Development In Review

Abstracts arising from this thesis

Madawala R.J, Murphy C.R. caveolin 1 and 2 in the uterus during early pregnancy. Poster presented at the Society for Reproductive Biology Conference 2010 Sydney, NSW, Australia Abstract: #310

Madawala R.J, Day M.L, Murphy C.R Caveolin and focal adhesion proteins talin and paxillin during early pregnancy in the rat and in human Ishikawa cells. Poster presented at the Society for the Study of Reproduction Conference 2011 Portland, Oregon, USA. Abstract: #476

Madawala R.J, Murphy C.R. Actin crosslinking protein filamin a during early pregnancy in the rat uterus_Poster presented at Society for Reproductive Biology Conference 2012_Gold Coast, QLD, Australia. Abstract: #294

Madawala R.J, Dowland S.N, Lindsay L.A, Murphy C.R. Caveolins 1 and 2 are redistributed in the uterus during early pregnancy and are associated with integrin β1 turnover. Platform presentation at the Society for Reproduction and Fertility Conference 2013. Cambridge, England, UK. Abstract: #O028

Madawala R.J, Murphy C.R Actin crosslinking protein filamin A during early pregnancy in the rat uterus_Poster presented at Society for Study of Reproduction Conference 2013. Montreal, Canada. Abstract: #463

Madawala R.J, Dowland S.N, Lindsay L.A, Murphy C.R. Little Caves: *Caveolins 1 and 2 are redistributed in the rat uterus during early pregnancy* Poster presented at the Society for Reproductive Biology Conference 2013 Sydney, NSW, Australia Abstract #256

Author Contribution to Published Works

Selected peer-reviewed and submitted manuscripts have been used to compose parts of this thesis. Below are descriptions of co-authors contributions to these manuscripts

Madawala R.J, Dowland S.N, Lindsay L.A, Murphy C.R. Caveolins redistribute in uterine epithelial cells during early pregnancy in the rat: *An epithelial polarisation strategy?* Histochemistry and Cell Biology; Accepted.

Samson N. Dowland: Transmission microscopy of uterine tissue

Laura A. Lindsay: Intellectual input, manuscript editing

Christopher R. Murphy: Intellectual input, manuscript editing

Madawala R.J, Poon C.E, Dowland S.N, Murphy C.R. Actin Crosslinking Protein Filamin A During Early Pregnancy In The Rat Uterus. Molecular Reproduction and Development. In Submission

Connie E. Poon: Assistance in ovariectomy surgery

Samson N. Dowland: Assistance in ovariectomy surgery

Christopher R. Murphy: Intellectual input, manuscript editing

Author Contribution to Thesis Chapter

Thesis chapter 7 is yet to be submitted for publication. Below is the description of co-author contribution to this chapter.

Madawala R.J, Dowland S.N, Murphy C.R. Afadin an actin filament binding protein is localised at apical cell-cell junctions in uterine epithelial cells during early pregnancy

Samson N. Dowland: Transmission microscopy of uterine tissue

Co-author signatures

The co-authors listed below certify that they have contributed to publications and/or manuscripts in submission as outlined on the previous page and that they agree to use those publications/manuscripts in this thesis.

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CHAPTER 1: INTRODUCTION

1.1 OPENING REMARKS

Uterine epithelial cells form a barrier lining the uterine lumen which is normally hostile to blastocyst implantation. At the time of implantation these cells allow for successful adhesion and invasion of the blastocyst into the underlying endometrium. This period of time in which these events occur is short and is termed the 'window of receptivity'.

Uterine epithelial cells undergo many changes in order for them to be receptive to blastocyst implantation, these changes are collectively known as the 'plasma membrane transformation' (Murphy 2001), and are regulated by the ovarian hormones oestrogen and progesterone. At the time of fertilisation the apical plasma membrane of uterine epithelial cells is lined by microvilli. Intercellular junctions are present and include the *zonula occludens* and *zonula adherens* (Murphy and Shaw 1994). The basolateral plasma membrane of luminal uterine epithelial cells is smooth with basal focal adhesions that anchor the cell to the underlying extracellular matrix. These features described are representative of a typical polarised simple columnar epithelial cell (Shion and Murphy 1995). By the time of implantation many of these structures are lost or altered. During the 'window of receptivity' the apical plasma membrane flattens out, and apical plasma membrane cholesterol is increased. On the lateral plasma membrane cell-cell junctions are altered, including an increase in depth of the tight junction (Luxford and Murphy 1992b; Murphy 2004; Murphy and Dwarte 1987; Murphy and Martin 1985). At the basal plasma membrane, a loss of basal focal adhesions and an increase plasma membrane tortuosity is observed (Shion and Murphy 1995; Kaneko et al. 2009; Kaneko et al. 2008). All these changes are associated with general actin cytoskeletal remodelling. Despite all the changes that these cells undergo, the uterine epithelial cells yet maintain a polarised epithelial phenotype.

The proteins investigated in this thesis; caveolin, PTRF, SDPR, filamin A, afadin, talin and paxillin, not only have individual functions at the plasma membrane but are also generally associated with the actin cytoskeleton. The actin cytoskeleton in uterine epithelial cells undergoes extensive remodelling leading to receptivity, as to how the actin cytoskeleton contributes to a polarised epithelium is yet to be determined.

Talin and paxillin have been previously investigated in the uterus of the rat during early pregnancy, and were localised to the basal plasma membrane on day 1 of pregnancy (Kaneko et al. 2008). Talin and paxillin were reduced at the time of implantation (Kaneko et al. 2008), which correlated with the loss of ultrastructural focal adhesions at this time (Shion and Murphy 1995).

The molecules being investigated in this thesis contribute to cellular polarisation in many cell types but have yet to be studied in UECs. As UECs undergo dramatic morphological changes leading to receptivity Therefore, the aim of this thesis is to investigate the protein expression, localisation and hormone regulation of caveolin-1, caveolin-2, PTRF, SDPR, filamin A, and afadin during early pregnancy in the rat, as well as talin and paxillin in a polarised uterine epithelial cell line to understand how these molecules contribute to the major changes which occur in UECs during the plasma membrane transformation of early pregnancy.

The following sections of this chapter will now discuss the relevant organs and proteins investigated in this thesis.

1.2 THE UTERUS

The uterus is a hormone receptive organ that receives the developing blastocyst from the uterine tube. In rats, mice and humans, the blastocyst invades into the uterine wall and foetal development continues during gestation.

The rat uterus is comprised of two uterine horns bounded by a common cervix and vagina (Mossman 1977). The uterine wall consists of three layers as observed in figure 1.1. The outer layer is the perimetrium, a serous covering of the uterus which is continuous with the abdominal and pelvic peritoneum. The myometrium is comprised of two layers of smooth muscle, an inner circular and outer longitudinal layer which are separated by connective tissue and blood vessels. The innermost layer of the uterus is the endometrium, which consists of a connective tissue stroma and uterine glands lined by simple columnar epithelium which is continuous with the luminal epithelium. The luminal uterine epithelium is important for successful embryo implantation and only makes up 5-10% of the uterus (Martin et al. 1973; Das and Martin 1973).

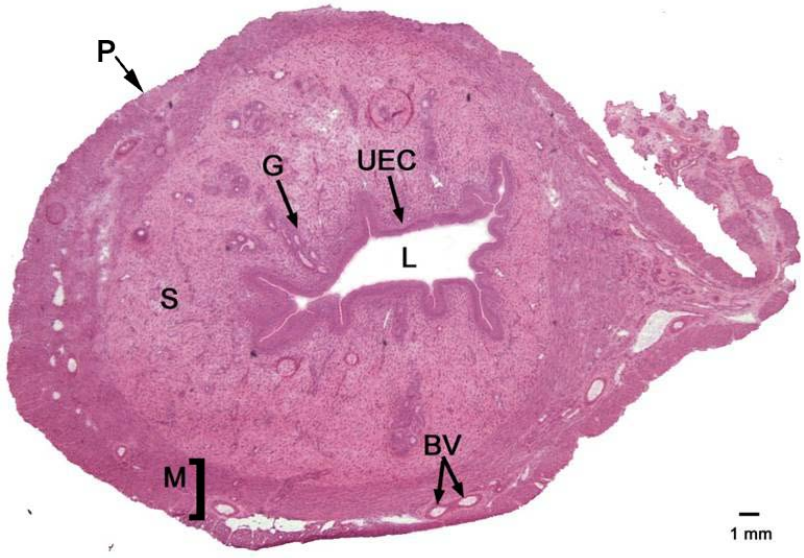

Figure 1.1 Cross section of the rat uterus

Histological cross section of a rat uterine horn stained with haematoxylin and eosin. The layers of the uterus are the perimetrium (P), two muscle layers that form the myometrium (M), the endometrium which is comprised of a connective tissue stroma (S) and endometrial glands (G). A central lumen (L) is lined by uterine epithelial cells (UECs).

1.3 THE OESTROUS CYCLE

The reproductive cycle of the female rat is an oestrous cycle, 4-5 days in length and consists of 4 stages known as pro-oestrus, oestrous, early di-oestrus, and late di-estrous (Long and Evans 1922). Each stage of the oestrous cycle are characterised by changes in the morphology and biochemistry of the uterine tube, uterine endometrium, and vaginal mucosa (Lawn 1973; Long and Evans 1922). As such, the distinctive histology of the vaginal epithelium is used to monitor the stage of the oestrous cycle by vaginal smearing (Long and Evans 1922), [figure 1.2]. Vaginal smears obtained at pro-oestrus are characterised by nucleated round epithelial cells of uniform size, this stage lasts for 12-14 hours. The stage of oestrus lasts for 25-27 hours and is characterised anucleated cornified cells present in the vaginal smear (Hubscher et al. 2005; Long and Evans 1922; Marcondes et al. 2002). Early di-oestrous is also referred to as metoestrus and lasts for 6-8 hours; the vaginal smear at this stage of the oestrous cycle consists of leukocytes and both cornified and nucleated epithelial cells in equal proportions. The vaginal smear at late di-oestrus consists predominantly of leukocytes, this stage of the oestrous cycle

persists for 55-57 hours; which comprises more than half of the oestrous cycle. (Marcondes et al. 2002; Long and Evans 1922; Hubscher et al. 2005)

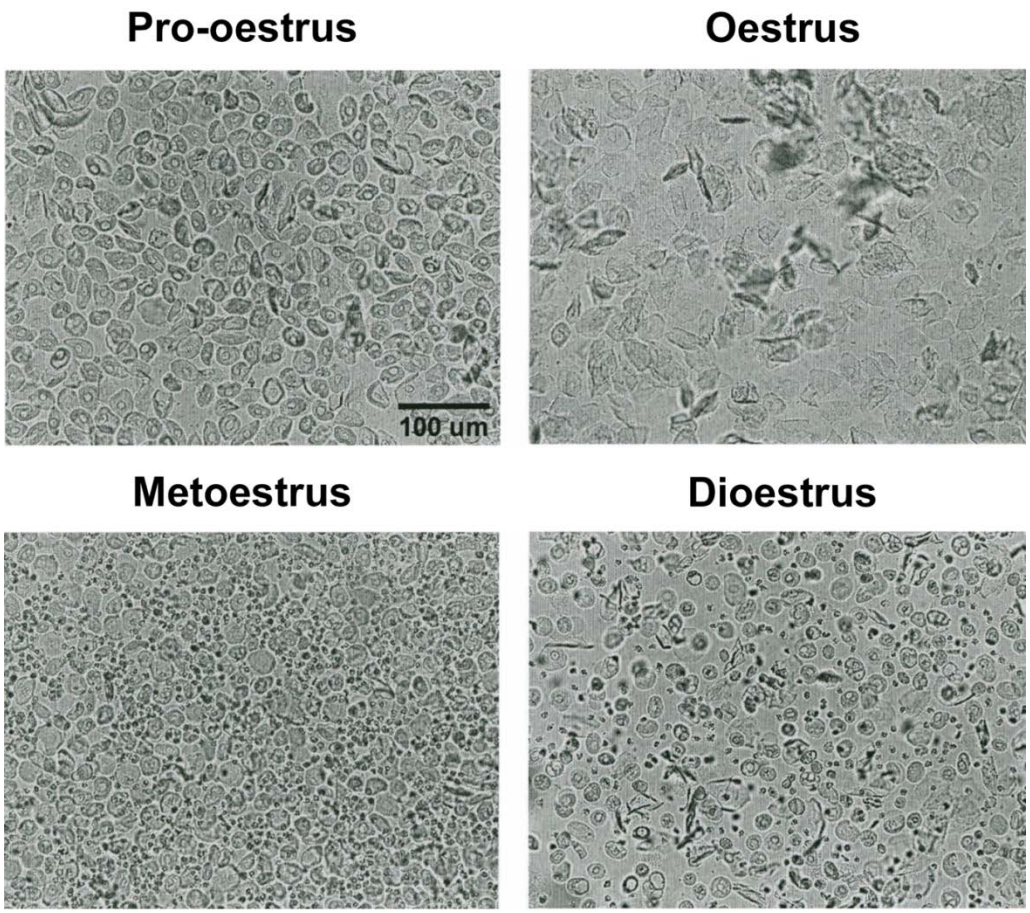

Figure 1.2 Representative vaginal smear profiles during the oestrous cycle

A representative vaginal smear showing the characteristic cell profile for each stage of the oestrous cycle in the rat. Adapted from Hubscher et al 2005.

1.3.1 ENDOCRINE CONTROL OF THE OESTROUS CYCLE

During the oestrous cycle serum levels of prolactin, luteinising hormone (LH) and follicle stimulating hormone (FSH) remain low except at pro-oestrous, where an increase in oestrogen is followed by a peak in prolactin, progestin, follicle stimulating hormone (FSH) and luteinising hormone (LH) (Smith et al. 1975; Spornitz et al. 1999; Austin and Short 1984). A graph representing these changes is shown in figure 1.3. If rats are mated during oestrus a vaginal plug is observed the following morning, and can remain for 24 hours (Long and Evans 1922). Ovulation occurs spontaneously in the rat, however, copulation is required to produce a fully functional corpus luteum (Zarrow and Clark 1968; Young et al. 1941).

Pregnancy occurs when spermatozoa and ova meet in the uterine tube (Finn and Martin 1974). This is confirmed by the presence of spermatozoa in the vaginal smear after mating. The day after mating where there are spermatozoa in the vaginal smear is designated as day 1 of pregnancy. A pseudo pregnant state can be induced if mating occurs with an infertile male, or by cervical stimulation, this state persists for 13 days (Schwartz et al. 1955; Kopia et al. 1974).

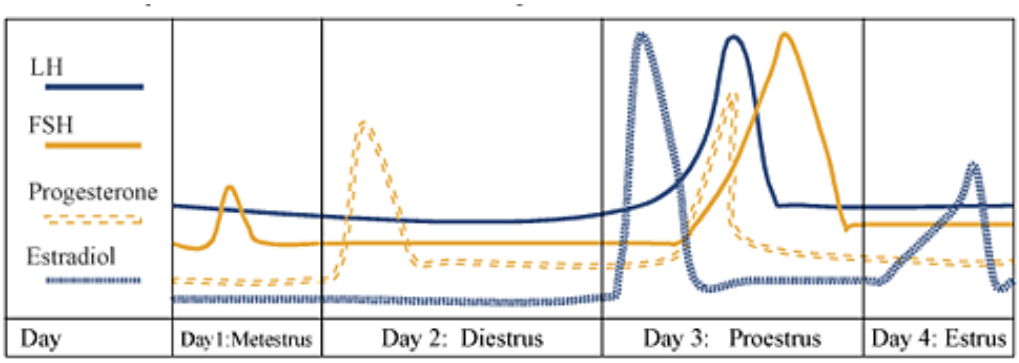

Figure 1.3 Hormonal patterns in blood plasma during the rat oestrous cycle

Ovarian hormones (oestrogen and progesterone) fluctuate during the oestrous cycle, under the control of pituitary gonadotropins (LH and FSH). Adapted from Austin and Short, (1984)

1.4 PREGNANCY

The gestation period of the rat is 22 days (Austin and Short 1984), and during the early stages of pregnancy the endometrium of the uterus undergoes many changes while the embryo develops. The uterus is primed with maternal hormones oestrogen and progesterone (Psychoyos 1973; Schlafke and Enders 1963; Psychoyos and Mandon 1971; Psychoyos 1993) which particularly affects the luminal uterine epithelial cells (UECs). These cells transition to a receptive state for a short period of time, known as the 'window of receptivity' (Psychoyos 1973). Blastocyst studies have demonstrated the 'window of receptivity' to be a short time in which blastocysts can successfully implant in the endometrium of the uterus. Blastocysts transferred to pre-receptive uteri implant successfully, while those that are transferred after this period of receptivity fail to implant (Doyle et al. 1963; Yoshinaga 1988).

The effect of progesterone and oestrogen on the uterus has been established in ovariectomised rats treated with exogenous ovarian hormones (Psychoyos 1973;

Martel et al. 1991). Ovariectomised rats treated with progesterone followed by a single dose of oestrogen have been demonstrated to be the minimal hormonal requirement for successful implantation (Psychoyos 1973; Martel et al. 1991). A state of pseudo-pregnancy can be induced in rats by mating with a sterile male or through artificial cervical stimulation (Kopia et al. 1974; Schwartz et al. 1955). During this state the corpora lutea are functional for the first 13 days of pseudo-pregnancy, after which time they degenerate due to the absence of an implanting blastocyst (Austin and Short 1984). There are structural and ultrastructural similarities between pregnant and pseudo-pregnant uteri that have been recognised (Kopia et al. 1974; Lee et al. 2007; Allen 1931; Ljungkvist and Nilsson 1971) and as such they have been used for the study of early pregnancy in this thesis.

1.5 IMPLANTATION

Implantation is a highly regulated event, and is comprised of three stages, known as apposition, adhesion and invasion (Enders and Schlafke 1967; Abrahamsohn and Zorn 1993). In each of these stages intimate contact between foetal and maternal tissues is accomplished by uterine closure and blastocyst swelling (Abrahamsohn and Zorn 1993). Different species undergo different modes of implantation, in rodents and humans invasive implantation occurs, along with displacement implantation which is seen particularly in rats and mice (Enders and Schlafke 1967; Schlafke et al. 1985; Parr et al. 1987). A schematic of the stages of implantation can be seen in figure 1.4.

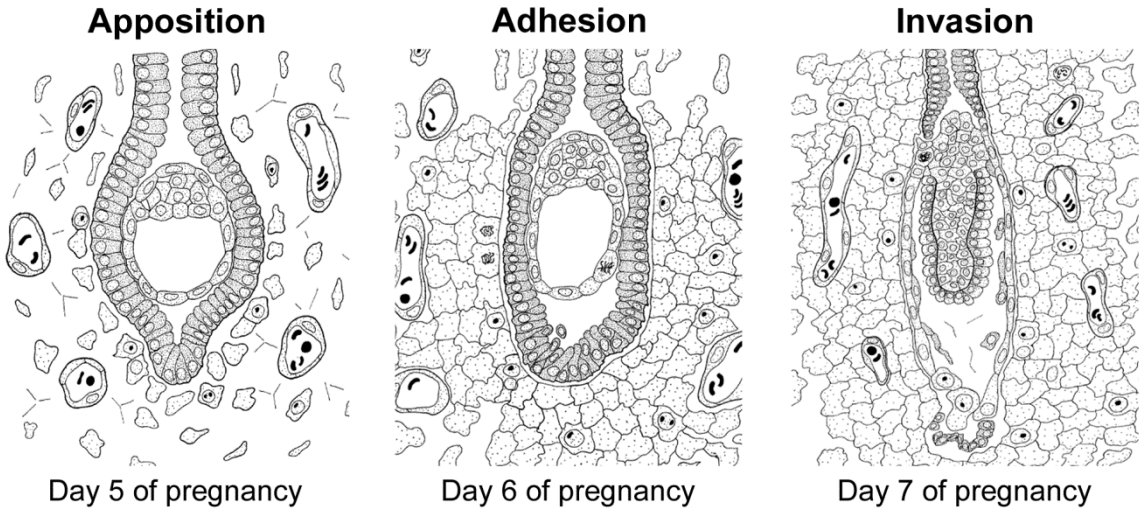

Figure 1.4 The stages of implantation

Diagram representing the implantation chamber during the three stages of implantation in the rat apposition, adhesion and invasion. Adapted from Enders and Schlafke (1967).

1.5.1 Apposition

The developing blastocyst enters the uterine lumen on day 5 of pregnancy in the rat. At this time the blastocyst loses its zona pellucida, and comes into close contact with the luminal uterine epithelium. This is facilitated by uterine lumen closure which forms the implantation chamber (Enders 1975; Enders and Schlafke 1967). Uterine closure allows for communication between blastocyst and uterine epithelial cells (Hill 2001). Blastocysts at this stage of implantation are still free in the uterine lumen as they are easily flushed without any damage to the UECs (Enders and Schlafke 1967).

1.5.2 Adhesion

Adhesion of the blastocyst to the UECs occurs on day 6 of pregnancy. Blastocysts attach to the anti-mesometrial surface of the uterine lumen with their inner cell mass orientated mesometrially (Warren and Enders 1964; Enders and Schlafke 1967; Enders et al. 1980). Adhesion between the blastocyst and UECs is accomplished by surface projections of the blastocyst which interdigitate with apical projections of the luminal uterine epithelium and later flatten out (Enders 1975) The adhesion is mediated by adhesion molecules on either the trophoblast cells of the blastocyst or the UECs or both (Aplin 1997). At this time removal of the

blastocyst from the implantation chamber will cause damage to the UECs (Enders and Schlafke 1969; Enders and Schlafke 1967). Also at this time the fibroblasts of the endometrial stroma immediately under the luminal uterine epithelium undergo cellular changes, these will be discussed later in this chapter.

1.5.3 Invasion

Invasion of the blastocyst into the underlying endometrium occurs on day 7 of pregnancy in the rat. The UECs are removed by the blastocyst to allow the trophoblast to penetrate the basal lamina and invade the underlying stroma (Tachi et al. 1970). Invasion of the blastocyst requires remodelling of the underlying stromal cells of the implantation chamber into large decidual cells which serve to restrict excessive trophoblast invasion (Enders and Schlafke 1969; Chavez et al. 1984; Abrahamsohn and Zorn 1993).

The invasive capabilities of the blastocyst have been demonstrated in other organs such as the kidney (Fawcett 1950), spleen and testis (Kirby 1963). However the uterus is not receptive to blastocyst implantation outside the window of receptivity as demonstrated in experiments involving blastocyst transfer outside this window; in which blastocysts were no longer able to implant (Doyle et al. 1963). The UECs outside the window of receptivity thus serve as an impenetrable barrier.

1.6 Epithelial Polarity

Polarised epithelial cells are characterised by the organisation of plasma membrane lipids and proteins into distinct domains (Rodriguez-Boulan and Nelson 1989). These domains, both structural and functional, consist of an apical plasma membrane that faces the lumen, a basolateral plasma membrane where junctional complexes are found. These cellular domains of UECs undergo dramatic changes, both morphological and biochemical, between non receptive and receptive states, and during this transition they lose many epithelial junctional structures and associated cytoskeletal components (Kaneko et al. 2009; Kaneko et al. 2008; Luxford and Murphy 1992b; Murphy 2001; Murphy 1995; Murphy and Dwarte 1987; Preston et al. 2004). Despite these cellular changes UECs still maintain a polarised organisation of lipids and proteins in the various membrane domains.

1.7 THE PLASMA MEMBRANE TRANSFORMATION

The UECs are the first site of contact between maternal and foetal tissues and undergo dramatic changes in their plasma membrane both morphological and molecular in order to be receptive to blastocyst implantation, these changes are regulated by ovarian hormones progesterone and oestrogen (Luxford and Murphy 1992a; Murphy et al. ; Murphy et al. 1981; Murphy and Rogers 1981). Collectively these changes are known as the 'plasma membrane transformation' and key features are shown in figure 1.5.

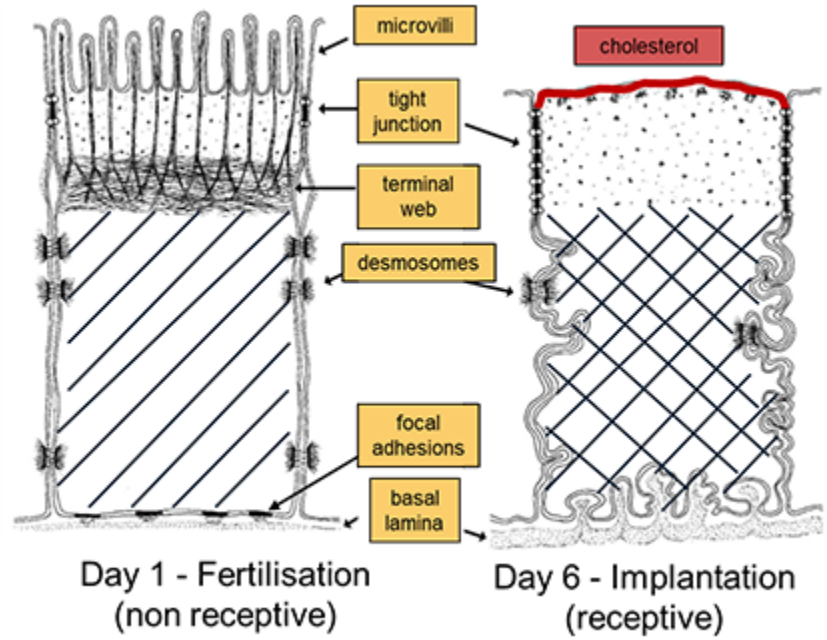

Figure 1.5 The plasma membrane transformation of uterine epithelial cells

Schematic diagram representing the changes in the morphology of a uterine epithelial cell on days 1 and 6 of pregnancy. These changes include the loss of the microvilli, alteration of the lateral junctional complex, change in plasma membrane curvature and cholesterol content but also remodelling of the actin cytoskeleton. Adapted from Murphy (2004).

On day 1 of pregnancy UECs are non-receptive to blastocyst implantation. At this time these cells have apical microvilli and associated terminal web. A glycocalyx rich in anti-adhesive molecules such as MUC-1 (Isaacs and Murphy 2003) adds to an epithelium that is not receptive to blastocyst implantation. The lateral junctions of UECs such as the adherens and tight junction (Orchard and Murphy 2002; Nicholson et al. 2010; Murphy et al. 1982), and basal focal adhesions (Rousselle et al. 1991; Shion and Murphy 1995; Kaneko et al. 2008) being intact in UECs in

addition to the appearance of the basolateral membrane being smooth are features of a non-receptive UECs.

On day 6 of pregnancy UECs are receptive to blastocyst implantation. At this time a number of morphological and molecular changes have occurred. The actin cytoskeleton has undergone extensive remodelling (Luxford and Murphy 1989, 1992b, 1993; Murphy 1995) where apical microvilli are no longer present, instead irregular protrusions are observed (Luxford and Murphy 1989, 1993). There is a loss of the apical glycocalyx and a reduction in MUC-1 protein expression (Isaacs and Murphy 2003), along with an increase in intramembranous particles and apical membrane cholesterol (Murphy and Martin 1985; Murphy and Dwarte 1987; Luxford and Murphy 1993) as observed from freeze fracture studies. The basolateral membrane at this time is highly tortuous (Shion and Murphy 1995), where there is an increase in the depth and complexity of tight junctions and their associated proteins - occludin and ZO-1 at this time (Murphy et al. 1982; Luxford and Murphy 1992a; Orchard and Murphy 2002), as well as a loss of basal focal adhesions (Kaneko et al. 2008; Rousselle et al. 1991; Shion and Murphy 1995).

The UECs during early pregnancy are not the only cells that contribute to a uterus that is receptive to blastocyst implantation. The underlying stromal cells also undergo a number of changes for successful blastocyst implantation and pregnancy, which are known as decidualisation.

1.8 DECIDUALISATION

Decidualisation involves remodelling of the endometrial stromal fibroblasts surrounding the implanting blastocyst. This process is unique to the uterus as only fibroblasts of the endometrium react in this manner to a blastocyst (Bevilacqua et al. 1991; Abrahamsohn and Zorn 1993). In humans decidualisation occurs spontaneously in a hormonally primed uterus in the absence of the blastocyst (Cornillie et al. 1985), while in other species such as the rat, decidualisation only occurs around the blastocyst. The decidual tissue is essential for the continued growth and development of the embryo (Abrahamsohn and Zorn 1993) as it serves not only to provide nourishment but also separates adjacent embryos and restricts excessive embryo invasion. The latter is achieved through two mechanisms including alterations of the extracellular matrix (Carson et al. 1992; Finn 1971) or

by the release of soluble growth regulating factors and proteases (Weiss et al. 2007; Mikhaĭlov and Malygin 1981). The decidua also provides an immunosuppressive environment where maternal immunoglobulins are excluded from the implanting blastocyst by decidual cells of the primary decidual zone (Parr et al. 1986; Tung et al. 1986).

Oedema in the endometrial stroma is the first sign of decidualisation as demonstrated by the vascular leakage from post capillary fenestrated venules of injected dyes such as pontamine sky blue (PSB); this is referred to as the PSB reaction and is used as a marker of decidualisation (Abrahamsohn et al. 1983; Christofferson and Nilsson 1988). The primary decidualisation zone refers the region where sub-epithelial stromal cells undergo alterations prior to the PSB reaction. Decidualisation of these endometrial stromal cells (ESCs) involves the transition from long thin fibroblast cells to large and rounded epitheliod like cells known as decidual cells (Parr et al. 1986; Finn 1971). This process involves formation of adherens type junctional structures at sites of contact between adjacent decidual cells (Finn and Keen 1963; Finn and Lawn 1967; Lundkvist and Ljungkvist 1977; Tung et al. 1986) and an accumulation of glycogen, lipids and fibrillar material arranged into a filamentous meshwork inside decidual cells (Kearns and Lala 1983). These changes also involve remodelling of the actin cytoskeleton, which is mediated by actin binding proteins such as α-actinin in decidual cells (Shaw et al. 1998; Terry et al. 1996; Loo et al. 1998). As pregnancy progresses decidualisation of the endometrium continues outwards to the myometrium (Abrahamsohn et al. 1983), with a fully functional decidua present by day 10 of pregnancy in the rat (Welsh and Enders 1985). A diagram of the decidualisation zones seen on day 8 of pregnancy is shown in figure 1.6.

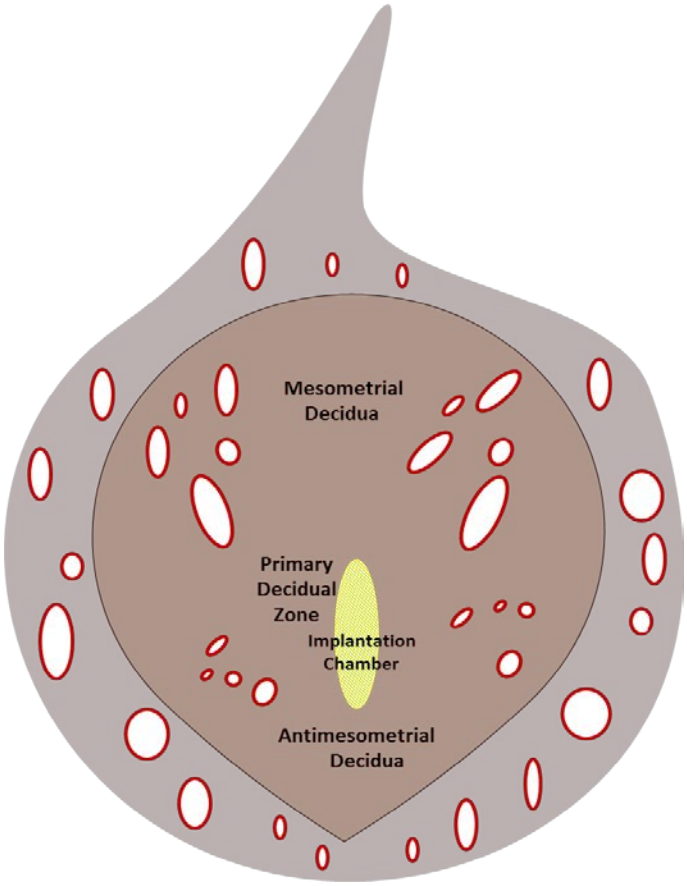

Figure 1.6 Illustration of developed decidua of pregnant rats on day 8 of pregnancy.

An illustration depicting the primary decidual zone, mesometrial and antimesometrial decidua on day 8 of pregnancy. Adapted from Abrahamsohn and Zorn (1993); Lundkvist and Ljungkvist (1977).

The mechanism by which these agents induce decidualisation is unclear. Artificial deciduogenic agents such as oil require a nidatory oestrogen surge for the development of deciduomata (Finn and Keen 1963). However, it does cause some damage to the uterine mucosa following application and as such may result in the release of inflammatory mediators which may initiate decidualisation (Shaw et al. 1998; Lundkvist and Ljungkvist 1977; Lundkvist 1978).

Both embryo and artificially induced deciduomata are similar in structure as well as the period of sensitivity to decidualisation and minimal hormonal requirements (Jollie and Bencosme 1965; FINN and HINCHLIFFE 1965; Finn 1971; Lundkvist and Ljungkvist 1977; Lundkvist and Nilsson 1982). Thus, artificial induction of decidualisation is a useful tool to investigate the decidualisation reaction.

1.9 THESIS FOCUS

The process of how UECs maintain epithelial polarity during the plasma membrane transformation when there is loss of numerous membrane junctional structures and related cytoskeletal elements of these junctions (Murphy 2001; Murphy 2004; Murphy and Shaw 1994) is intriguing. This thesis will focus on the changes that these cells undergo and the associated molecules and structures which serve to maintain a polarised epithelial state. The following sections of this introduction will discuss relevant structures and molecules investigated in this thesis.

1.9.1 Lipid Raft and Lipid Distribution

Lipid rafts are membrane microdomains that are enriched in cholesterol and saturated glycosphingolipids (Simons and Sampaio 2011; Causeret et al. 2005). Lipid rafts enable proteins to cluster at the plasma membrane, facilitating increased ligand affinities (Lang et al. 1998) and as such are involved in cell polarisation (Gómez-Moutón et al. 2004; Viola and Gupta 2007). UECs exhibit an elevated concentration of glycosphingolipids and cholesterol at the apical surface at the time of implantation which may provide a rigid membrane (Petrache et al. 2005; Tabas 2002). Flotillin, a lipid raft marker, has been found to shift apically at the time of implantation from the basal plasma membrane (Lecce et al. 2013), suggesting that a polarised distribution of lipids and lipid rafts may be a mechanism to maintain epithelial integrity at a time when junctional structures such as focal adhesions (Kaneko et al. 2011; Kaneko et al. 2009) and desmosomes (Preston et al. 2004) and associated proteins are down regulated to allow successful implantation.

1.9.1.1 Caveolae

Caveolae are omega (Ω) shaped invaginations of the plasma membrane that are 100 nm in diameter. They were first observed in endothelial cells (Palade 1953) and also the epithelial cells of the gall bladder (Yamada 1955). Caveolae are considered to be specialised plasma membrane subdomains that are enriched in cholesterol, glycosphingolipids and GPI anchored proteins. These structures have been observed in a number of cell types and are involved in endocytosis, signal transduction, and cholesterol and lipid transport (Sargiacomo et al. 1993; Parton

et al. 1994; Murata et al. 1995; Smart et al. 1995; Fra et al. 1995; Schlegel et al. 1998).

1.9.2 Actin Cytoskeleton

The actin cytoskeleton plays an important role in the formation of a number of key cellular structures such as the terminal web, microvilli, and cellular junctions, which form the basis of cell polarity and plasma membrane stability (Li and Gundersen 2008).

Remodelling of the actin cytoskeleton occurs in UECs during the transition from a non-receptive to a receptive state (Luxford and Murphy 1992b; Terry et al. 1996). Actin cytoskeletal changes are mediated by actin binding proteins, a number of which have been investigated in the uterus during early pregnancy including plectin and α-actinin, which both localise apically in UECs at the time of implantation (Terry et al. 1996; Murphy and Shaw 1994)

1.9.3 Cellular Junctions

Cellular junctions such as the adherens junction and the tight junction serve to separate the apical domain from the rest of the cell. In addition to this, these structures also contribute to a polarised epithelial phenotype.

1.9.3.1 Tight Junction

The tight junction (*zonula occludens*) of polarised epithelial cells is responsible for the formation of an apical seal between adjacent epithelial cells (Fanning et al. 1999). Tight junctions control the diffusion of proteins and molecules between apical and basolateral cell membranes and regulate ion, water and molecular transport through the paracellular pathway (Tsukita et al. 2009). Claudin, occludin and tricellulin are key proteins of the tight junctional complex. Tight junctions in UECs have been shown to increase in depth at the time of implantation when compared to the time of fertilisation (Murphy et al. 1982).

1.9.3.2 Adherens Junction

The adherens junction (*zonula adherens*) is a lateral junction that links the actin cytoskeleton of adjacent UECs and functions to maintain cell to cell contact and resists mechanical stresses (Hartsock and Nelson 2008). The adherens junction is comprised of two adhesion complexes which are the E Cadherin-Catenin complex

and the Nectin-Afadin-Ponsin (NAP) complex (Asakura et al. 1999). At cell-cell adherens junctions cadherins interact with each other at the extracellular surface in a Ca^{2+} dependent manner as well as with the actin cytoskeleton at the cytoplasmic interface through F-actin binding peripheral membrane proteins α-actinin, α/β-catenin, and vinculin (Asakura et al. 1999; Li et al. 2002).

1.9.3.3 Focal adhesions

Focal adhesions were observed as electron dense plaques between the cell and the substratum in cultured cells and were considered at first to be an artefact, as they were not so easily observed *in vivo* (Abercrombie and Dunn 1975; Abercrombie et al. 1971; Burridge et al. 1988). Focal adhesions are comprised of a number of structural, signalling adaptor and scaffolding proteins which serve to link cytoskeletal actin fibres and the extracellular matrix (ECM) (Burridge and Chrzanowska-Wodnicka 1996). Adhesion to the ECM is mediated primarily by integrins which cluster with other proteins at focal adhesions. Focal adhesions have been observed in many cell types including fibroblasts, endothelial, and epithelial cells (Burridge et al. 1988). There is similarity between cells adhering to substratum *in vitro* and those adhering to an extracellular matrix (ECM) *in vivo* (Burridge et al. 1988). This is evident in smooth muscle cells that display dense plaques where focal adhesion proteins vinculin, and talin were identified (Burridge et al. 1988; Burridge and Chrzanowska-Wodnicka 1996; Burridge and Connell 1983a, b; Geiger et al. 1984; Elad et al. 2013). Focal adhesion proteins vinculin, talin and paxillin have been used as markers of focal adhesions and hence allow visualisation of these structures (Burridge et al. 1988; Turner et al. 1990; Burridge and Tsukita 2001; Dubash et al. 2009; Geiger et al. 1984; Elad et al. 2013). A schematic representation of focal adhesion is shown in figure 1.7.

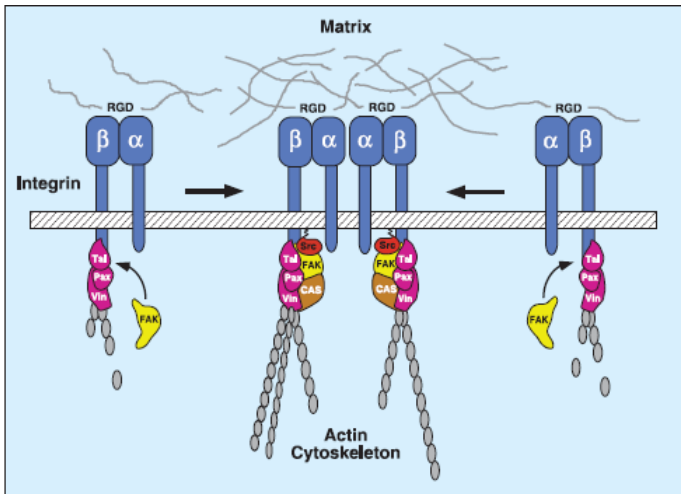

Figure 1.7 Schematic representation of focal adhesions

The focal adhesion is comprised of an adhesion complex, which provides a structural link between the intracellular actin stress fibres and the ECM. Upon integrin mediated activation, numerous focal adhesion proteins are recruited to sites of focal adhesions, and thus provide a central site for signal transduction. Adapted from Giancotti and Ruoslahti (1999)

1.10 CAVEOLIN

Caveolin is an integral membrane protein which is a major component of omega shaped invaginations of the plasma membrane known as caveolae. There are three members of the caveolin family caveolin-1, caveolin-2 and caveolin-3 (Williams and Lisanti 2004). Caveolin-1 and caveolin-2 are expressed ubiquitously, and caveolin 3 is expressed in striated muscle cell types (cardiac and skeletal) (Song et al. 1996).

1.10.1 Structure

When inserted in the plasma membrane, caveolin assumes a hairpin like orientation with both the N- and C-termini cytoplasmically orientated (Rothberg et al. 1992; Sargiacomo et al. 1995; Scherer et al. 1995). Caveolin consists of a membrane bound domain and a scaffolding domain in addition to the N and C termini (Stan 2005). The scaffolding domain of caveolin is essential for the formation of both homo and hetero oligomers of caveolin which range between 350-450 kDa in size; and is also the region responsible for the interaction of caveolin with cholesterol and signalling molecules (Murata et al. 1995; Sargiacomo et al. 1995; Engelman et al. 1998). Post-translational modifications of caveolin include palmitoylation on three cysteine residues, phosphorylation on tyrosine 14

and 19 residues and serine 22 and 23 residues (Okamoto et al. 1998; Sargiacomo et al. 1993; Li et al. 1996). A schematic diagram of caveolin and its isoforms can be seen in figure 1.8.

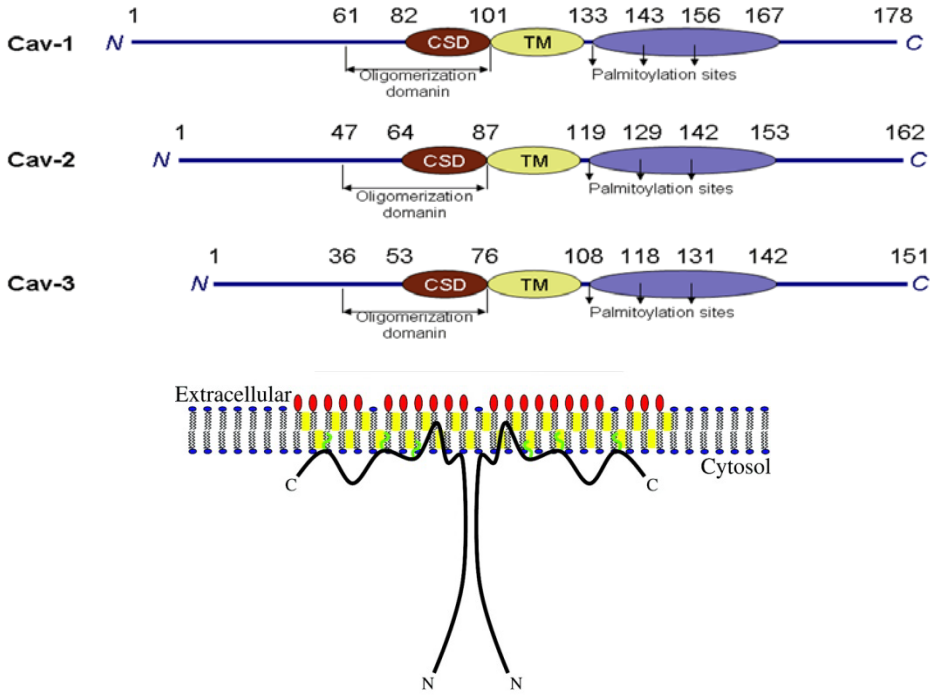

Figure 1.8 Schematic Diagram of domain structure of Caveolin proteins and membrane insertion.

Caveolin proteins differ in the length of their N-terminus. They all share a scaffolding domain (CSD) and transmembrane domain. When caveolin is inserted in the membrane both N and C termini are cytoplasmically oriented. Adapted from Branza-Nichita et al. (2012); Cohen et al. (2004).

1.10.2 In Epithelia

Caveolin 1 and 2 have been found in a number of tissues. Polarised epithelial cells of the retina, small intestine, kidney, and lung all have morphological caveolae and caveolin proteins (Breton et al. 1998; Andoh et al. 2001; Orlichenko et al. 2005; Mora et al. 2006; Hackett et al. 2013; Krasteva et al. 2006). The function of caveolin proteins and morphological caveolae include endocytosis, cholesterol transport throughout the cells, and also signal transduction (Sargiacomo et al. 1993; Parton et al. 1994; Murata et al. 1995; Smart et al. 1996).

1.10.3 In Reproduction

Caveolin is present in a number of polarised epithelial cells (Schlegel et al. 1998), , given the functions of caveolin mentioned earlier, caveolin may be involved in the

development of uterine receptivity. Caveolin has only been investigated in the myometrium of ovariectomised rats with regards to its mRNA expression (Turi et al. 2001).

Given this and previous studies documenting caveolin in epithelia of other tissues, this thesis focused on the cellular localisation and protein expression of caveolin 1 and 2 in the endometrium during early pregnancy and the hormonal control of these proteins in the rat.

1.11 CAVINS

Cavins are a family of peripheral membrane proteins (Hayer et al. 2010) of which there are four members. PTRF (RNA pol I transcription factor) also known as cavin 1; SDPR (serum deprivation protein response) also known as cavin 2; SBRC (sdr related gene product that binds to C kinase) also known as cavin 3; and MURC (muscle restricted coiled-coiled protein) also known as cavin 4. Cavin proteins are conserved among mammals and the homology between family members is found within the N terminus (Bastiani et al. 2009). All members of this family have been found to be present in caveolar membranes (Bastiani et al. 2009; Liu and Pilch 2008; Hill et al. 2008; Hansen et al. 2009; McMahon et al. 2009) where they serve to regulate caveolae formation and dynamics (Briand et al. 2011).

1.11.1 Structure

1.11.1.1 PTRF

PTRF was originally identified as a nuclear factor that regulates transcription in cells *in vitro* (Jansa and Grummt 1999) where it induces the dissociation of transcription complexes and facilitates the reinitiation of RNA polymerase I (Aboulaich et al. 2004; Jansa et al. 2001; Jansa and Grummt 1999). It was found to be associated with caveolae in adipocytes at first, and later in other tissues (Voldstedlund et al. 2001) where it stabilised caveolae at the plasma membrane (Hill et al. 2008; Liu and Pilch 2008). PTRF consists of two PEST domains (sequence enriched in proline, glutamic acid, serine and threonine) which are known to serve as a recognition domain for proteolysis; a nuclear localisation signal domain and also putative leucine zippers which are involved in dimerization and protein-protein interactions associated with DNA binding (Hansen and

Nichols 2010; Aboulaich et al. 2004; Tan and Richmond 1990). The structural domains of PTRF are shown in figure 1.9.

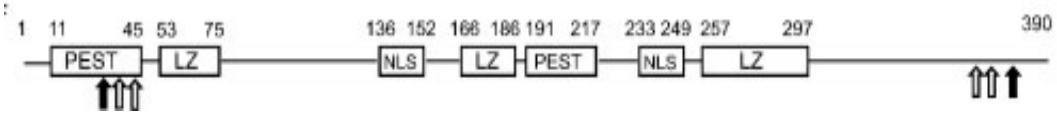

Figure 1.9 Schematic diagram showing the domain structure of PTRF

PTRF is comprised of two PEST domains (sequence enriched in proline, glutamic acid, serine and threonine); a nuclear localisation signal domain (NLS) and also putative leucine zippers (LZ). Adapted from Aboulaich et al (2004).

1.11.1.2 SDPR

SDPR is a protein kinase C (PKC) binding protein involved in the targeting of αPKC to caveolae (Mineo et al. 1998). SDPR shares more than twenty percent homology with PTRF and has been found to co-localise with caveolin-1 and is enriched in isolated caveolar membranes (Briand et al. 2011; Hansen et al. 2009; Hansen and Nichols 2010). While SDPR alone does not promote caveolar formation it does affect the morphology of caveolae due to its targeting of αPKC which regulates membrane invagination (Mineo et al. 1998; Aboulaich et al. 2004). SDPR consists of two PEST domains, and a putative leucine zipper (Hansen and Nichols 2010; Aboulaich et al. 2004; Tan and Richmond 1990). The structural domains of SDPR are shown in figure 1.10**.**

Figure 1.10 Schematic diagram showing the domain structure of SDPR

SDPR is comprised of two PEST domains (sequence enriched in proline, glutamic acid, serine and threonine), and a putative leucine zipper (LZ) Adapted from Aboulaich et al (2004).

1.11.2 In Epithelia

1.11.2.1 PTRF

PTRF has been observed in prostate epithelium and the androgen-independent prostate cancer cell line (PC3) (Gould et al. 2010; Moon et al. 2013), as well as in epithelial cells of the lung (Liu et al. 2008) where it was associated with morphological caveolae. Caveolae formation in cells lacking PTRF has been shown to be reduced; as such, PTRF expression has been demonstrated to be important for the formation of caveolae where association with caveolin at the plasma

membrane generated membrane curvature (Vinten et al. 2005; Hill et al. 2008; Liu and Pilch 2008; Chadda and Mayor 2008).

1.11.2.2 SDPR

SDPR has been observed in epithelial cells of the mammary and prostate glands as well as kidney tubules (Li et al. 2008; Mineo et al. 1998). SDPR expression in these tissues was found to be suppressed in cancerous epithelial cells of breast, kidney and prostate (Li et al. 2008). SDPR has been demonstrated to induce membrane curvature and caveolae membrane tubulation (Hansen et al. 2009).

1.11.3 In Reproduction

PTRF and SDPR have not been previously investigated in the uterus, and have yet to be investigated during pregnancy. PTRF and SDPR have been documented to induce membrane curvature in other cells (Hansen et al. 2009). At the time of implantation there is an increase in tortuosity of the basolateral plasma membrane of UECs (Murphy and Shaw 1994; Shion and Murphy 1995; Murphy 2001), PTRF and SDPR are likely candidates that facilitate this increase in membrane curvature and as such this is the first study to investigate PTRF and SDPR in the uterus during early pregnancy.

1.12 Focal Adhesion Proteins

1.12.1 Structure

1.12.1.1 Talin

Talin is a high molecular weight homodimer (Beckerle et al. 1987) that binds to multiple focal adhesion proteins including vinculin, integrins, focal adhesion kinase (FAK) and actin (Hemmings et al. 1996; Bubeck et al. 1997; Patil et al. 1999; Calderwood et al. 1999; Tadokoro et al. 2003; Nayal et al. 2004). Talin consists of a C-terminal rod domain and N-terminal head domain which includes the FERM domain with three subdomains F1, 2 and 3 (Calderwood et al. 2003; Garcia-Alvarez et al. 2003). The F3 domain in particular is the region that binds to cytoplasmic tails of β integrins for integrin activation (Calderwood et al. 1999; Garcia-Alvarez et al. 2003). The structural domains of Talin are shown in figure 1.11.

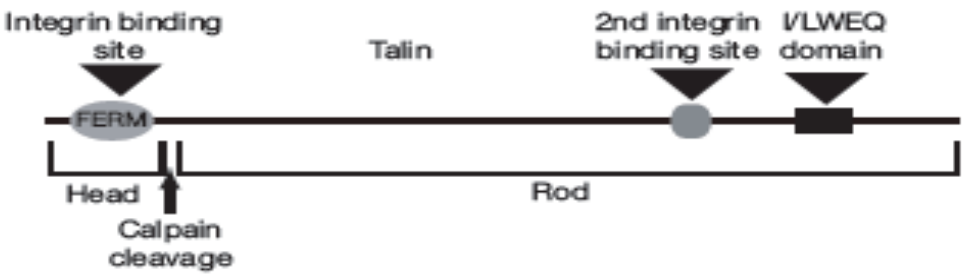

Figure 1.11 Schematic diagram of showing the domain structure of talin.

Talin is comprised of a 47 kDa N-terminus head and a 190 kDa C-terminus rod domain, the junction between these two regions is the site for cleavage of talin by calpain 2. The FERM domain is responsible for binding with β integrin tails. Adapted from Tanentzapf and Brown (2006).

1.12.1.2 Paxillin

Paxillin was identified as a substrate for the non-receptor tyrosine kinase oncogene pp60v-Src on Rous sarcoma virus transformed fibroblasts (Glenney 1989). Paxillin is comprised of five leucine rich LD motifs at the N terminus, which are important in protein recognition. The C-terminus contains four LIM domains, which serve to mediate protein-protein interactions (Brown et al. 2005; Brown and Turner 2004; Tumbarello et al. 2005; Turner 2000b). Phosphorylation of paxillin at multiple tyrosine and serine/threonine sites is responsible for regulating the localisation of paxillin to focal adhesions; particularly serine/threonine at LIM 2 and 3 domains (Dawid et al. 1998; Brown and Turner 2004; Turner 2000b). There are four alternative splice variants of paxillin, paxillin α, β, γ and δ (Brown and Turner 2004; Mazaki et al. 1997; Yamaguchi et al. 1997). Paxillin β and γ have an additional short sequence at the N-terminus of paxillin α, while paxillin δ lacks two tyrosine phosphorylation sites (Tumbarello et al. 2005). The domain structure of paxillin is shown in figure 1.12.

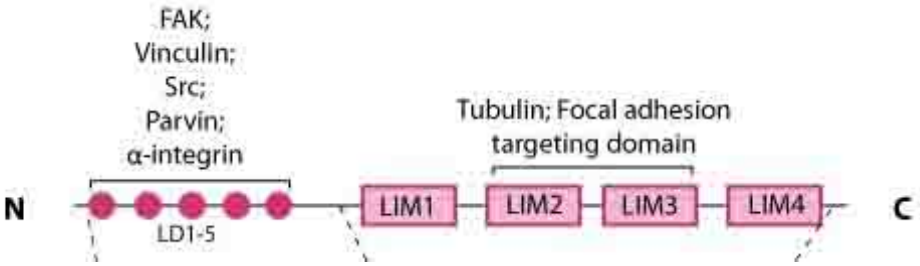

Figure 1.12 Schematic diagram showing the domain structure of paxillin

Paxillin is comprised of five leucine rich LD motifs at the N terminus which are important in protein recognition, the C-terminus contains four LIM domains which serve to mediate protein-protein interactions and target paxillin to focal adhesions. Adapted from Turner (2000).

1.12.2 In Epithelia

1.12.2.1 Talin

Talin has been observed in epithelia from a number of organs such as the cornea (Paallysaho and Williams 1991), kidney (Nuckolls et al. 1990), skin (Hentula et al. 2001) and mammary glands (Wang et al. 2011).

1.12.2.2 Paxillin

Paxillin has been observed in epithelia of the cornea (Kimura et al. 2008; Teranishi et al. 2009), stomach, colon, mammary and salivary glands and skin (Yuminamochi et al. 2003). Paxillin has been shown to play an important role in cell migration, a process that requires remodelling of the actin cytoskeleton (Turner 2000a, b).

1.12.3 In Reproduction

Talin and paxillin have been investigated previously in the rat uterus during early pregnancy; where both talin and paxillin were found to be concentrated at the basal plasma membrane at the time of fertilisation, and were lost from sites of focal adhesions at the time of implantation (Kaneko et al. 2009; Kaneko et al. 2008).

1.13 Filamin A

Filamin A (FLNA) is an F-actin cross linking protein that forms orthogonal networks of actin *in vitro* and *in vivo* (Davies et al. 1978; Feng and Walsh 2004; van der Flier and Sonnenberg 2001).

1.13.1 Structure

FLNA is comprised of two 280 kDa subunits that form homodimers at the C-terminus, the N- terminus contains an actin binding domain where orthogonal actin networks are arranged *in vitro* (Boxer and Stossel 1976; Gorlin et al. 1990; Hartwig and Stossel 1975; Stossel et al. 2001). FLNA has been reported to interact with a number of proteins (van der Flier and Sonnenberg 2001) that are membrane receptors for cell signalling molecules such as integrins (Cunningham et al. 1992; Gorlin et al. 1990; Stossel et al. 2001). The domain structure of filamin A is seen in figure 1.13.

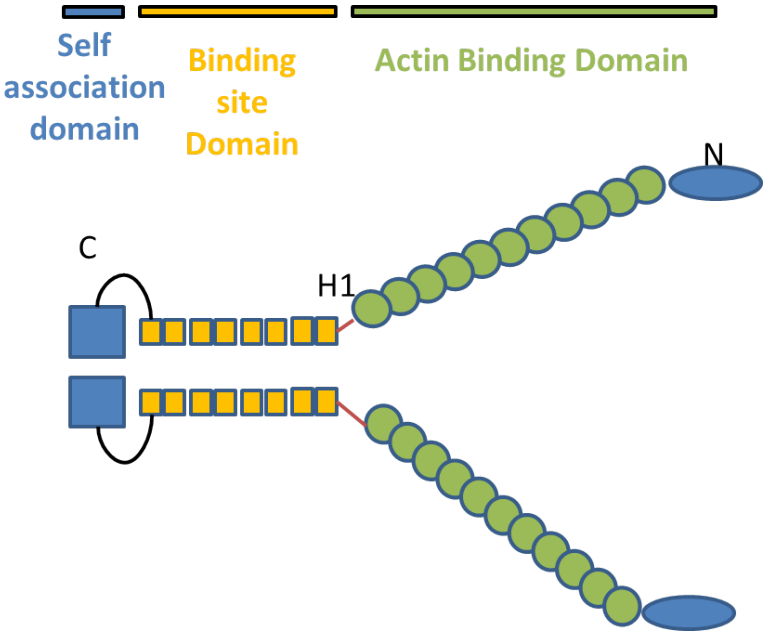

Figure 1.13 Schematic diagram showing the domain structure of filamin A.

FLNA is comprised of two 280 kDa subunits that form homodimers at its self-association domain at the C-terminus, and interacts with actin via the actin binding domain in the N- terminus. Adapted from van der Flier and Sonnenberg (2001)

1.13.2 In Epithelia

FLNA has a wide tissue distribution. It has been found in epithelial cells of the lung intestine (Bretscher and Weber 1978), kidney (Torrado et al. 2004), mammary gland (Gehler et al. 2009; Donaldson et al. 2002) and testis (Mittal et al. 1987). FLNA in these cells cross links F-actin to form orthogonal networks, and hence contributes to the structural integrity of cells (Esue et al. 2009).

1.13.3 In Reproduction

FLNA has not been investigated in the uterus and has yet to be studied with regards to implantation. Considering the number of actin cytoskeletal changes that luminal uterine epithelial cells and stromal cells undergo leading to implantation (Luxford and Murphy 1992a, 1989; Shaw et al. 1998; Venuto et al. 2008), FLNA is likely to be involved in facilitating remodelling of the actin cytoskeleton, and hence contribute to the maintenance of a polarised epithelium during early pregnancy.

1.14 Afadin

Afadin is an F-actin binding protein found to be associated with the adherens junction where it forms a complex with nectin and ponsin (Tanaka-Okamoto et al. 2011; Mandai et al. 1997; Hartsock and Nelson 2008).

1.14.1 Structure

The largest afadin protein is called I-afadin; it binds to F-actin through its F-actin binding domain in the C-terminus. I-afadin associates with other proteins through its other protein domains, which include two Ras-associated domains, fork head-associated domain, dilute domain, PDZ domain, and three proline rich domains (Takai et al. 2008; Toyoshima et al. 2014). The domain structure of afadin is shown in figure 1.14.

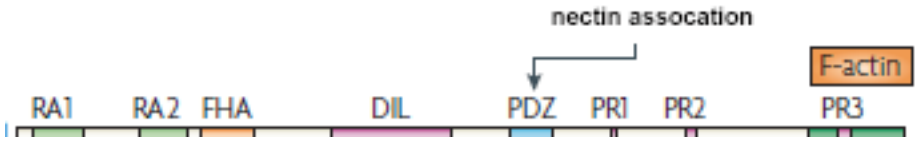

Figure 1.14 Schematic diagram showing the domain structure of afadin.

Afadin interacts with a number of proteins through its many protein domains these include two Ras-associated domains (RA1, RA2), fork head-associated domain (FHA), dilute domain (DIL), PDZ domain, and three proline rich domains (PR1, PR2, PR3). Binding to F-actin is mediated by the F-actin binding domain in the C-terminus. Adapted from Takai et al (2008).

1.14.2 In Epithelia

Afadin is localised to the apical junctions of luminal epithelial cells of the small intestine (Ikeda et al. 1999; Tanaka-Okamoto et al. 2011) and human colon cancer cell lines (Sun et al. 2014). In particular afadin has been investigated in polarised epithelia due to its association with cell-cell junctions such as the adherens junction. As was mentioned earlier, lateral membrane junctions are a characteristic of a polarised epithelial cell phenotype (Tsukita et al. 2009).

1.14.3 In Reproduction

Afadin has not been investigated in the uterus; however, due to its association with F-actin as well as α catenin and β catenin, two proteins that are localised to the adherens junction (Hartsock and Nelson 2008), afadin has been purported to be associated with such cell-cell contacts in uterine epithelium (Singh and Aplin

2009). The association of afadin to the adherens junction in other cells where it binds to nectin, is of interest in UECs where the morphological adherens junction is lost at the time of implantation.

1.15 Aims

Each of the molecules mentioned here participates in cell polarity in other epithelial cells; and as such their potential involvement in the changes that UECs undergo during early pregnancy is investigated in this thesis. This thesis aims to investigate the localisation, expression and hormonal regulation of caveolin-1, caveolin-2, PTRF, SDPR, filamin A, and afadin during early pregnancy in the rat. The results of this study will help elucidate how the UECs maintain epithelial polarity during early pregnancy.

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CHAPTER 2: MATERIALS AND METHODS

2.1 ANIMALS

All Wistar rats used in the following studies were housed in plastic cages in a controlled environment, which was kept at 21°C and were subjected to a 12 hour light/dark cycle. Rats had access to food and water *ad libitum* and all procedures were approved by the Animal Care and Ethics Committee of the University of Sydney.

2.2 VAGINAL SMEARING

Vaginal smearing was carried out on virgin female Wistar rats 10-14 weeks of age at 1630 hours to determine whether they were in pro-oestrus, which is when they are receptive to mating. Vaginal smearing was accomplished by flushing a small amount of water into the vagina and pulled back into a rounded glass pipette. The vaginal smear was then examined under a light microscope to determine the stage of the oestrus cycle as has been documented previously (Long and Evans 1922). Rats that displayed a vaginal smear characteristic of pro-oestrus were placed in the same cage with a male Wistar rat of proven fertility, or a vasectomised male for mating to induce pregnancy or pseudo-pregnancy respectively.

2.3 PREGNANCY

The following morning at 0900 hours, rats were examined for a vaginal plug and subjected to vaginal smearing to detect the presence of sperm. If no sperm was detected, cells in the vaginal smear would indicate that the oestrus cycle had progressed into di-oestrus which is characterised by some large, round nucleated epithelial cells and many small leukocytes. If sperm was present in the vaginal smear obtained this was designated as the morning of day 1 of pregnancy. Rats were then sacrificed on the desired day of pregnancy required for the following studies.

2.4 PSEUDO PREGNANCY

Female Wistar rats used for pseudo pregnancy were mated overnight with a vasectomised male. The following morning rats were checked to a vaginal plug which signified that mating had occurred and represented morning of day 1 of pseudo pregnancy. Vaginal smears were taken each day of pseudo pregnancy to

ensure that a pseudo pregnant state remained. Rats were then sacrificed on day 6 of pseudo pregnancy.

2.5 OVARIECTOMY

Female Wistar rats 12 weeks of age within the weight range of 250 – 300 g were bilaterally ovariectomised under anaesthetic by an intraperitoneal injection of Ketamine (75 mg/kg) and Xylazine (4 mg/mL). Fur was removed using an electric shaver, skin was cleaned with 70% ethanol and a small incision of 1 cm was made laterally, below the ribs. The ovary was exposed and removed with scissors. The uterine horn was replaced into the abdominal cavity and the incision was closed using braided, 25 mm siliconised silk thread on a round bodied needle. The skin was then stapled using 9 mm stainless steel wound clips. Rats were left to recover for 3 weeks at which point hormones were administered by a subcutaneous injection into the rump of the rat. Rats were randomly assigned to four different hormone regimes summarised in Table 2.1. All hormones were dissolved in a solution of benzyl alcohol and peanut oil made in a 1:4 ratio to achieve doses within the normal physiological range (Ljungkvist and Nilsson 1971; Ljungkvist 1971a, 1972). All animals were injected for three consecutive days with either 0.1 mL peanut oil and benzyl alcohol alone; 0.1 mL peanut oil and 0.5 μg 17β – oestradiol alone; 0.1 mL peanut oil with 5 mg of progesterone alone; or 0.1 mL peanut oil containing 5 mg progesterone for 3 days and an additional injection of 0.5 μg 17β-oestradiol on day 3 of treatment. The combined hormonal regime of both progesterone and oestrogen administered to rats is the minimal hormonal conditioning required to facilitate a receptive uterine epithelium (Ljungkvist 1971a, b). All animals were killed on the morning of the fourth day 24 hours after the last injection.

Table 2.1 Hormonal regimes administered to ovariectomised rats

Treatment	**Day 1**	**Day 2**	**Day 3**	**Day 4**
Vehicle alone (C)	Vehicle	Vehicle	Vehicle	Sacrifice
Progesterone alone (P)	Progesterone	Progesterone	Progesterone	Sacrifice
Oestrogen alone (E)	Oestrogen	Oestrogen	Oestrogen	Sacrifice
Combined Progesterone and Oestrogen (PE)	Progesterone	Progesterone	Progesterone Oestrogen	Sacrifice

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CHAPTER 3: Caveolins redistribute in uterine epithelial cells during early pregnancy in the rat: *An epithelial polarisation strategy?*

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3.1 ABSTRACT

At the time of implantation, uterine luminal epithelial cells undergo a dramatic change in all plasma membrane domains. Changes in the basolateral plasma membrane at the time of implantation include progression from smooth to highly tortuous, as well as a loss of integrin based focal adhesions. Another aspect of the basolateral plasma membrane which has not been studied in uterine epithelial cells are caveolae which are omega shaped invaginations of the plasma membrane, and are known to be involved in endocytosis and contribute to membrane curvature. The current study investigated caveolin, a major protein of caveolae, to explore the possible roles that they play in the remodelling of the basolateral plasma membrane of uterine epithelial cells during early pregnancy in the rat. Morphological caveolae were found at the time of implantation and were significantly increased compared to day 1 of pregnancy. Caveolin 1 and 2 were found to shift to the basolateral plasma membrane of uterine epithelial cells at the time of implantation as well as when treated with progesterone alone, and in combination with oestrogen. A statistically significant increase in the amount of caveolin 1 and a decrease in caveolin 2 protein in uterine epithelial cells was observed at the time of implantation. Caveolin 1 also co-immunoprecipitated with integrin β1 on day 1 of pregnancy, which is a protein that has been reported to be found in integrin-based focal adhesions at the basolateral membrane on day 1 of pregnancy. The localisation and expression of caveolin 1 at the time of implantation is consistent with the presence and increase of morphological caveolae seen at this time. The localisation and expression of caveolin 1 and 2 in luminal uterine epithelium at the time of implantation suggest a role in trafficking proteins and the maintenance of a polarised epithelium.

3.2 INTRODUCTION

The luminal uterine epithelial cells (UECs) are the first site of contact between maternal and foetal tissues, and as such they undergo a series of alterations in order for them to become receptive to blastocyst implantation and subsequent invasion. These changes from a non-receptive columnar epithelial cell to a receptive cell include an increase in basolateral membrane tortuosity, a loss of basal focal adhesions, a redistribution of lipid and cholesterol and also the presence of vesicles of various sizes which are at their greatest number during the pre-implantation period (Kaneko et al. 2008; Murphy 2004; Murphy and Martin 1985).

Caveolin is a major protein present in omega-shaped invaginations of the plasma membrane known as caveolae and are considered to be specialised plasma membrane subdomains that are enriched in cholesterol, glycosphingolipids and GPI (Glycosylphosphatidylinositol) anchored proteins. There are three members of the caveolin family of proteins, caveolin -1, 2, and 3. Caveolin 1 and 2 expression in tissues is ubiquitous with caveolin 3 being restricted to muscle and astrocytes (Williams and Lisanti 2004). Caveolin 1 is considered to be necessary for the formation of morphological caveolae (Sowa 2011) and also for the transport of caveolin 2 from the golgi complex to the plasma membrane(Parolini et al. 1999; Mora et al. 1999). Caveolin sits in the plasma membrane in a hairpin like arrangement with both N and C termini being cytoplasmically oriented. There is a membrane spanning domain, and also a scaffolding domain which is the domain in which caveolin forms homo- or hetero-oligomers, and is also the region for the interaction of caveolin and other proteins, whereby caveolin interacts and sequesters them in their inactive state (Lo et al. 2004).

Given the involvement of these structures and molecules in membrane curvature in the several cell types mentioned above and the known changes in the basal plasma membrane of uterine epithelial cells during early pregnancy, we examined them during early pregnancy. We report here the first study to investigate the localisation and expression of caveolins 1 & 2 and ultrastructural caveoli, as well as

the hormonal control of both, in the basal plasma membrane of UEC during the plasma membrane transformation of early pregnancy in the rat.

3.3 MATERIALS AND METHODS

3.3.1 Animals

Female virgin Wistar rats (10–12 weeks of age) were housed in plastic cages at 21°C with a 12-h light–dark cycle and were fed and watered *ad libitum*. Vaginal smearing was performed to determine stage of the oestrous cycle, and those rats showing a vaginal smear characteristic of pro-oestrus were caged overnight with a male of proven fertility. The presence of sperm in the vaginal smear taken the following morning indicated mating and was designated day 1 of pregnancy. Uterine horns from days 1, 6, and 9 were collected to correspond to the time of fertilisation, time of implantation and after implantation respectively. Five rats were collected for each day of pregnancy. All experimental procedures were approved by The University of Sydney ethics committee.

3.3.2 Ovariectomy

Female virgin Wistar rats were bilaterally ovariectomised while anaesthetised with an intraperitoneal injection of xylazine (4 mg/kg; TROY laboratories Pty. Ltd., Smithfield, NSW, Australia) and ketamine (75 mg/kg; Parrell Laboratories (AUST) Pty. Ltd., Alexandria, NSW, Australia) and were allowed to recover for at least 3 weeks. Progesterone (Sigma, St Louis, MO, USA) and 17-β-oestradiol (Sigma) were dissolved in benzyl alcohol (Sigma) and peanut oil in 1:4 v/v and injected as previously described (Murphy and Rogers 1981). Five animals were randomly allocated to each of the following hormonal regimes. Group 1 (control) was injected with 0.1 ml of carrier (benzyl alcohol and peanut oil) alone for three consecutive days. Group 2 (PPP) received 5 mg progesterone dissolved in 0.1 ml of carrier for three consecutive days. Group 3 (EEE) received 0.5 µg 17-β-oestradiol in 0.1 ml of carrier for 3 consecutive days. Group 4 (PPPE) received 5 mg of progesterone in 0.1 ml of carrier for 3 consecutive days and also 0.5 µg of 17-β-oestradiol dissolved in 0.1 ml of carrier on the opposite side of the neck on the third day. PPPE is the minimal requirement in obtaining a receptive uterus (Murphy and Rogers 1981; Psychoyos 1973). Injections were administered in the morning and animals were killed 24 h after the last injection.

All animals in pregnancy and hormone treated groups were killed with an intraperitoneal injection of sodium pentobarbitone (Nembutal; Merial Australia, Parramatta, NSW, Australia). Uterine horns were excised and randomly allocated for either immunofluorescence microscopy (IF), transmission electron microscopy (TEM) or isolation of uterine epithelial cells for western blotting analysis fractionation, or co- immunoprecipitation.

3.3.3 Immunofluorescence microscopy

Uterine tissue from normal pregnant and ovariectomised rats treated with ovarian hormones, were embedded in O.C.T compound (Tissue-Tek; Sakura Firetek, Torrance, CA, USA), snap frozen in supercooled isopentane (BDH Laboratory supplies, Poole Dorset, England, UK) and stored under liquid nitrogen until use. Sections, 7 μm, were cut using a Leica LM 3050 cryostat (Leica, Heerbrugg, Switzerland). Tissue sections were air dried on gelatine-chrome alum-coated glass slides at room temperature and blocked in 1% bovine serum albumin (BSA; Sigma) in PBS for 30 min. All primary and secondary antibodies were diluted with this blocking solution. Sections were incubated with primary antibody of rabbit polyclonal anti-caveolin 1 antibody (0.8 μg/ml; Santa Cruz Biotechnology, Dallas, Texas, USA: sc-894), rabbit polyclonal anti-caveolin 2 antibody (2 μg/ml; Sigma, Castle Hill, NSW, Australia: C9992), or rabbit polyclonal anti-phospho caveolin -1 (Y14) antibody (2 μg/ml; Abcam, Cambridge, England, UK: ab38468) overnight at 4 °C. Sections were washed in PBS 3 × 5 min, and incubated with fluorescein isothiocyanate (FITC)-conjugated AffiniPure goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at a concentration of 2.5 μg/ml for 1 h in the dark followed by 3 × 5 min PBS wash. Sections were then mounted with vectashield containing DAPI (Vector, Burlingame, CA, USA), cover slipped and examined with a Zeiss Deconvolution microscope (Carl Zeiss Inc., Jena, Germany). Immunofluorescence micrographs were taken using a Zeiss AxioCamHR digital monochrome CCD camera (Carl Zeiss Inc., Jena, Germany) and Zeiss AxioVision version 4.0 image-acquisition software. Non-immune controls were carried out with experimental runs where sections were incubated with rabbit IgG purified immunoglobulin (2 μg/ml; Sigma) in place of the primary antibody.

3.3.4 Transmission electron microscopy

Uteri were excised and cut into 5mm pieces then immediately immersed in freshly prepared Karnovsky's fixative solution (pH 7.4) comprised of 2.5% glutaraldehyde (ProSciTech, Queensland, Australia), 2% paraformaldehyde (ProSciTech, Queensland, Australia) in 0.1M Sorenson's phosphate buffer (PB, pH 7.4) for 45 mins at room temperature. Tissue was then cut into 0.5-1mm slices on dental wax under a droplet of fixative using a double-edged razor blade, then returned to fresh fixative for a further 45 mins. The tissue was washed in PB then postfixed for 1hr in a solution of 1% osmium tetroxide (OsO_4) and 0.8% potassium ferricyanide in PB, to enhance the contrast of the plasma membrane (Karnovsky 1971). This solution was rinsed off with PB, before a 2% OsO_4 solution (in PB) was added for 10 min to remove any unreacted potassium ferricyanide, which can cause infiltration problems (Hoshino et al. 1976). The tissue was then washed extensively with MilliQ water before being dehydrated in a graded series of alcohols and infiltrated with Spur's resin (SPI supplies, Leicestershire, England, UK). Tissue slices were embedded in fresh Spur's resin in BEEM® capsules (ProSciTech, Australia) and polymerised at 60°C for 24hrs. Two randomly-selected blocks per animal were cut using a Leica Ultracut T ultra-microtome (Leica, Heerbrugg, Switzerland) and 60-70 nm sections were mounted onto 400-mesh copper grids. Sections were post-stained with a saturated solution of uranyl acetate in 50% ethanol for 45mins, followed by Reynold's lead citrate for 10mins. These sections were viewed with a Jeol 1011 transmission electron microscope (Jeol Ltd., Tokyo, Japan) at 80kV and imaged with a Gatan SC200 Orius CCD Camera (Gatan Inc., California, USA).

3.3.5 Counting of caveolae

For each sample, about 25 images were taken at 50 000 X magnification and caveolae were counted in a single blind study, where images selected were counted by an individual who did not know which image was from which day of pregnancy (Vogel et al. 1998). Basolateral membranes of UECs from day 1 and day 6 of pregnancy were measured using Image J software and the number of morphological caveolae (80-100 nm) with a neck connecting them to the basolateral membrane were counted. Smooth uncoated vesicles within 200 nm of

the basal plasma membrane were also counted. Numbers of caveolae were reported as number of caveolae/μm.

3.3.6 Isolation of uterine luminal epithelial cells

Uterine luminal epithelial cells were isolated from each animal as previously described (Kaneko et al. 2008). The uterine horn was opened longitudinally and surface luminal epithelial cells were scraped off using sterile surgical blades (Livingstone International, Rosebery, NSW, Australia) and immediately placed into lysis buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.1% SDS, 0.5% Deoxycholic acid, 1% Igepal and protease inhibitor cocktail; Sigma Mammalian Cell lysis kit, Sigma). The cell lysate was passed through a 23 gauge needle using a 1 ml syringe (Livingstone, International, Rosebery, NSW, Australia) and briefly centrifuged at 8,000g at 4 °C. The supernatant was collected and frozen immediately in liquid nitrogen and stored at −80°C until use.

3.3.7 Membrane and cytosolic separation of isolated of uterine luminal epithelial cells

Isolated uterine luminal epithelial cells collected from rat uterine horns of days 1 and 6 of pregnancy were immediately placed in fractionation buffer (50 mM TRIS–HCl, 1 mM EDTA, 150 mM NaCl, 0.5% Igepal, 130 mM KCl, 1% deoxycholic acid sodium salt solution) and passed through a 23 gauge needle using a 1 ml syringe and lysates ultracentrifuged at 40,000g at 4 °C for 1 h (Beckman and Coulter Ultracentrifuge TLX 120). The supernatant (cytosolic component) was collected and the pellet (membrane component) was resuspended in 25 μL of fractionation buffer, and stored at -80 °C until use.

3.3.8 Western blotting analysis

Protein concentrations were determined using the BCA protein assay (Micro BCA™ Protein assay kit; Quantum Scientific, Murarrie, QLD, Australia) and POLAR Star Galaxy microplate reader (BMG LabTech, Durham, NC, USA) according to the manufacturer's instructions. Protein samples (20 μg) and sample buffer (8% glycerol, 50 mM Tris–HCl, pH 6.8, 1.6% SDS, 0.024% bromophenol blue, 4% β-2-mercaptoethanol) were boiled at 95°C for 5 minutes prior to loading onto a 12% SDS-polyacrylamide gel. Gels were subjected to electrophoresis at 200 V for 40

minutes and proteins transferred to a polyvinylidene dilfluoride (PVDF) membrane (Immunobilon™ transfer membrane; Millipore, Bedford, MA, USA) at 100 V for 1 hour. Membranes were blocked by incubating in a solution of 5% skim milk powder in TBS-t (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature with constant agitation, followed by primary antibody incubation with rabbit polyclonal anti-caveolin-1 antibody (0.13 µg/ml, Santa Cruz: sc-894), rabbit polyclonal anti-caveolin 2 antibody (0.5 µg ml/1, Sigma: C9992), or phospho-caveolin 1 (Y14) (1 µg/ml, Cell Signalling Technologies: 3251) overnight at 4°C. All antibodies were diluted with 1% skim milk powder in TBS-t. The membranes were washed 3 × 10 min in TBS-t and subsequently incubated for 2 h with goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (0.5 µg/ml; Dako, VIC, Australia) at room temperature with constant agitation. The proteins were detected by enhanced chemiluminescence (ECL Plus Western Blotting Detection System; Amersham, GE Healthcare, NSW, Australia) and unsaturated images were captured using the Alpha Innotech Digital Imaging System (Alpha Innotech, San Leandro, CA, USA). Membranes were subsequently rinsed in TBS-t and antibodies removed by heating at 60°C for 45 min in stripping buffer (62.5 mM Tris–HCl, pH 6.7, 2% SDS and 100 mM β-2-mercaptoethanol) and reprobed with mouse monoclonal anti-β-actin antibody (0.4 µg/ml; Sigma, NSW, Australia) to ensure equal loading.

3.3.9 Co-immunoprecipitation

Beads were cross-linked with primary antibodies prior to immunoprecipitation (method adapted from Abcam). Magnetic beads (PureProteome™ Protein G Magnetic Beads, Millipore) were washed with PBS and incubated with 50 µL dilution buffer (0.1% BSA/PBS) with rotation for 10 minutes at 4°C. Dilution buffer was removed and incubated with rabbit anti-caveolin 1 (Santa Cruz sc-894) or goat anti-integrin β1 (Santa Cruz sc-6622), rabbit IgG and goat IgG non-immune antibodies (3 µg for all antibodies) for 1 h at 4°C with rotation. Antibody solution was removed and then beads were washed with dilution buffer for 5 minutes at 4°C, which was removed and then antibodies were cross-linked to beads by incubation with dimethyl pimelimidate (DMP) (Sigma Australia, 6.5mg/ml, 0.1 M triethanolamine diluent) for 30 minutes at room temperature with agitation. This

solution was then removed and beads washed with wash buffer (0.2 M triethanolamine, Sigma, Australia) for 5 minutes with rotation, this was repeated twice. Crosslinking reaction was quenched with 50 µL of 50 mM ethanolamine (Sigma, Australia) and rotated for 5 minutes at room temperature. Beads were washed with 50 µL PBS and excess unlinked antibody was removed by incubating beads with 50 uL of 1 M glycine (pH 3.0) for 10 minutes at room temperature with rotation. Beads were washed gently with 1% PBS-t for 5 minutes with rotation at room temperature and stored at 4°C until use.

Protein from isolated uterine epithelial cells (300 µg) from day 1 of pregnancy was incubated with cross-linked beads with rotation at 4°C for 3 hours. Unbound lysate was collected and stored for western blot analysis. Beads were then washed six times with 1% PBS-t and then heated at 95 °C for 10 minutes with 20 µL of sample buffer (8% glycerol, 50 mM Tris–HCl, pH 6.8, 1.6% SDS, 0.024% bromophenol blue, 4% β-2-mercaptoethanol) to separate the beads from the antibody-antigen complex. Supernatant was collected, subjected to SDS-PAGE as previously described and immunoblotted with rabbit anti-caveolin-1 (0.13 µg/ml; Santa Cruz Biotechnology, sc-894) or goat Integrin β1 (0.26 µg/ml; Santa Cruz Biotechnology, sc-6622).

3.3.10 Statistical analysis

The intensity of the bands detected from western blotting analysis was quantified using the two-dimensional spot density from the Alpha Innotech Digital System (Alpha Innotech). The integrated density value (IDV) was calculated using the AlphaEaseFC software. Statistical analysis was performed by student's t-test for early pregnancy and one-way ANOVA for comparison of hormone treatment groups with a Tukey's post hoc test. The mean ± SEM was calculated and $p < 0.05$ was considered to be significant. Student's t-test was also employed for statistical analysis of the number of caveolae, where the mean ± SEM was calculated and a $p < 0.05$ was considered to be significant.

3.4 RESULTS

"Caveolin 1 and 2 are relocated to the basal plasma membrane at the time of implantation"

Immunofluorescence microscopy of caveolin 1 **(figure 3.1a)** and 2 **(figure 3.1b)** demonstrated localisation on day 1 of pregnancy in UECs to the cytoplasm. A shift in localisation to the basal plasma membrane of caveolin 1 and 2 on day 6 of pregnancy **(figures 3.1c, 3.1d)** was observed with a return to a diffuse staining of caveolin 1 and punctate staining of caveolin-2 in the cytoplasm of UECs on day 9 of **pregnancy (figures 3.1e, 3.1f)** similar to that seen on day 1 of pregnancy. Caveolin 1 and 2 was also localised to blood vessels and also smooth muscle of the myometrium (not shown) on all days of pregnancy studied.

"Caveolin 1 protein abundance increases and caveolin 2 protein abundance decreases at the time of implantation"

Western blotting analysis of caveolin 1 (22 KDa) protein abundance in isolated uterine epithelial cells was significantly increased on day 6 of pregnancy **(figure 3.2a)** compared to day 1 of pregnancy [$p < 0.05$]; with subcellular fractionation showing a further significant increase seen at this time in the membrane fraction of isolated luminal uterine epithelia [$p < 0.05$] **(figure 3.2b).**

Expression of three isoforms of caveolin 2 (α - 22 KDa, β – 16 KDa, and γ – 11 KDa) was observed **(figure 3.2c)** in isolated UECs. The overall protein abundance of caveolin 2 is significantly reduced on day 6 of pregnancy in isolated uterine epithelial cells [$p < 0.05$]. An increase in the β isoform of caveolin 2 was observed in the membrane fraction of isolated uterine epithelial cells on day 6 of pregnancy **(figure 3.2d).**

"Morphological caveolae at the basal plasma membrane are increased at the time of implantation"

Transmission electron microscopy of luminal uterine epithelial cells on day 1 **(figure 3.3a)** and day 6 **(figure 3.3b)** of pregnancy shows the presence of morphological caveolae at the basal plasma membrane. Morphological caveolae at the basal plasma membrane were counted and were found to significantly increase 3.5 fold on day 6 of pregnancy [$p << 0.05$] **(figure 3.3c).**

''Caveolin 1 and 2 are localised to the basal plasma membrane under influence of progesterone''

The hormonal control of caveolin 1 and 2 localisation was determined using ovariectomised rats treated with various hormonal regimes. Caveolin 1 and 2 are localised cytoplasmically in luminal uterine epithelial cells under EEE treatment **(figures 3.4b, 3.4f).** Both caveolin 1 and 2 were localised to the basal plasma membrane of luminal uterine epithelial cells in PPP **(figures 3.4c, 3.4g)** and also PPPE **(figures 3.4d, 3.4h)** treated animals.

''Caveolin 1 protein abundance is increased and caveolin 2 protein abundance is decreased in response to progesterone.''

Western blotting analysis of isolated UECs of ovariectomised rats treated with various hormone regimes demonstrated caveolin 1 protein abundance to be significantly higher [$p < 0.05$] under the influence of progesterone in isolated luminal uterine epithelial cells **(figure 3.5a)** compared to oestrogen and vehicle treated groups. Caveolin 2 protein abundance however, is significantly lower [$p < 0.05$] under hormonal influences in isolated luminal uterine epithelial cells **(figure 3.5b)** compared to vehicle alone.

''Basal localisation of pY14 caveolin 1 and association with integrin β1 at the time of fertilisation.''

Post translational modifications of caveolin 1 such as phosphorylation at tyrosine 14 (pY14) residue have been reported to mediate integrin regulated membrane domain internalisation (del Pozo et al. 2005), and as such would have a localisation that would differ to the overall caveolin 1 localisation in UECs. Immunofluorescence microscopy of pY14 caveolin 1 showed a basal localisation in luminal uterine epithelial cells on both day 1 and day 6 of pregnancy (figure 3.6a, 3.6b). Western blotting analysis showed the protein abundance of pY14 caveolin 1 in isolated uterine epithelial cells was significantly increased on day 6 of pregnancy (figure 3.6c) compared to day 1 of pregnancy. Co-immunoprecipitation was employed to determine if there is an association between caveolin 1 and integrin β1 on day 1 of pregnancy in UECs. Caveolin 1 co-immunoprecipitated with integrin β1 on day 1 of pregnancy (figure 3.6d) but not on day 6 of pregnancy where integrin β1 is absent (Kaneko et al. 2011).

Figure 3.1 Immunofluorescence microscopy of caveolin 1 and 2 in the rat uterus during early pregnancy

Immunofluorescence micrographs of UECs stained with polyclonal anti-caveolin 1 antibody (green) or polyclonal anti–caveolin 2 antibody (green) and cell nuclei counterstained with dapi (blue). On day 1 of pregnancy **(a)** caveolin 1 displays a cytoplasmic localisation in UECs. On day 6 of pregnancy caveolin 1 **(c)** is localised basally in UECs (arrow head). On day 9 of pregnancy **(e)** caveolin 1 staining is similar to that seen on day 1 of pregnancy **(a)**. On day 1 of pregnancy **(b)** caveolin 2 displays a diffuse cytoplasmic staining in UECs. On day 6 of pregnancy caveolin 2 **(d)** is localised basally in UECs (arrow head). On day 9 of pregnancy **(f)** caveolin 2 staining is similar to that seen on day 1 of pregnancy **(b)**. Day 6 **(g)** and PPPE treated **(h)** Non Immune controls. E, luminal uterine epithelial cells; L, lumen; BV, blood vessel, arrow heads, basal plasma membrane. Bar = 30 μm

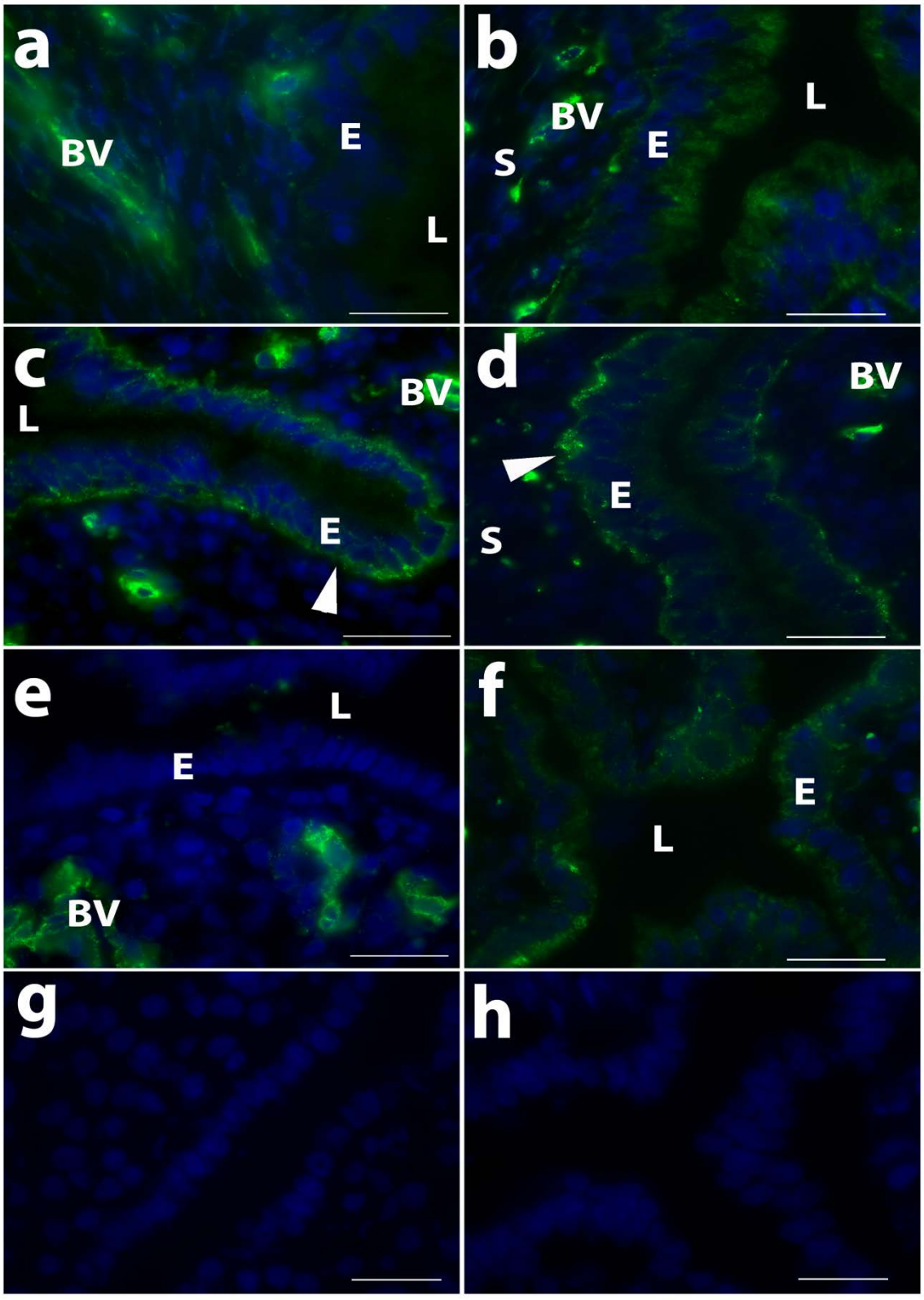

a
BV
E
L
b
BV
L
S
E
c
L
BV
E
d
BV
E
S
e
L
E
BV
f
E
L
g
h

Figure 3.2 Western blotting analysis of caveolin 1 and 2

Western blotting analysis of caveolin 1 expression from isolated UECs from day 1 and day 6 of pregnancy **(a)**, β actin was used as a loading control. Caveolin 1 was found to be significantly increased ($p < 0.05$, n=5) on day 6 of pregnancy with densitometric analysis. Integral density values (IDV) were calculated and are shown as ± SEM. Caveolin 1 protein abundance is significantly increased ($p < 0.05$, n=5) in the membrane (M) component of isolated uterine epithelial cells that have been separated into their membrane (M) and cytosolic (C) components **(b)**.

Western blotting analysis of caveolin 2 expression from isolated UECs from day 1 and day 6 of pregnancy **(c)**, β actin was used as a loading control. Caveolin 2 total protein abundance was found to be significantly reduced ($p < 0.05$, n=5) on day 6 of pregnancy with densitometric analysis. Integral density values (IDV) were calculated and are shown as ± SEM. Caveolin 2 total protein abundance was not found to be significantly different between the membrane (M) and cytosolic (C) component of isolated uterine epithelial cells **(d)**.

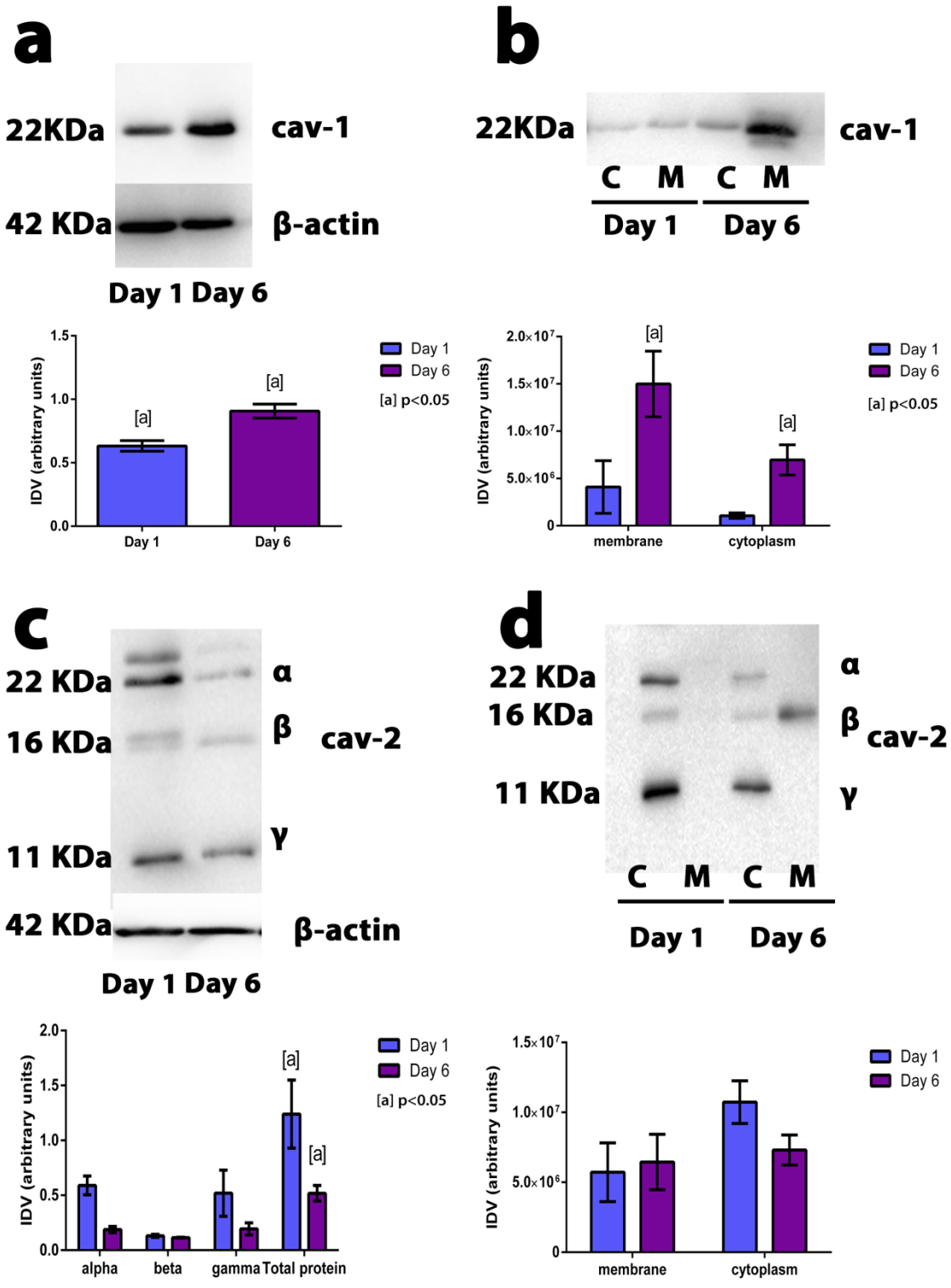
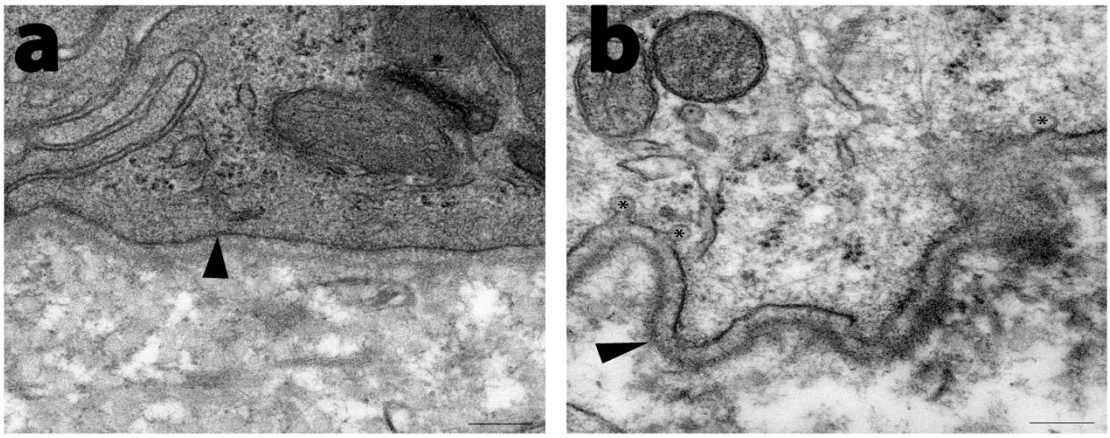

a
22KDa
cav-1
42 KDa
β-actin
Day 1 Day 6
IDV (arbitrary units)
1.5
1.0
0.5
0.0
[a]
[a]
Day 1
Day 6
Day 1
Day 6
[a] p<0.05
b
22KDa
cav-1
C M C M
Day 1 Day 6
IDV (arbitrary units)
2.0×10⁷
1.5×10⁷
1.0×10⁷
5.0×10⁶
0
[a]
[a]
membrane
cytoplasm
Day 1
Day 6
[a] p<0.05
c
22 KDa
16 KDa
11 KDa
α
β
γ
cav-2
42 KDa
β-actin
Day 1 Day 6
IDV (arbitrary units)
2.0
1.5
1.0
0.5
0.0
[a]
[a]
alpha
beta
gamma
Total protein
Day 1
Day 6
[a] p<0.05
d
22 KDa
16 KDa
11 KDa
α
β
γ
cav-2
C M C M
Day 1 Day 6
IDV (arbitrary units)
1.5×10⁷
1.0×10⁷
5.0×10⁶
0
membrane
cytoplasm
Day 1
Day 6

Figure 3.3 Transmission electron micrographs of the basal plasma membrane UECs

Transmission electron micrographs of luminal UECs on day 1 **(a)** and day 6 **(b)** of pregnancy. Morphological caveolae (*) are observed on the basal plasma membrane (arrow head) of luminal uterine epithelial cells on day 6 of pregnancy. There is a significant ($p<<0.05$ n=5, students T-test) increase in the number of morphological caveolae observed on day 6 of pregnancy **(c)**.


a
b

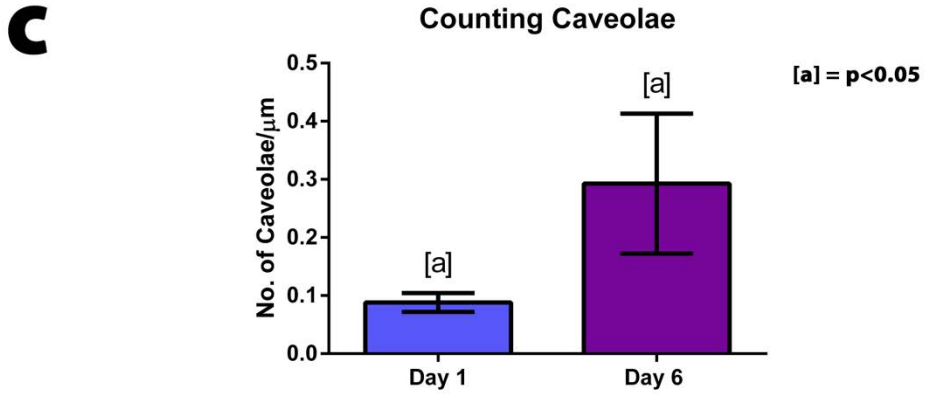

c
Counting Caveolae
No. of Caveolae/µm
0.5
0.4
0.3
0.2
0.1
0.0
[a]
[a]
Day 1
Day 6
[a] = p<0.05

Figure 3.4 Immunofluorescence microscopy of caveolin 1 and 2 in the rat uterus of ovariectomised rats

(a-d) Immunofluorescence micrographs of rat UECs (E) stained with caveolin 1 (green) and nuclei counterstained with dapi (blue) from ovariectomised rats treated with vehicle alone **(a)**, oestrogen alone **(b)**, progesterone alone **(c)** and combined progesterone and oestrogen **(d).** Caveolin 1 is localised basally in UECs under progesterone control **(c)**, and under combined progesterone and oestrogen treatment **(d)**. Localisation of caveolin 1 is cytoplasmic under oestrogen **(b)** and vehicle **(a)** treatment.

(e-h) Immunofluorescence micrographs of rat UECs (E) stained with caveolin 2 (green) and nuclei counterstained with dapi (blue) from ovariectomised rats treated with vehicle alone **(e)**, oestrogen alone **(f)**, progesterone alone **(g),** and combined progesterone and oestrogen **(h).** Caveolin 2 is localised basally in luminal uterine epithelial cells under progesterone control **(g)**, and under combined progesterone and oestrogen treatment **(h)**. Localisation of caveolin 2 is cytoplasmic under oestrogen **(f)** and vehicle **(a)** treatment. Scale bar 30 μm. L, lumen; E, epithelium; BV, blood vessel.

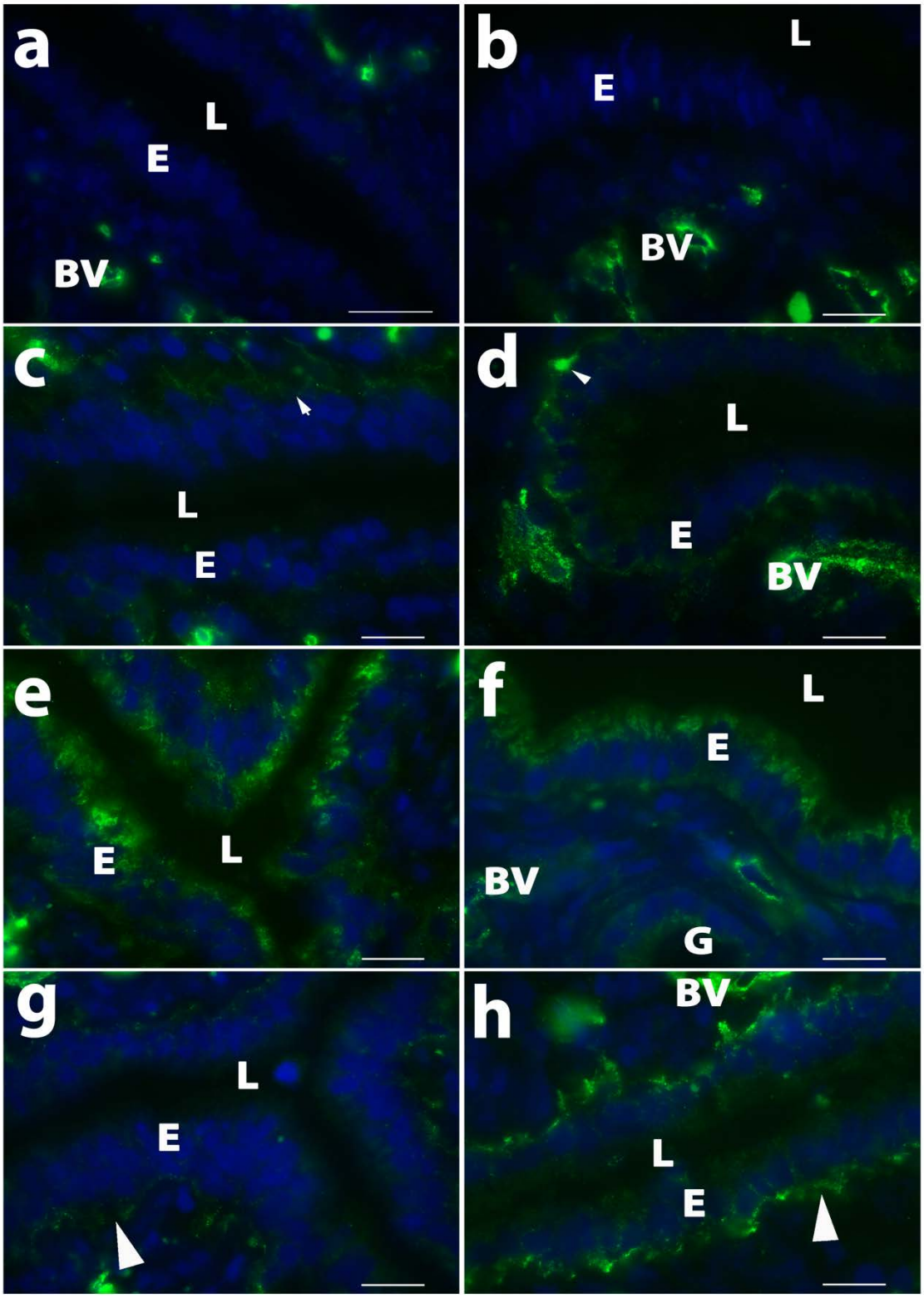

a
L
E
BV
b
L
E
BV
c
L
E
d
L
E
BV
e
E
L
f
L
E
BV
G
g
L
E
h
BV
L
E

Figure 3.5 Western blotting of isolated uterine epithelial cells from ovariectomised rats

Western blotting of isolated UECs from ovariectomised rats treated with progesterone alone (P), oestrogen alone (E), oestrogen and progesterone (PE), and vehicle alone (C). Densitometric analysis of western blotting and statistical analysis by one-way ANOVA demonstrated that caveolin 1 protein abundance is increased under progesterone treatment (p <0.05, n=5) **(a)**. Caveolin 2 total protein abundance decreased under hormonal treatment (p <0.05, n=5) and demonstrated by one way ANOVA analysis **(b)**. Integral density values (IDV) were calculated and are shown as ± SEM.

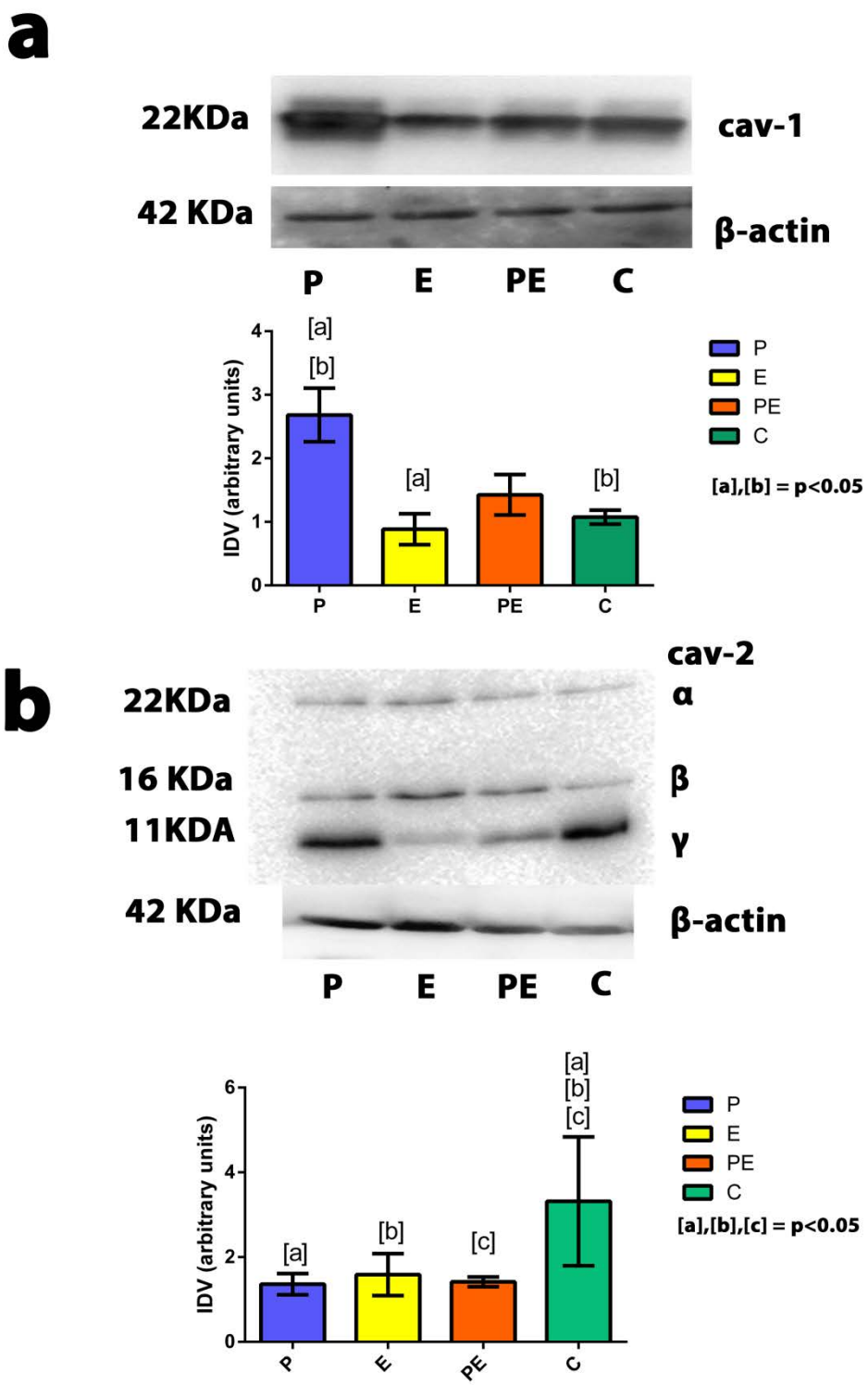
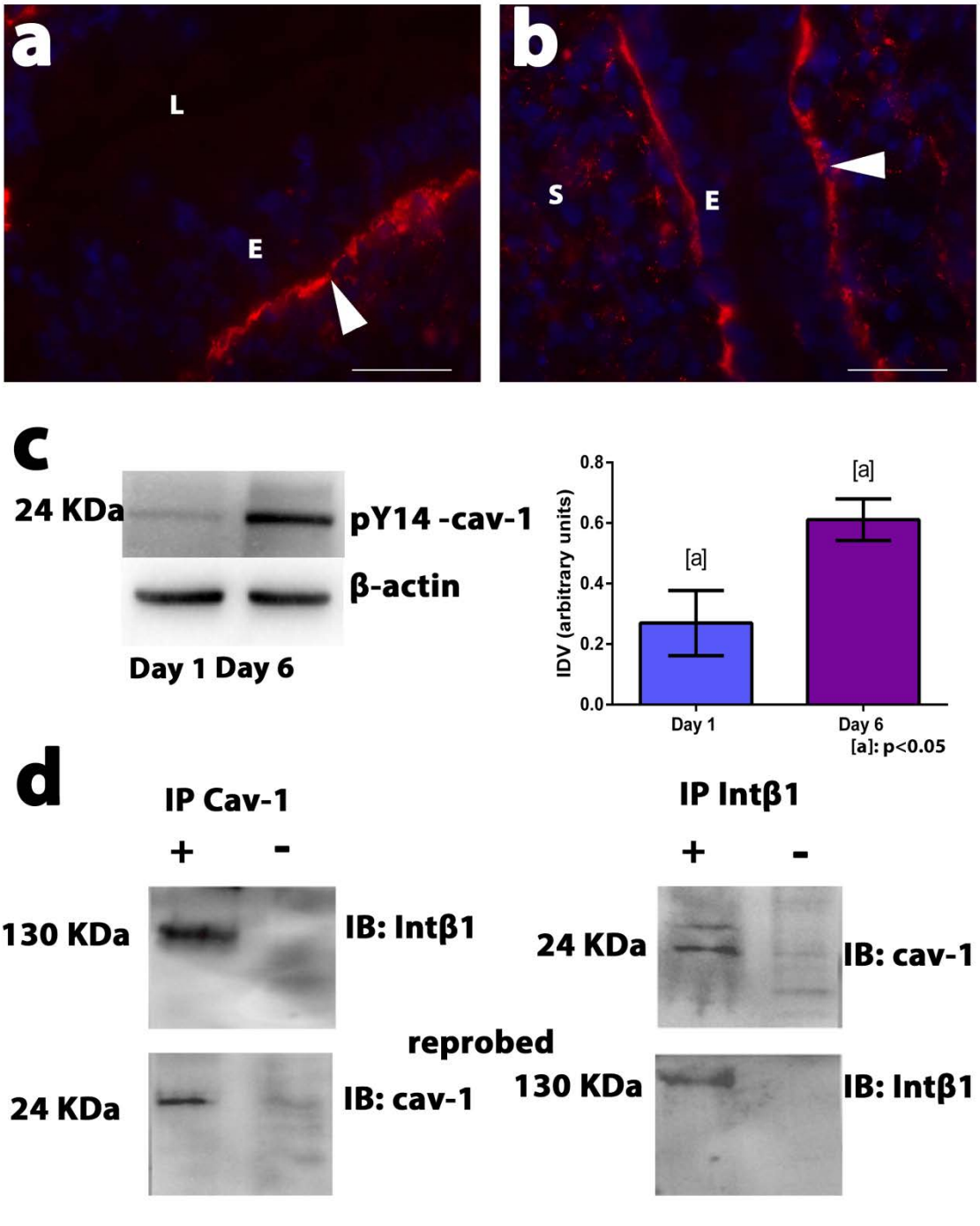

a
22KDa
cav-1
42 KDa
β-actin
P E PE C
IDV (arbitrary units)
[a]
[b]
[a]
[b]
P
E
PE
C
[a],[b] = p<0.05
b
cav-2
22KDa
α
16 KDa
β
11KDA
γ
42 KDa
β-actin
P E PE C
IDV (arbitrary units)
[a]
[b]
[c]
[a]
[b]
[c]
P
E
PE
C
[a],[b],[c] = p<0.05

Figure 3.6 Immunofluorescence microscopy and western blotting of p-cav 1 (Y14) in UECs during early pregnancy

(a, b) Immunofluorescence micrographs of UECs stained with polyclonal anti-pY14-caveolin 1 antibody (red) and cell nuclei counterstained with dapi (blue). On day 1 **(a)** and day 6 **(b)** of pregnancy pY14-caveolin 1 displays a basal localisation in luminal uterine epithelial cells (arrow head). E, luminal uterine epithelial cells; L, lumen; S, stroma. Bar = 30 μm

Western blotting analysis of pY14-caveolin 1 expression from UECs from day 1 and day 6 of pregnancy **(c)**, β actin was used as a loading control. pY14-caveolin 1 was found to be significantly increased ($p < 0.05$, n=5) on day 6 of pregnancy with densitometric analysis. Integral density values (IDV) were calculated and are shown as ± SEM.

Co-immunoprecipitation between caveolin 1 and integrin β1 **(d)** was performed to determine an association between these two proteins on day 1 of pregnancy in isolated luminal uterine epithelial cell lysates. Cell lysates from day 1 and day 6 of pregnancy were subjected to immunoprecipitation (IP) against antibodies caveolin 1 (+), rabbit IgG (-) and integrin β1 (+), goat IgG (-), and immunoblotted with integrin β1 and caveolin 1 antibodies respectively. Immunoblots were stripped and reprobed for immunoprecipitated antibody to confirm protein precipitate. No integrin β1 or caveolin 1 was detected in the respective IgG control lanes. An association between caveolin 1 and integrin β1 was observed on day 1 of pregnancy but not on day 6 of pregnancy (data omitted).


a
L
E
b
S
E
c
24 KDa
pY14 -cav-1
β-actin
Day 1 Day 6
IDV (arbitrary units)
0.8
0.6
0.4
0.2
0.0
[a]
[a]
Day 1
Day 6
[a]: p<0.05
d
IP Cav-1
+ -
130 KDa
IB: Intβ1
24 KDa
IB: cav-1
IP Intβ1
+ -
24 KDa
IB: cav-1
reprobed
130 KDa
IB: Intβ1

3.5 DISCUSSION

One aspect of uterine epithelial cells during the plasma membrane transformation of early pregnancy that has always been particularly intriguing is how they maintain their polarised state despite the loss of numerous membrane junctional structures and the related cytoskeletal elements of these junctions (Murphy 2001; Murphy 2004; Murphy and Shaw 1994). There have been suggestions that compensatory mechanisms involving the basal plasma membrane must exist but the present work is the first to provide evidence of one such mechanism. This study investigated the expression and localisation of caveolin 1 and 2 in UECs during early pregnancy and also under the influence of ovarian hormones. This study has documented for the first time the presence of morphological caveolae in luminal uterine epithelial cells at the time of implantation.

On day 1 of pregnancy caveolins 1 and 2 demonstrated a diffuse cytoplasmic localisation in luminal uterine epithelial cells. By day 6 of pregnancy there was redistribution in their localisation to the basal plasma membrane, with a corresponding significant increase in the number of morphological caveolae observed in these cells by TEM. The redistribution of caveolins to the basal plasma membrane at the time of implantation and also the increase of morphological caveolae correspond to the significant increase in caveolin 1 protein expression at this time, and the increase in the amount of caveolin 1 protein in the membrane fraction. However, a decrease in caveolin 2 protein abundance in uterine epithelial cells was also noted at the time of implantation. The appearance of caveolin 1 and 2 cytoplasmically at the time of fertilisation and more basally at the time of implantation may correspond to a change in vesicular trafficking at these times, as caveolae have been reported to be involved in transcytosis (Matveev et al. 2001; Millan et al. 2006; Navarro et al. 2004) in endothelial cells of both small and large molecules as well as potocytosis where caveolae utilize GPI anchored proteins that are enriched in their membranes to concentrate small molecules for translocation to the cytoplasm (Lisanti et al. 1995). The localisation of caveolins 1 and 2 after implantation (day 9) reverted to the pattern seen on day 1 of pregnancy. Hormonal studies showed that under oestrogen control the localisation of caveolin 1 and 2 was similar to day 1 of pregnancy, whereas progesterone alone or in combination with oestrogen led to the redistribution of caveolin 1 and 2 to the basal plasma

membranes similar to that seen at the time of implantation on day 6 of normal pregnancy.

Caveolin 1 and 2 were found to be located in endothelial cells of endometrial and myometrial blood vessels during all stages of pregnancy examined, and in response to all hormone regimes studied. Localisation of caveolins 1 and 2 in blood vessels has been observed in the endothelium of other organs (Hu and Minshall 2009; Minshall et al. 2003; Predescu et al. 1994) and is related to effects on eNOS (endothelial Nitric Oxide Synthase)(Huang 2003; Minshall et al. 2003) and hence in the endometrium, caveolin may play a role in angiogenesis during early pregnancy, as well as an important role in later stages of pregnancy.

The basal localisation of caveolin 1 and 2 at the time of implantation is a key location for the formation of caveolae, and involvement in vesicular transport in UECs. Luminal uterine epithelial cells use endocytotic vacuoles, and also pinocytosis to transport molecules throughout and into the cell across the luminal surface of the uterus (Parr 1982; Parr 1980). Vesicles of various sizes in luminal uterine epithelial cells have been reported to be increased between days 5 and 6 of pregnancy in the rat compared to day 1 of pregnancy (Parr 1982; Parr 1980). These vesicles, while not identified as caveolae at the time, may have been caveolae playing a pinocytotic function at the time of implantation where caveolae traffic proteins and other molecules from the underlying stromal cells to the epithelium. It is interesting however, that the protein abundance of caveolin 2 is reduced at the time of implantation unlike caveolin 1. The differences in protein abundance of the two proteins may be due to caveolin 2 being an accessory protein, and not essential for the formation of caveolae (Anderson 1993; Sowa 2011). The increase in morphological caveolae seen at the time of implantation further supports this conclusion. This is the first study to identify morphological caveolae at the basal plasma membrane of luminal uterine epithelial cells at the time of implantation and the increase in the number of caveolae at this time is consistent with the basal redistribution of caveolin 1 and 2 and also the increase in caveolin 1 protein abundance at the time of implantation.

Caveolin 1 and 2 in luminal uterine epithelial cells was found to be localised basally with both progesterone and combined progesterone and oestrogen treatment which was similar to what was observed on day 6 of pregnancy. This

was expected as this treatment is known to be sufficient for a morphologically receptive endometrium (Murphy and Rogers 1981; Murphy et al. 1981). Caveolin 1 protein abundance was found to be increased under progesterone treatment, while caveolin 2 protein abundance was significantly reduced under all hormonal influences. This may be due to the localisation of caveolin 2 being dependent on the localisation of caveolin 1, since caveolin 1 expression is required for caveolin 2 exit from the golgi apparatus to the plasma membrane (Parolini et al. 1999). Caveolae, and hence caveolin 1 expression has been reported to be down regulated in uterine smooth muscle under oestrogen treatment (Turi et al. 2001), where caveolin 1 binds directly to oestrogen receptor α by means of its scaffolding domain (Schlegel et al. 1999). The scaffolding domain (SCD) of caveolin facilitates the interaction and organisation of receptors and signalling molecules. The SCD of caveolin in caveolae is in the plane of the plasma membrane (Kirkham et al. 2008) which results in the inhibition of signalling molecules when bound to caveolin 1 in morphological caveolae; while molecules binding to the SCD of caveolin 1 in non caveolar or flattened caveolae would activate signalling. In this study caveolin 1 and 2 protein expression is seen to be significantly reduced under oestrogen treatment in comparison to progesterone and control. We suggest that caveolin 1 binds to the oestrogen receptor in luminal uterine epithelial cells as pregnancy progresses to the time of implantation thereby sequestering the receptor in its inactive state and aiding in the development of a receptive uterine epithelium.

Post translational modifications such as phosphorylation of caveolin 1 at tyrosine 14 residue have been reported to affect its subcellular localisation (del Pozo et al. 2005; Li et al. 1996; Orlichenko et al. 2005; Sun et al. 2009). Localisation of pY14 caveolin 1 was basal in luminal uterine epithelial cells at the time of fertilisation and implantation where morphological caveolae were found to be increased. While few caveolae were seen on day 1 of pregnancy, the localisation of pY14 caveolin 1 at the basal plasma membrane on day 1 of pregnancy suggests the presence of non caveolar regions or flattened caveolae at fertilisation. Previous studies of pY14 caveolin 1 have suggested roles in integrin β1 turnover (Salanueva et al. 2007; del Pozo et al. 2005) and other studies have shown that integrin β1 is lost from the basal plasma membrane of luminal uterine epithelial cells at the time of implantation (Kaneko et al. 2008; Kaneko et al. 2011). This study has

demonstrated an association between caveolin 1 and integrin β1 on day 1 of pregnancy**,** where the SCD of caveolin 1 may serve to bind to integrin β1 in non caveolar or flattened caveolar domains, and then progress to internalise integrin β1 as the membrane invaginates to form caveolae, thereby aiding in the disassembly of integrin based focal adhesions (Cheng et al. 2006; del Pozo et al. 2005; Salanueva et al. 2007).

The apical and basolateral membranes of UECs undergo dramatic changes both morphologically and biochemically between non receptive and receptive states, and lose many epithelial junctional structures and associated cytoskeletal components (Kaneko et al. 2009; Kaneko et al. 2008; Luxford and Murphy 1992; Murphy 2001; Murphy 1995; Murphy and Dwarte 1987; Preston et al. 2004). Despite these cellular changes UECs still maintain a polarised organisation of lipids and proteins, and a number of proteins are dependent on specific lipid rafts for their localisation and function (Fabbri et al. 2005; Head et al. 2006; Ikonen 2001; Lee et al. 2009). An elevated concentration of glycosphingolipids and cholesterol at the apical surface in UECs may provide a rigid membrane (Petrache et al. 2005; Tabas 2002) that does not allow for membrane curvature or invaginated caveolae, while patches of glycosphingolipids and cholesterol at the basal plasma membrane may provide a more flexible membrane that contributes to both membrane curvature and invaginated caveolae (Mora et al. 1999; Mora et al. 2006; Parolini et al. 1999). Earlier work on flotillin – another lipid raft marker which was found to be localised apically at the time of implantation (Lecce et al. 2013) and the work in this study shows a basal localisation of caveoli and caveolin at the time implantation in UECs which suggests that a polarised distribution of lipids and lipid rafts at the time of implantation may be a mechanism to maintain epithelial integrity where junctional structures such as focal adhesions (Kaneko et al. 2011; Kaneko et al. 2009) and desmosomes (Preston et al. 2004) and associated proteins are down regulated at the time of implantation for successful implantation.

In summary this work has shown that morphological caveoli are present at the basal plasma membrane of uterine epithelial cells during early pregnancy and also that caveolins redistribute to this region during the same period. We suggest that the increased caveolar formation and the accompanying increased turnover and

pinocytosis in the basal plasma membrane is a mechanism to compensate for the loss of membrane junctional structures in the basolateral plasma membrane and so maintain epithelial cell polarity.

3.6 REFERENCES

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CHAPTER 4: PTRF IS ASSOCIATED WITH CAVEOLIN-1 IN UTERINE EPITHELIAL CELLS AT THE TIME OF IMPLANTATION, BUT SDPR IS LOST AT THE TIME OF IMPLANTATION.

4.1 ABSTRACT

The plasma membrane of uterine epithelial cells undergoes a number of changes during early pregnancy. The changes in the basolateral membrane at the time of implantation in particular change from being smooth to highly tortuous in morphology, along with a dramatic increase in the number of morphological caveolae at this time. The major protein of caveolar membranes is caveolin, however, previous studies have shown that PTRF (RNA pol I transcription factor) and SDPR (serum deprivation protein response) are two members of the cavin protein family. These proteins are known to be involved in caveolae biogenesis, where they directly bind to cholesterol and lipids and have been reported to promote membrane curvature.

As there is an increase in membrane tortuosity and caveolae at the time of implantation, this study investigated PTRF and SDPR to explore the possible roles that they play in the morphology of the uterine epithelium during early pregnancy. PTRF protein abundance did not change in uterine epithelial cells during early pregnancy or in response to ovarian hormones. At the time of implantation in uterine epithelial cells PTRF co-immunoprecipitated with caveolin 1 thereby demonstrating an association with caveolin-1 at the basal plasma membrane in caveolae. SDPR protein was observed to be present only at the time of fertilisation, and also under the influence of oestrogen alone, where a cytoplasmic localisation in uterine epithelial cells was observed.

The localisation and expression PTRF and SDPR in uterine epithelial cells during early pregnancy suggest that they have roles in the maintenance of lipids and cholesterol in the plasma membrane. PTRF and SDPR may contribute not only to the morphology of the basal plasma membrane as observed at the time of implantation, but also the maintenance of epithelial polarity during early pregnancy.

4.2 INTRODUCTION

Luminal uterine epithelial cells (UECs) undergo extensive molecular and morphological changes during early pregnancy in order for them to be receptive to blastocyst implantation. The changes that these cells undergo include a redistribution of lipid and cholesterol in the plasma membrane (Murphy and Dwarte 1987; Lecce et al. 2013) and also the presence of vesicles of various sizes which are increased during the pre-implantation period (Parr 1982; Parr 1980). The basolateral membrane in particular loses integrin based focal adhesions, and becomes more tortuous at the time of implantation (Kaneko et al. 2008; Demir et al. 2002; Shion and Murphy 1995)

Cavins are a family of peripheral membrane proteins (Hayer et al. 2010) of which there are four members; PTRF (RNA pol I transcription factor) also known as cavin 1; SDPR (serum deprivation protein response) also known as cavin 2; SBRC (sdr related gene product that binds to C kinase) also known as cavin 3; and MURC (muscle restricted coiled-coiled protein) also known as cavin 4. All members of this family have been found to be present in caveolar membranes (Bastiani et al. 2009; Liu and Pilch 2008; Hill et al. 2008; Hansen et al. 2009; McMahon et al. 2009) where they serve to regulate caveolae dynamics (Briand et al. 2011). Caveolae are omega shaped invaginations of the plasma membrane and function as scaffolds for signalling as well as intracellular transport vesicles and also serve to regulate lipid composition of the plasma membrane.

Initially caveolae were thought to contain only caveolin-1, however, recent evidence found a role for PTRF and SDPR in caveolae formation (Fra et al. 1995; Bauer and Pelkmans 2006). Caveolin, PTRF and SDPR have been found to associate with each other and form protein complexes, in which one member of the complex regulates the expression of the other members of the complex, (McMahon et al. 2009; Mineo et al. 1998; Hill et al. 2008; Hill et al. 2012; Nabi 2009) thereby affecting the formation and morphology of caveolae.

Morphological caveolae at the basolateral membrane have been observed at the time of implantation along with an increase in caveolin -1 protein in luminal uterine epithelial cells at the time of implantation (Thesis chapter 3). Given this data and that both PTRF and SDPR are associated with both the biogenesis and shape of caveolae and this in turn contributes to membrane curvature, this study

investigated the localisation and expression of PTRF and SDPR during early pregnancy, and also the association of PTRF with caveolin-1 at the time of implantation.

4.3 MATERIALS AND METHODS

4.3.1 Animals

Female virgin Wistar rats (10–12 weeks of age) were housed in plastic cages at 21°C with a 12-h light–dark cycle and were fed and watered *ad libitum*. Vaginal smearing was performed to determine stage of the oestrous cycle, and those rats showing a vaginal smear characteristic of pro-oestrus were caged overnight with a male of proven fertility. The presence of sperm in the vaginal smear taken the following morning indicated mating and was designated day 1 of pregnancy. Uterine horns from days 1 and 6 were collected to correspond to the time of fertilisation and implantation. Five rats were collected for each day of pregnancy. All experimental procedures were approved by The University of Sydney ethics committee.

4.3.2 Immunofluorescence microscopy

Uterine tissue from normal pregnant and ovariectomised rats treated with ovarian hormones, were embedded in O.C.T compound (Tissue-Tek; Sakura Firetek, Torrance, CA, USA), snap frozen in supercooled isopentane (BDH Laboratory supplies, Poole Dorset, England, UK) and stored under liquid nitrogen until use. Sections, 7 μm, were cut using a Leica LM 3050 cryostat (Leica, Heerbrugg, Switzerland). Tissue sections were air dried on gelatine-chrome alum-coated glass slides at room temperature and fixed with 4% PFA for 10 minutes at room temperature, washed in PBS 3 x 5 min, and subsequently blocked in 1% bovine serum albumin (BSA; Sigma) in PBS for 30 min. All primary and secondary antibodies were diluted with this blocking solution. Sections were incubated with primary antibody of goat polyclonal SDPR (N19) (0.8 μg, Santa Cruz Biotechnology, Dallas, Texas, USA: sc-162162) , overnight at 4 ◦C. Sections were washed in PBS 3 × 5 min, and incubated with Alexafluor 488 (A488)-conjugated AffiniPure donkey anti goat IgG secondary antibody (Life Technologies, Carlsbad, California, USA) at a concentration of 2.5 μg/mL for 1 h in the dark followed by 3 × 5 min PBS washes. Sections were then mounted with vectashield containing DAPI (Vector, Burlingame, CA, USA), cover slipped and examined with a Zeiss Deconvolution microscope (Carl Zeiss Inc., Jena, Germany). Immunofluorescence micrographs were taken using a Zeiss AxioCamHR digital monochrome CCD camera (Carl Zeiss

Inc., Jena, Germany) and Zeiss Zen 2011 image-acquisition software. Non-immune controls were carried out with experimental runs where sections were incubated with goat IgG purified immunoglobulin (0.8 μg/mL; Sigma) in place of the primary antibody.

4.3.3 Isolation of uterine luminal epithelial cells

Uterine luminal epithelial cells were isolated from each animal as previously described (Kaneko et al. 2008). The uterine horn was opened longitudinally and surface luminal epithelial cells were scraped off using sterile surgical blades (Livingstone International, Rosebery, NSW, Australia) and immediately placed into lysis buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.1% SDS, 0.5% Deoxycholic acid, 1% Igepal and protease inhibitor cocktail; Sigma Mammalian Cell lysis kit, Sigma). The cell lysate was passed through a 23 gauge needle using a 1 ml syringe (Livingstone, International, Rosebery, NSW, Australia) and briefly centrifuged at 8,000g at 4 °C. The supernatant was collected and frozen immediately in liquid nitrogen and stored at −80°C until use.

4.3.4 Western blotting analysis

Protein concentrations were determined using the BCA protein assay (Micro BCA™ Protein assay kit; Quantum Scientific, Murarrie, QLD, Australia) and POLAR Star Galaxy microplate reader (BMG LabTech, Durham, NC, USA) according to the manufacturer's instructions. Protein samples (20 μg) and sample buffer (8% glycerol, 50 mM Tris–HCl, pH 6.8, 1.6% SDS, 0.024% bromophenol blue, 4% β-2-mercaptoethanol) were boiled at 95°C for 5 minutes prior to loading onto a 12% SDS-polyacrylamide gel. Gels were subjected to electrophoresis at 200 V for 40 minutes and proteins transferred to a polyvinylidene dilfluoride (PVDF) membrane (Immunobilon™ transfer membrane; Millipore, Bedford, MA, USA) at 100 V for 1 hour. Membranes were blocked by incubating in a solution of 5% skim milk powder in TBS-t (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature with constant agitation, followed by primary antibody incubation with mouse polyclonal anti-PTRF antibody (0.2 μg/mL, Santa Cruz: sc-136300), or goat polyclonal anti-SDPR (N-19) antibody (0.13 μg/mL, Santa Cruz sc-162162) overnight at 4°C. All antibodies were diluted with 1% skim milk powder in TBST. The membranes were washed 3 × 10 min in TBST and

subsequently incubated for 2 h with sheep anti mouse (0.4 μg/mL, Amersham, GE healthcare, Buckinghamshire, UK) and goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (0.5 μg/mL; Dako, VIC, Australia) respectively at room temperature with constant agitation. The proteins were detected by enhanced chemiluminescence (ECL Plus Western Blotting Detection System; Amersham, GE Healthcare, NSW, Australia) and unsaturated images were captured using the Alpha Innotech Digital Imaging System (Alpha Innotech, San Leandro, CA, USA). Membranes were subsequently rinsed in TBST and antibodies removed by heating at 60°C for 45 min in stripping buffer (62.5 mM Tris–HCl, pH 6.7, 2% SDS and 100 mM β-2-mercaptoethanol) and reprobed with mouse monoclonal anti-β-actin antibody (0.4 μg/mL; Sigma, NSW, Australia) to ensure equal loading.

4.3.5 Co-immunoprecipitation

Beads were cross-linked with primary antibodies prior to immunoprecipitation (method adapted from Abcam). Magnetic beads (PureProteome™ Protein G Magnetic Beads, Millipore) were washed with PBS and incubated with 50 μL dilution buffer (0.1% BSA/PBS) with rotation for 10 minutes at 4°C. Dilution buffer was removed and incubated with rabbit anti-caveolin-1 (Santa Cruz sc-894) or mouse PTRF (Santa Cruz sc-136300), rabbit IgG and mouse IgG non-immune antibodies (3 μg for all antibodies; Sigma, Australia) for 1 h at 4°C with rotation. Antibody solution was removed and then beads were washed with dilution buffer for 5 minutes at 4°C, which was removed and then antibodies were cross-linked to beads by incubation with dimethyl pimelimidate (DMP) (Sigma Australia, 6.5mg/mL, 0.1 M triethanolamine diluent) for 30 minutes at room temperature with agitation. This solution was then removed and beads washed with wash buffer (0.2 M triethanolamine, Sigma, Australia) for 5 minutes with rotation, this was repeated twice. Crosslinking reaction was quenched with 50 μL of 50 mM ethanolamine (Sigma, Australia) and rotated for 5 minutes at room temperature. Beads were washed with 50 μL PBS and excess unlinked antibody was removed by incubating beads with 50 uL of 1 M glycine (pH 3.0) for 10 minutes at room temperature with rotation. Beads were washed gently with 1% PBS-t for 5 minutes with rotation at room temperature and stored at 4°C until use.

Protein from isolated uterine epithelial cells (300 µg) from day 1 of pregnancy was incubated with cross-linked beads with rotation at 4°C for 3 hours. Beads were then washed six times with 1% PBS-t and then heated at 95 °C for 10 minutes with 20 µL of sample buffer (8% glycerol, 50 mM Tris–HCl, pH 6.8, 1.6% SDS, 0.024% bromophenol blue, 4% β-2-mercaptoethanol) to separate the beads from the antibody-antigen complex. Supernatant was collected, subjected to SDS-PAGE as previously described and immunoblotted with rabbit anti-caveolin-1 (0.13 µg/mL; Santa Cruz Biotechnology, sc-894) or mouse PTRF (0.2 µg/mL; Santa Cruz Biotechnology, sc-136300).

4.3.6 Statistical analysis

The intensity of the bands detected from western blotting analysis was quantified using the two-dimensional spot density from the Alpha Innotech Digital System (Alpha Innotech). The integrated density value (IDV) was calculated using the AlphaEaseFC software. Statistical analysis was performed by student's t-test for early pregnancy and one-way ANOVA for comparison of hormone treatment groups with a Tukey's post hoc test. The mean ± SEM was calculated and $p < 0.05$ was considered to be significant.

4.4 RESULTS

SDPR is localised and expressed in luminal UECs at the time of fertilisation but not at the time of implantation

Immunofluorescent sections showed the presence of SDPR in UECs and also in glandular epithelium. Immunofluorescent labelling of SDPR in UECs showed a cytoplasmic localisation on day 1 of pregnancy **(figure 4.1a).** On day 6 of pregnancy there was absence in staining of SDPR **(figure 4.1b)** compared to day 1 of pregnancy. SDPR immunolabelling in endometrial glands was cytoplasmic on both days of pregnancy studied. Immunolabelling of UECs with PTRF was unsuccessful despite troubleshooting efforts.

Western blotting analysis of SDPR of isolated UECs showed the presence of SDPR on day 1 of pregnancy but not on day 6 of pregnancy. Two bands of SDPR were observed on day 1 of pregnancy at 110 kDa and also at its monomeric weight of 49 kDa **(figure 4.2B).** Equal loading was confirmed with β-actin staining.

SDPR localisation and protein expression in luminal UECs is dependent on oestrogen

The hormonal control of SDPR localisation was determined using ovariectomised rats treated with PPP, EEE, PPPE, and vehicle. SDPR localisation in UECs was found to be dependent on EEE treatment **(figure 4.1d)**. SDPR labelling in luminal UECs under other hormonal regimes (PPP, PPPE, and vehicle) was absent **(figure 4.1c, e, f)**. SDPR localisation in endometrial glandular epithelium was cytoplasmic under all hormonal regimes studied.

Western blotting analysis of isolated luminal UECs from ovariectomised rats treated with above mentioned hormonal regimes showed SDPR expression under EEE treatment only at 100 kDa and 49 kDa **(figure 4.2B).** Monoclonal β-actin was used as a loading control to ensure equal loading.

PTRF protein abundance is unaltered during early pregnancy and in response to ovarian hormones

Western blotting analysis of PTRF during early pregnancy displayed a 60 kDa band of PTRF from isolated luminal uterine epithelial cells on both days 1 and 6 of pregnancy. Protein abundance of PTRF is not significantly altered between day 1 and day 6 of pregnancy ($p>0.05$, **figure 4.2A**). Western blotting analysis of isolated

UECs of ovariectomised rats treated with various hormone regimes showed no significant change in the protein abundance of PTRF **(figure 4.2A)** under these regimes. Monoclonal β-actin was used as a loading control.

PTRF associates with caveolin-1 at the time of implantation

PTRF has been found in caveolar membranes and has been shown to drive caveolar formation. Co-immunoprecipitation was used to determine if there is an association between PTRF and caveolin-1 on day 6 of pregnancy in UECs and gauge the localisation of PTRF in UECs based upon this association. Caveolin-1 co-immunoprecipitated with PTRF in isolated UECs on day 6 of pregnancy **(figure 4.3).**

Figure 4.1 Immunofluorescence microscopy of SDPR in the uterus during early pregnancy

Immunofluorescence micrographs of endometrium stained with polyclonal SDPR antibody (green) and counterstained with dapi (blue). On day 1 of pregnancy **(a)** SDPR is localised to the cytosol of luminal UECs. On day 6 of pregnancy **(b),** SDPR staining is absent in luminal UECs. In uteri of ovariectomised rats treated with progesterone only **(c)** combination of progesterone and oestrogen **(e)** and with vehicle control SDPR staining was absent in luminal UECS. Only under oestrogen alone treatment **(d)** did SDPR localise to the cytosol of luminal UECS. Glandular staining of SDPR was present during early pregnancy and in uteri of ovariectomised rates treated with hormones **(f)**. Randomised goat IgG control of day 6 uterus **(g).** E, luminal epithelial cells; L, lumen; S, stroma; G, gland. Scale bar = 30 μm.

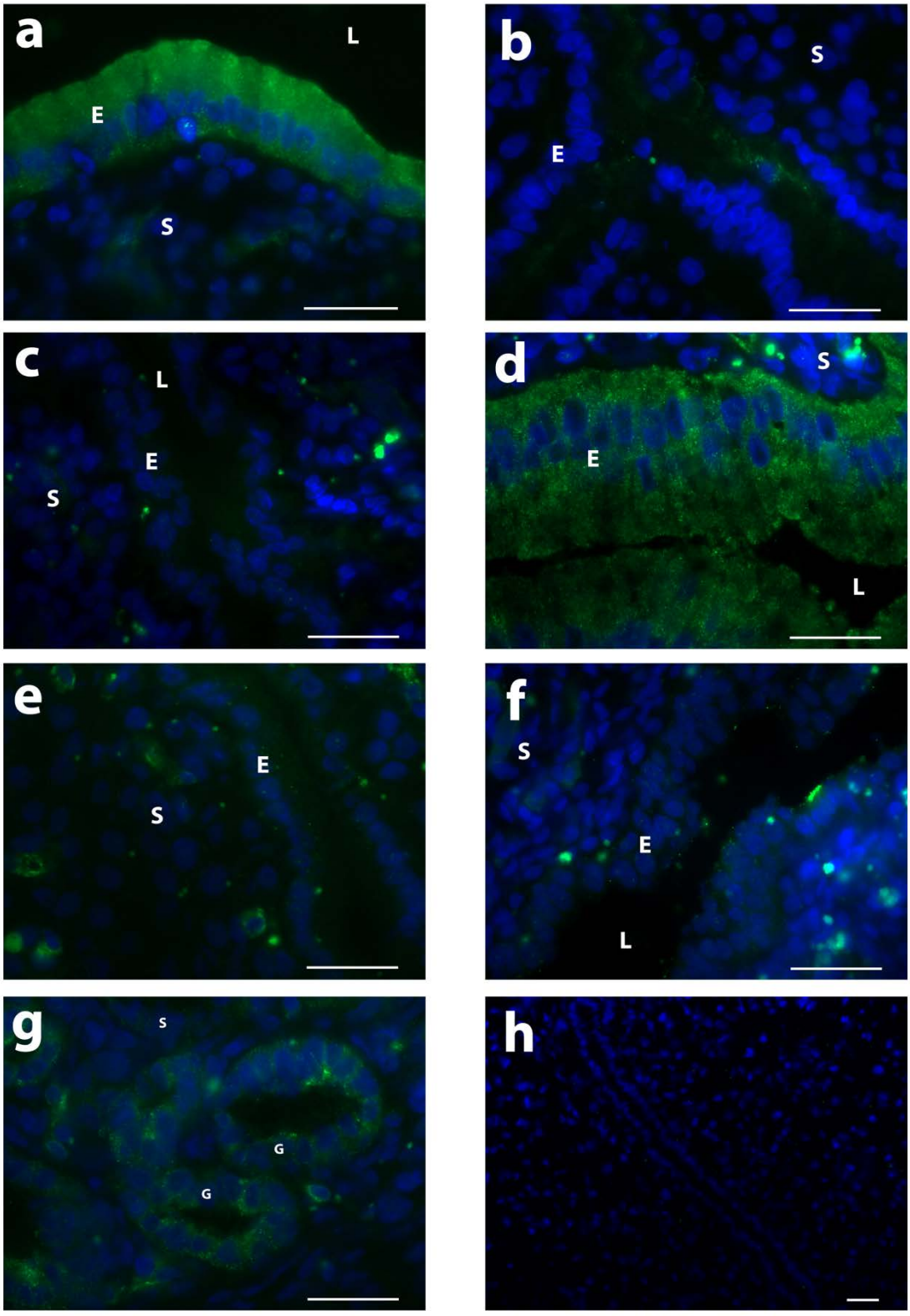
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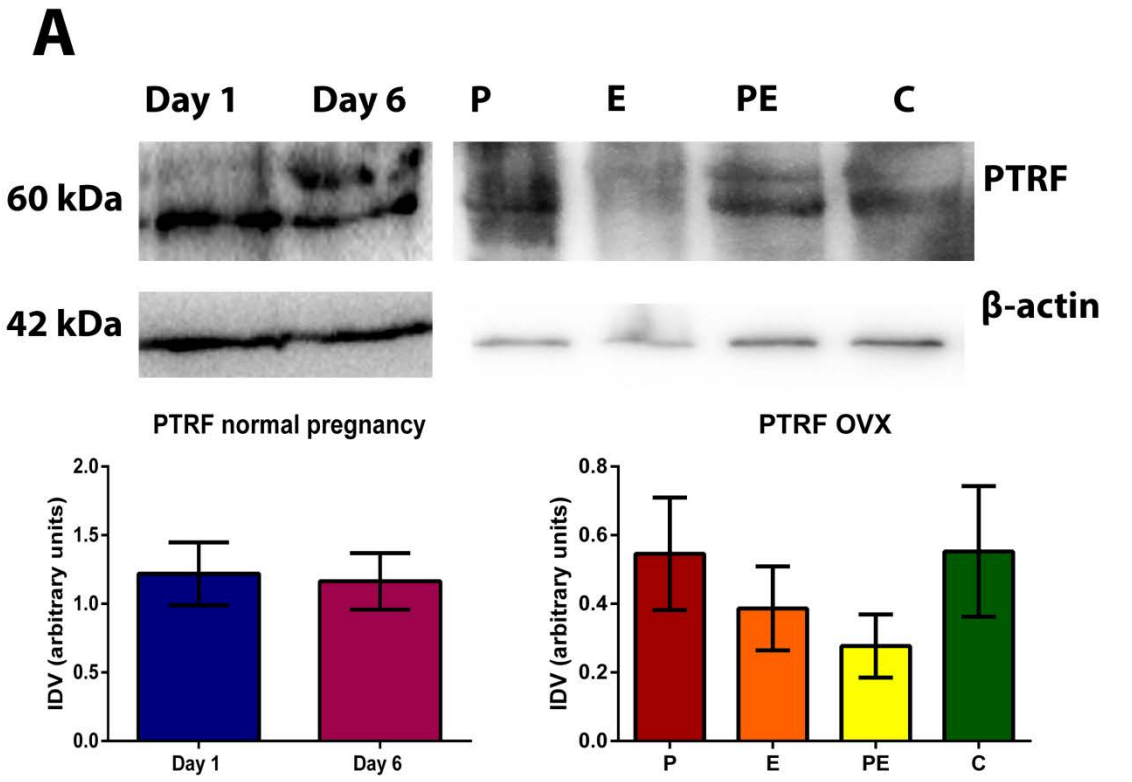
Figure 4.2 PTRF and SDPR western blotting of isolated uterine epithelial cells

Western blotting analysis of PTRF (60 kDa) **(A)** from isolated UECs on day 1 and day 6 of pregnancy, and ovariectomised rats treated with progesterone alone (P), oestrogen alone (E), progesterone and oestrogen (PE), and vehicle alone (C). Densitometric analysis of western blotting and statistical analysis by student's t-test and also one way ANOVA showed no significant difference in PTRF protein abundance between day 1 and day 6 of pregnancy, or under the influence of ovarian hormones. Integral density values (IDV) were calculated and are shown as ± SEM.

Western blotting analysis of SDPR **(B)** from isolated UECs on day 1 and day 6 of pregnancy and also ovariectomised rats treated with progesterone alone (P), oestrogen alone (E), progesterone and oestrogen (PE), and vehicle alone (C), showed SDPR to be present on day 1 of pregnancy only at 49 kDa and also at a higher molecular weight of 110 kDa. SDPR was present only under the influence of oestrogen at 100 kDa in isolated UECs.


A
Day 1
Day 6
P
E
PE
C
60 kDa
PTRF
42 kDa
β-actin
PTRF normal pregnancy
IDV (arbitrary units)
2.0
1.5
1.0
0.5
0.0
Day 1
Day 6
PTRF OVX
IDV (arbitrary units)
0.8
0.6
0.4
0.2
0.0
P
E
PE
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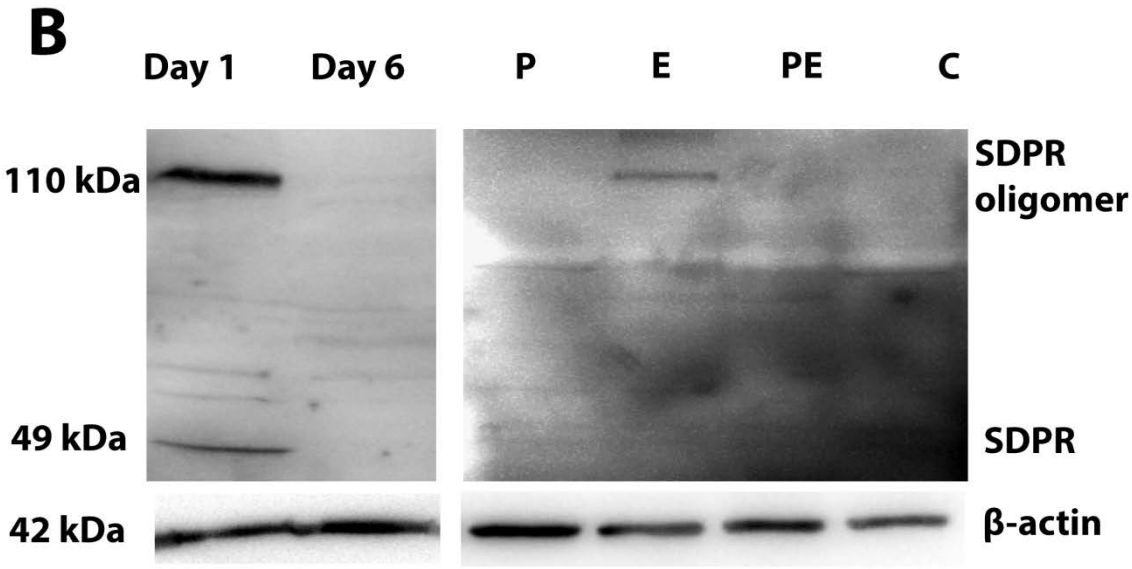
B
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Day 6
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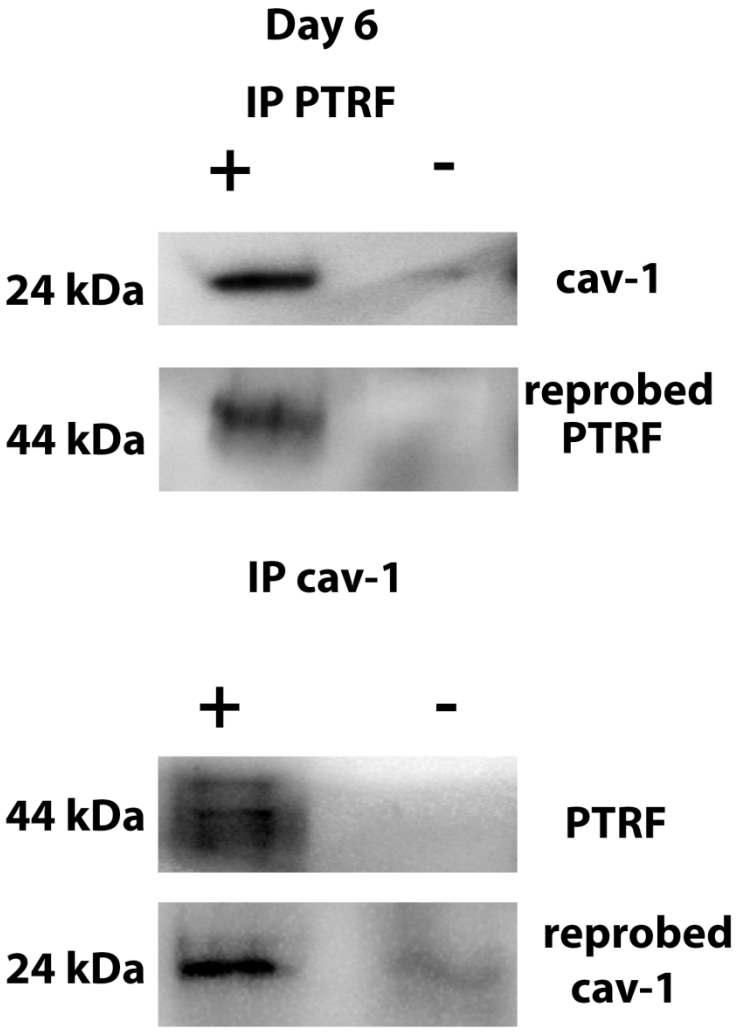
110 kDa
SDPR
oligomer
49 kDa
SDPR
42 kDa
β-actin

Figure 4.3 Co-immunoprecipitation between caveolin 1 and PTRF

Co-immunoprecipitation between caveolin 1 and PTRF was performed to determine an association between these two proteins on day 1 of pregnancy in isolated luminal uterine epithelial cell lysates. Cell lysates from day 1 and day 6 of pregnancy were subjected to immunoprecipitation (IP) against antibodies caveolin 1 (+), rabbit IgG (-) and PTRF (+), mouse IgG (-), and immunoblotted with PTRF and caveolin 1 antibodies respectively. Immunoblots were stripped and reprobed for immunoprecipitated antibody to confirm protein precipitate. No PTRF or caveolin 1 was detected in the respective IgG control lanes. An association between caveolin 1 and PTRF was observed on day 6 of pregnancy.


Day 6
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+
-
24 kDa
cav-1
44 kDa
reprobed
PTRF
IP cav-1
+
-
44 kDa
PTRF
24 kDa
reprobed
cav-1

4.5 DISCUSSION

Morphological caveolae have been observed at the basal plasma membrane in luminal UECs at the time of implantation. PTRF and SDPR are proteins that have been isolated from caveolar membranes (Vinten et al. 2005; Voldstedlund et al. 2001), and have been found to be associated with caveolar formation and membrane curvature. This present study investigated the localisation of SDPR and also the protein expression of PTRF and SDPR in UECs during early pregnancy and their hormonal regulation. PTRF protein abundance did not significantly change during early pregnancy or under the influence of ovarian hormones. However, at the time of implantation, PTRF co–immunoprecipitated with caveolin-1 in isolated UECs. SDPR protein expression was restricted to the time of fertilisation and also under the influence of oestrogen. This was evident in the cytoplasmic localisation of SDPR in luminal UECs at both the time of fertilisation, and under the influence of oestrogen.

PTRF has been shown to be essential for caveolae formation in adipocytes where it stabilises caveolae at the plasma membrane (Hill et al. 2008; Liu and Pilch 2008) and down regulation of PTRF causes an increase in caveolin-1 mobility, where it is released from the plasma membrane and degraded (Chadda and Mayor 2008; Liu and Pilch 2008). PTRF binds to phosphatidylserine *in vitro* and as such is thought to be associated with the enrichment and/or the organisation of lipid at the plasma membrane (Hill et al. 2012). PTRF protein expression was not altered during early pregnancy or under the influence of ovarian hormones. In this study PTRF was found to associate with caveolin-1 at the time of implantation; at which time caveolin-1 is localised to the basal plasma membrane of UECs. Since PTRF associates with caveolin-1 at the time of implantation we suggest that PTRF is also localised at the basal plasma membrane with caveolin-1 to promote caveolar formation, and to potentially generate membrane curvature in UECs at the time of implantation.

All members of the cavin family interact in an oligomeric complex not only at the plasma membrane where it associates with caveolin-1; but also in the cytosol of cells (Bastiani et al. 2009) independently of caveolin 1 expression. Formation of SDPR/PTRF complex has been shown to promote PTRF to caveolar membranes (Hansen et al. 2009; Hansen and Nichols 2010). This study has shown the

localisation of SDPR and also the expression of both PTRF and SDPR in UECs at the time of fertilisation where the observation of both the predicted molecular weight of SDPR at 49 kDa and also at 110 kDa. The higher molecular weight of SDPR at 110 kDa may be a protein complex of both PTRF and SDPR at this time that may be cytosolic at the time of fertilisation.

Previous studies have shown that SDPR colocalised with caveolin-1 and can be isolated from caveolar membranes (Mineo et al. 1998). While SDPR alone does not promote the formation of caveolae, it does affect the morphology of caveolae, where it promotes tubular morphology of caveolae (Hansen et al. 2009). This study found that SDPR protein expression is lost at the time of implantation and also under the influence of progesterone alone and in combination with oestrogen. From this finding we suggest that as the morphology of caveolae observed at the basal plasma membrane in UECs at the time of implantation does not appear to be tubular in shape; the caveolar shape in UECs may only require the expression of PTRF with caveolin-1 and not SDPR at the time of implantation.

Epithelial polarity is an important feature of UECs that is maintained throughout pregnancy. Despite the cellular changes that UECS undergo during the transition from receptive to non-receptive states, UECs maintain a polarised organisation of lipids and proteins. The difference in the lipid and cholesterol compositions at the apical and basolateral membranes in UECs at both the time of fertilisation and implantation suggest that cholesterol concentrations in the basal plasma membrane in particular is lower than what has been observed at the apical plasma membrane (Murphy and Dwarte 1987; Murphy and Martin 1985). As such, a difference in membrane rigidity, with the apical plasma membrane being more rigid than that of the basal plasma membrane would allow for a more flexible membrane which could account for the increase in membrane curvature basally at the time of implantation. PTRF and SDPR have been implicated in generating membrane curvature in cells where they bind both lipid and cholesterol and also associate with caveolin-1; in this study PTRF was observed to associate with caveolin-1 at the time of implantation suggesting that they contribute to the basolateral membrane tortuosity that is observed in UECs at the time of implantation.

In summary this work has shown an association between PTRF and caveolin 1 at the time of implantation with subsequent loss of SDPR at this time. We suggest that PTRF is involved in the formation of caveolae at the time of implantation and the loss of SDPR at this time may contribute to the morphology of caveolae observed basally in UECs. As PTRF and SDPR have previously been reported to be involved in the generation of membrane curvature in other cells (Hansen et al. 2009; Hill et al. 2008; Chadda and Mayor 2008) both PTRF and SDPR may mediate lipid and cholesterol in UECs at the time of fertilisation. However, expression of PTRF and its association with caveolin-1 at the time of implantation suggest that PTRF contributes to membrane curvature and caveolar formation at this time thereby contributing to the maintenance of epithelial cell polarity.

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CHAPTER 5 CAVEOLIN-1 AND FOCAL ADHESION PROTEINS TALIN AND PAXILLIN IN HUMAN ISHIKAWA CELL

5.1 ABSTRACT

Adhesion of cells to the extracellular matrix is mediated primarily by integrins, which cluster with other proteins at focal adhesions. Caveolin-1 has also been reported to associate with integrins facilitating focal adhesion turnover. In rat luminal uterine epithelial cells (UECs), paxillin and talin disassemble from basal focal adhesions at the time of implantation. Focal adhesions play an important role in normal uterine receptivity; and are an important structure that facilitates cellular adhesion to the underlying extracellular matrix (ECM) proteins such as fibronectin. Due to the importance of fibronectin in the uterus at the time of implantation *in vivo* the influence of fibronectin on the expression and localisation of talin and paxillin was also investigated. Caveolin-1 was also investigated in these cells as it has been reported to be involved in focal adhesion dynamics through its association with integrin β1. The actin cytoskeleton plays an important role in epithelial polarity, since talin, paxillin and caveolin-1 associate with the actin cytoskeleton; this study investigated the role of the actin cytoskeleton, by observing the effects of cytochalasin D, on the localisation and expression of paxillin, talin and caveolin-1 in Ishikawa cells.

Confocal immunofluorescence microscopy of Ishikawa cells grown on glass coverslips coated with Matrigel™ or fibronectin showed that caveolin-1, talin and paxillin are localised apically in Ishikawa cells when provided with an extracellular matrix. This is in contrast to the localisation of these proteins in UECs *in vivo* where they are localised basally. Actin disruption experiments showed a decrease in apical staining of paxillin and talin in Ishikawa cells grown on either Matrigel™ or fibronectin, however punctate staining of caveolin-1 was observed in cytochalasin D treated Ishikawa cells grown on Matrigel™. Protein abundance of talin and paxillin was greater in Ishikawa cells treated with cytochalasin D grown on fibronectin, whereas caveolin-1 protein abundance was greater in cytochalasin D treated cells grown on Matrigel™ compared to untreated cells.

The present results suggest that the presence of fibronectin influences the expression and apical localisation of these proteins in Ishikawa cells, and hence the adhesive properties that are attributed to this cell line.

5.2 INTRODUCTION

Uterine luminal epithelial cells (UEC) are the first site of contact between maternal and foetal tissue and undergo dramatic changes to be receptive to the implanting embryo. These changes, both morphological and molecular, and are known as the 'plasma membrane transformation' (Murphy 2001; Murphy 2004). One of the changes which occur in these cells is the loss of basal focal adhesions at the time of implantation.

Focal adhesions have been observed as electron dense plaques similar to immature hemi- desmosomes (Rousselle et al. 1991) a morphological structure that has been observed during early pregnancy between the basal plasma membrane and the underlying basal lamina of UECs at the time of fertilisation are absent at the time of implantation (Shion and Murphy 1995). Focal adhesions are comprised of a number of proteins, which have structural, signalling adaptor and scaffolding functions. These proteins together serve to link cytoskeletal actin fibres and the extracellular matrix (ECM) (Burridge and Chrzanowska-Wodnicka 1996). Adhesion to the ECM is mediated primarily by integrins which cluster with other proteins at focal adhesions. Integrins β1 and β3 disassemble from basal focal adhesions of UECs at the time of implantation in the rat, with integrin β3 being localised apically in UECs *in* vivo and also in Ishikawa cells (Kaneko et al. 2011a; Kaneko et al. 2011b). Both talin and paxillin are key proteins of the focal adhesion complex which serve to link the ECM to the actin cytoskeleton of cells (Nemethova et al. 2008). They have been investigated previously in the rat uterus during early pregnancy where both talin and paxillin were found to be concentrated at sites of focal adhesions at the basal plasma membrane at the time of fertilisation, and were absent at the time of implantation (Kaneko et al. 2009; Kaneko et al. 2008).

Caveolin-1 is another protein which interacts with the focal adhesion complex, which has been shown to be localised to the basal plasma membrane at the time of implantation together with an increase in morphological caveolae (Chapter 3). Caveolin-1 has been observed to interact with integrin β1 in UECs *in vivo* (Chapter 3) where it may facilitate focal adhesion turnover as has been suggested by previous studies of caveolin-1 and integrin β1 interactions (Salanueva et al. 2007; del Pozo et al. 2005).

The ECM plays a role in the morphology and growth of cells, this has been observed in previous studies where human endometrial cells grown on basement membrane such as Matrigel™ mimic the *in vivo* morphology of the endometrium (White et al. 1990). Matrigel™ is rich in type IV collagen, laminin, and heparin sulphate proteoglycan and entactin, but lacks fibronectin. Fibronectin is a major ECM ligand that has been reported to interact with integrins β1 and β3 via its RGD sequence (Schultz and Armant 1995; Yelian et al. 1995). Fibronectin is present in the basement membrane of rat UECs and in the underlying stromal cells *in vivo* and also the trophoblast cells of the blastocyst. Fibronectin expressed on the trophoblast cells of the blastocyst interacts with apically localised integrins in UECs such as integrin β3 where it serves as a bridging ligand facilitating adhesion between trophoblast cells of mouse and rat blastocysts and luminal UECs (Kaneko et al. 2012; Thorsteinsdóttir 1992; Wartiovaara et al. 1979; Yohkaichiya et al. 1988). Despite fibronectin being present in the basement membrane of UECs throughout early pregnancy in the rat, fibronectin is absent in Ishikawa cells which suggests that the source of fibronectin is the underlying endometrium *in vivo* (Kaneko et al. 2012).

Cellular attachment to the underlying extracellular matrix is important for epithelial polarity (Glasser and Mulholland 1993; van der Wouden et al. 2003). Ishikawa cells lack fibronectin, unlike what is observed *in vivo* where fibronectin is present in the basement membrane and most likely arises from the underlying endometrium (Kaneko et al. 2012). As the extracellular matrix is important for epithelial polarity, this study aimed to investigate the localisation and expression of focal adhesion proteins talin and paxillin in the Ishikawa cell line when grown on a basement membrane or fibronectin alone to examine the influence of fibronectin on the expression of these proteins in this cell line. Caveolin-1 localisation and expression was also investigated as it was observed to be associated with integrin β1 (Chapter 3) *in vivo*, and is also associated with maintenance of lipid polarity in UECs.

In addition, the actin cytoskeleton plays an important role in epithelial polarity (Li and Gundersen 2008) and talin, paxillin and caveolin-1 associate with the actin cytoskeleton *in vivo.* Thus disruption of the actin cytoskeleton with cytochalasin D in Ishikawa cells was used to study the role of the actin cytoskeleton in the

localisation and expression of these proteins when grown on a basement membrane or fibronectin alone.

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5.3 Materials and Methods

5.3.1 Culture of Ishikawa cells

Ishikawa cells are an endometrial adenocarcinoma cell line that was used in this study, and was a gift from Professor Lois Salamomsen, Prince Henry's Institute Melbourne, Australia. Coverslips (10 mm diameter) were coated with a thin coat of Matrigel™ (356234; BD Biosciences, Franklin Lakes, NJ, USA) or human fibronectin (5 μm/cm^2; 356008, BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Ishikawa cells were seeded on these coverslips and were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS; Bovogen Biologicals, Essendon, Vic, Australia) and 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA) and grown until confluent. '

5.3.2 Cytochalasin D Treatment of Ishikawa Cells

After Ishikawa cells were 80% confluent on coverslips coated with either Matrigel™ (356234; BD Biosciences, Franklin Lakes, NJ, USA) or human fibronectin (5 μm/cm^2; 356008, BD Biosciences, Franklin Lakes, NJ, USA) cytochalasin D (2 μg/mL, Sigma, Sydney, NSW, Australia) (Stevenson and Begg 1994) diluted in media was added and incubated for a further 24 hours. Cells were then collected for either immunofluorescence or western blotting analysis.

5.3.3 Immunofluorescence Microscopy of Ishikawa Cells

Ishikawa cells were fixed in 4% paraformaldehyde /PBS for 30 minutes at room temperature. Cells were washed with PBS, and subsequently permeabilised with 0.3% Triton X100 in PBS for 30 minutes. Cells were then blocked with 0.7% BSA diluted in PBS for 30 minutes at room temperature. Both primary and secondary antibodies were diluted in this blocking solution. Cells were incubated with primary antibody of mouse anti-Talin antibody (0.02 mg/mL, Sigma, Sydney, NSW, Australia, T3287); mouse anti Paxillin antibody (0.02 mg/mL, Sigma Sydney, NSW, Australia, P1093); or rabbit anti caveolin-1 antibody (0.8 μg/mL; Santa Cruz Biotechnology, Dallas, Texas, USA: sc-894) overnight at 4°C followed by incubation with secondary antibody fluorescein isothiocyanate (FITC)-conjugated AffiniPure goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories,

West Grove, PA, USA) or anti-rabbit IgG F(ab')$_2$ fragment-cy3 secondary antibody (0.5 mg/ml; Sigma, Sydney, NSW, Australia) for 2 hours at room temperature. Coverslips were inverted onto 5 µL of vectashield containing DAPI on a clean slide. Cells were examined with a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss Inc., Jena, Germany), and images were acquired using Zeiss LSM software (Carl Zeiss Inc., Jena, Germany). Z-series optical sections were taken from the bottom to top Z-plane (25 µm thick). Non immune controls were also executed, where cells were incubated with mouse or rabbit IgG purified immunoglobulin (Sigma, Sydney, NSW, Australia) instead of primary antibody. Cells seeded from five individual cell vials, with 5 wells per vial per treatment used in this study (n=25).

5.3.4 Western Blotting Analysis of Ishikawa Cells

Cells were seeded in a 6 well plate coated with a thin coat of Matrigel™ (356234; BD Biosciences, Franklin Lakes, NJ, USA) or human fibronectin (5 µm/cm^2; 356008, BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Cells were grown until confluent or when 80% confluent was treated with cytochalasin D for 24 hours. Cells were rinsed with PBS, collected from wells and centrifuged for 5 minutes at 1000*g*. The supernatant was discarded and cells resuspended in lysis buffer (50 mM Tris–HCl, pH7.5, 1 mM EDTA, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholic acid, 1% Igepal and protease inhibitor cocktail; Mammalian Cell Lysis kit; Sigma) and passed through a 23 G needle using a 1 ml syringe (Livingstone International, NSW, Australia). Protein concentrations were determined using the DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

Protein samples (40 µg) and sample buffer (8% glycerol, 50 mM Tris–HCl, pH 6.8, 1.6% SDS, 0.024% bromophenol blue, 4% β-2-mercaptoethanol) were boiled at 95°C for 5 minutes prior to loading onto a 4-20% precast SDS-polyacrylamide gel (Bio-Rad Inc. Hercules, CA, USA). Gels were subjected to electrophoresis at 200 V for 40 minutes and proteins transferred to a polyvinylidene dilfluoride (PVDF) membrane (Immunobilon™ transfer membrane; Millipore, Bedford, MA, USA) at 100 V for 1 h 15 min. Membranes were blocked by incubating in a solution of 5% skim milk powder in TBS-t (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature with constant agitation, followed by primary

antibody incubation with mouse anti-Talin antibody (70 μg/mL, Sigma, Sydney, NSW, Australia, T3287); mouse anti Paxillin antibody (0.83 μg/mL, Sigma Sydney, NSW, Australia, P1093); or rabbit anti caveolin-1 antibody (0.13 μg/mL; Santa Cruz Biotechnology, Dallas, Texas, USA: sc-894) overnight at 4°C. All antibodies were diluted with 1% skim milk powder in TBST. The membranes were washed 3 × 10 min in TBST and subsequently incubated for 2 h with sheep anti mouse (0.4 ug/mL, Amersham, GE healthcare, Buckinghamshire, UK) or goat anti-rabbit horseradish peroxidase-conjugated secondary antibody IgG (0.5 μg/mL; Dako, VIC, Australia) at room temperature with constant agitation. The proteins were detected by enhanced chemiluminescence (ECL Plus Western Blotting Detection System; Amersham, GE Healthcare, NSW, Australia) and unsaturated images were captured using the Alpha Innotech Digital Imaging System (Alpha Innotech, San Leandro, CA, USA). Membranes were subsequently rinsed in TBST and antibodies removed by heating at 60°C for 45 min in stripping buffer (62.5 mM Tris–HCl, pH 6.7, 2% SDS and 100 mM β-2-mercaptoethanol) and reprobed with mouse monoclonal anti-β-actin antibody (0.4 μg/mL; Sigma, NSW, Australia) to ensure equal loading.

5.3.5 Statistical analysis

The intensity of the bands detected from western blotting analysis was quantified using the two-dimensional spot density from the Alpha Innotech Digital System (Alpha Innotech). The integrated density value (IDV) was calculated using the AlphaEaseFC software. Statistical analysis was performed by student's t-test ; the mean ± SEM was calculated and $p < 0.05$ was considered to be significant.

5.4 RESULTS

Talin is localised apically in Ishikawa cells grown on an extracellular matrix.

Ishikawa cells grown on fibronectin (HFN) **(figure 5.1A)** or Matrigel™ **(figure 5.1C)** were stained for talin and examined under the confocal microscope, taking Z-series optical sections from the basal to the apical surface of the cell. Talin staining was present at the apical surface but absent from the basal surface under both extracellular matrix conditions. A decrease in apical staining of talin was observed from Z-series optical sections of cytochalasin D treated Ishikawa cells grown on fibronectin **(figure 5.1B)** or Matrigel™.**(figure 5.1D).**

Two protein bands of talin were observed at 225 kDa and a calpain cleaved product at 190 kDa in Ishikawa cells grown on either fibronectin (HFN) **(figure 5.2A)** or Matrigel™ **(figure 5.2B)** by western blotting analysis. The addition of cytochalasin D to Ishikawa cells grown on fibronectin alone led to an increase in the protein abundance of both full length and cleaved talin **(figure 5.2C)**. However, the addition of cytochalasin D to Ishikawa cells grown on Matrigel™ **(figure 5.2D)** resulted in no change in either full length or cleaved talin. Protein abundance of talin was compared between Ishikawa cells grown on Matrigel™ and fibronectin with or without cytochalasin D treatment. Talin whole protein (225 kDa) protein abundance showed no significant difference between Matrigel™ and fibronectin ECM **(figure 5.2E)**, however, when actin is disrupted with cytochalasin D the protease cleaved product of talin (190kDa) (Franco et al. 2004) **(figure 5.2F)** protein abundance was increased significantly when Ishikawa cells were grown on fibronectin.

Paxillin is localised apically in Ishikawa cells grown on an extracellular matrix.

Ishikawa cells grown on fibronectin (HFN) **(figure 5.3A)** or Matrigel™ **(figure 5.3C)** were stained for paxillin and examined under the confocal microscope, taking Z-series optical sections from the basal to the apical surface of the cell. Paxillin staining was present at the apical surface but absent from the basal surface under both extracellular matrix conditions. A decrease in apical staining of paxillin was observed from Z-series optical sections of cytochalasin D treated Ishikawa cells grown on fibronectin **(figure 5.3B)** or Matrigel™.**(figure 5.3D).**

Paxillin protein was observed at 68 kDa and also a 46 kDa isoform of paxillin which corresponds to paxillin δ (Tumbarello et al. 2005) in Ishikawa cells grown on both fibronectin (HFN) **(figure 5.4A)** and Matrigel™ **(figure 5.4B).** The addition of cytochalasin D to Ishikawa cells grown on Matrigel™ alone led to an increase in the protein abundance of both paxillin and paxillin δ **(figure 5.4D)**. However, the addition of cytochalasin D to Ishikawa cells grown on fibronectin **(figure 5.4C)** resulted in no change in either paxillin or paxillin δ. Protein abundance of paxillin was compared between Ishikawa cells grown on Matrigel™ and fibronectin and also when these cells were treated with cytochalasin D. Paxillin protein (68 kDa) was found to have no difference between Matrigel™ or fibronectin ECM substrates **(figure 5.4E)**; while abundance of paxillin δ protein (46 kDa) was significantly greater in Ishikawa cells grown on Matrigel™ and with cytochalasin D treatment **(figure 5.4F)**.

Caveolin-1 is localised apically in Ishikawa cells grown on an extracellular matrix and protein abundance is increased with actin disruption when grown on Matrigel™ only.

Ishikawa cells grown on fibronectin (HFN) **(figure 5.5A)** or Matrigel™ **(figure 5.5C)** were stained for caveolin 1 and examined under the confocal microscope, taking Z-series optical sections from the basal to the apical surface of the cell. Caveolin-1 staining was present at the apical surface but absent from the basal surface under both extracellular matrix conditions. A decrease in apical staining of caveolin-1 was observed from Z-series optical sections of cytochalasin D treated Ishikawa cells grown on fibronectin **(figure 5.5B)** Punctate staining of caveolin-1 apically in Ishikawa cells grown on Matrigel™ was observed when treated with cytochalasin D **(figure 5.5D)**.

Caveolin-1 protein was observed at 24 kDa in Ishikawa cells grown on both fibronectin (HFN) **(figure 5.6A)** or Matrigel™ **(figure 5.6B)**. The addition of cytochalasin D to Ishikawa cells grown on fibronectin alone **(figure 5.6C)** showed no significant change in the protein abundance of caveolin-1. However, caveolin-1 protein abundance was increased in Ishikawa cells grown on Matrigel™ when treated with cytochalasin D **(figure 5.6D)**. Protein abundance of caveolin was compared between Ishikawa cells grown on Matrigel™ and fibronectin and also when these cells were treated with cytochalasin D. Caveolin-1 showed no

significant difference in protein abundance between Ishikawa cells grown on Matrigel™ or fibronectin alone **(figure 5.6E)**. However, caveolin-1 protein abundance was significantly increased in cytochalasin D treated cells when grown on Matrigel™ compared to treated cells grown on fibronectin **(figure 5.6F)**.

Figure 5.1 Confocal Z-series optical sections of an Ishikawa cell monolayer grown on an extracellular matrix and stained with talin antibody (green) and counterstained with dapi (blue). Localisation of talin is observed apically in an Ishikawa cell monolayer grown on fibronectin (A), showing the apical and the basal surface of Ishikawa cells. Ishikawa cells grown on fibronectin and treated with cytochalasin D (B) display a reduced apical localisation of talin. Talin is observed apically in Ishikawa cells grown on Matrigel™ (C) and this localisation is reduced when Ishikawa cells grown on Matrigel are treated with cytochalasin D (D).

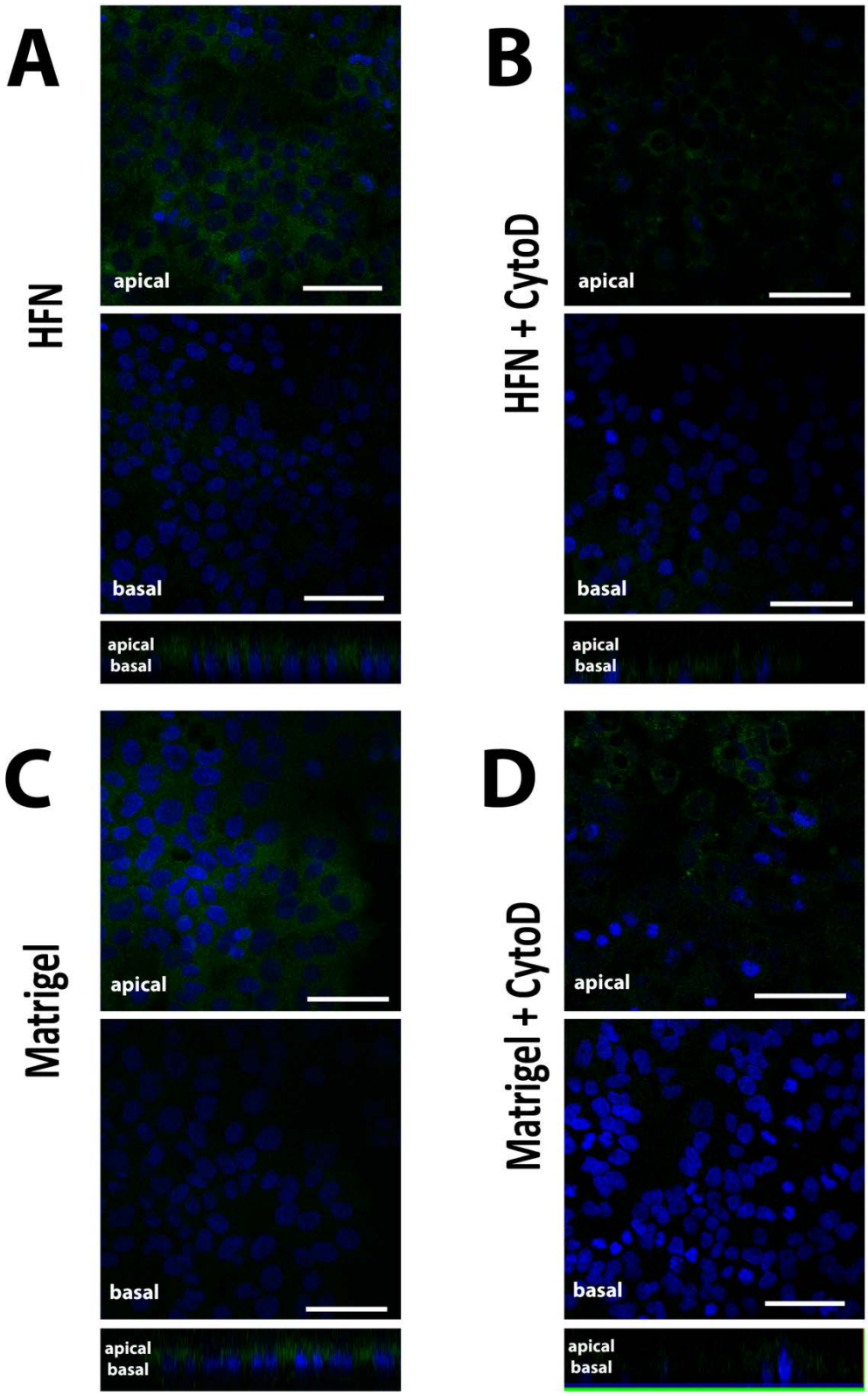
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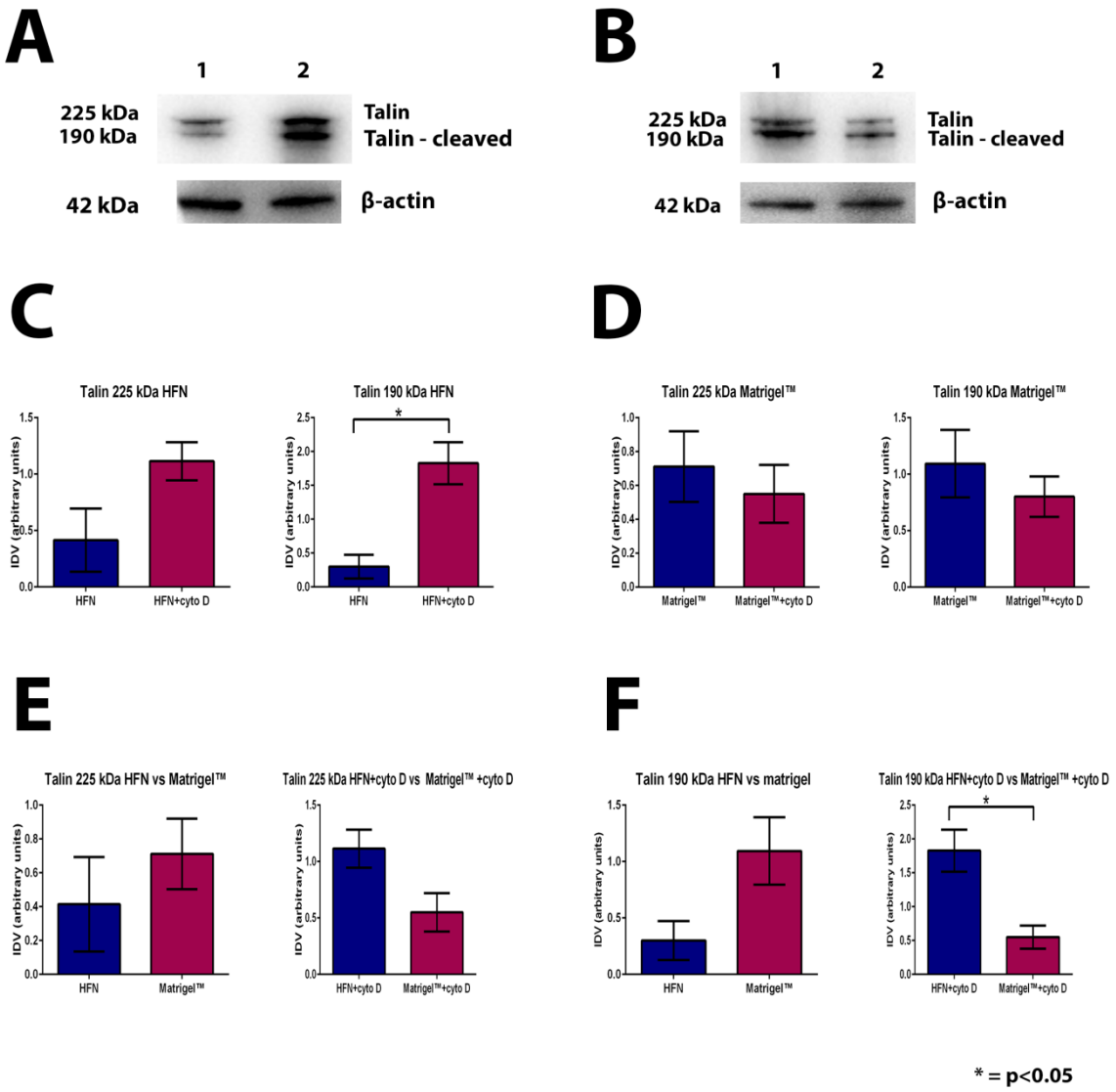
Figure 5.2 Western blotting analysis of talin from Ishikawa cells grown on fibronectin (A) or Matrigel™ (B) in both untreated (1) and cytochalasin D treated (2) cells. Talin is observed at 225 kDa and also a protease cleaved product at 190 kDa. Densitometric analysis of western blotting and statistical analysis by Student's t-test showed a significant difference in protein abundance of the 190kDa protease cleaved product of talin in Ishikawa cells grown on fibronectin when compared to untreated Ishikawa cells grown also on fibronectin (C). Comparison of talin protein abundance between cytochalasin D treated and untreated cells grown on Matrigel™ showed no significant difference (D). No significant difference of whole talin (225 kDa) protein abundance between Ishikawa cells grown on fibronectin or Matrigel™ (E). Protease cleaved talin (190 kDa) protein abundance was greater in cytochalasin D treated Ishikawa cells grown on fibronectin compared to Matrigel™ (F). Integral density values (IDV) were calculated and are shown as ± SEM.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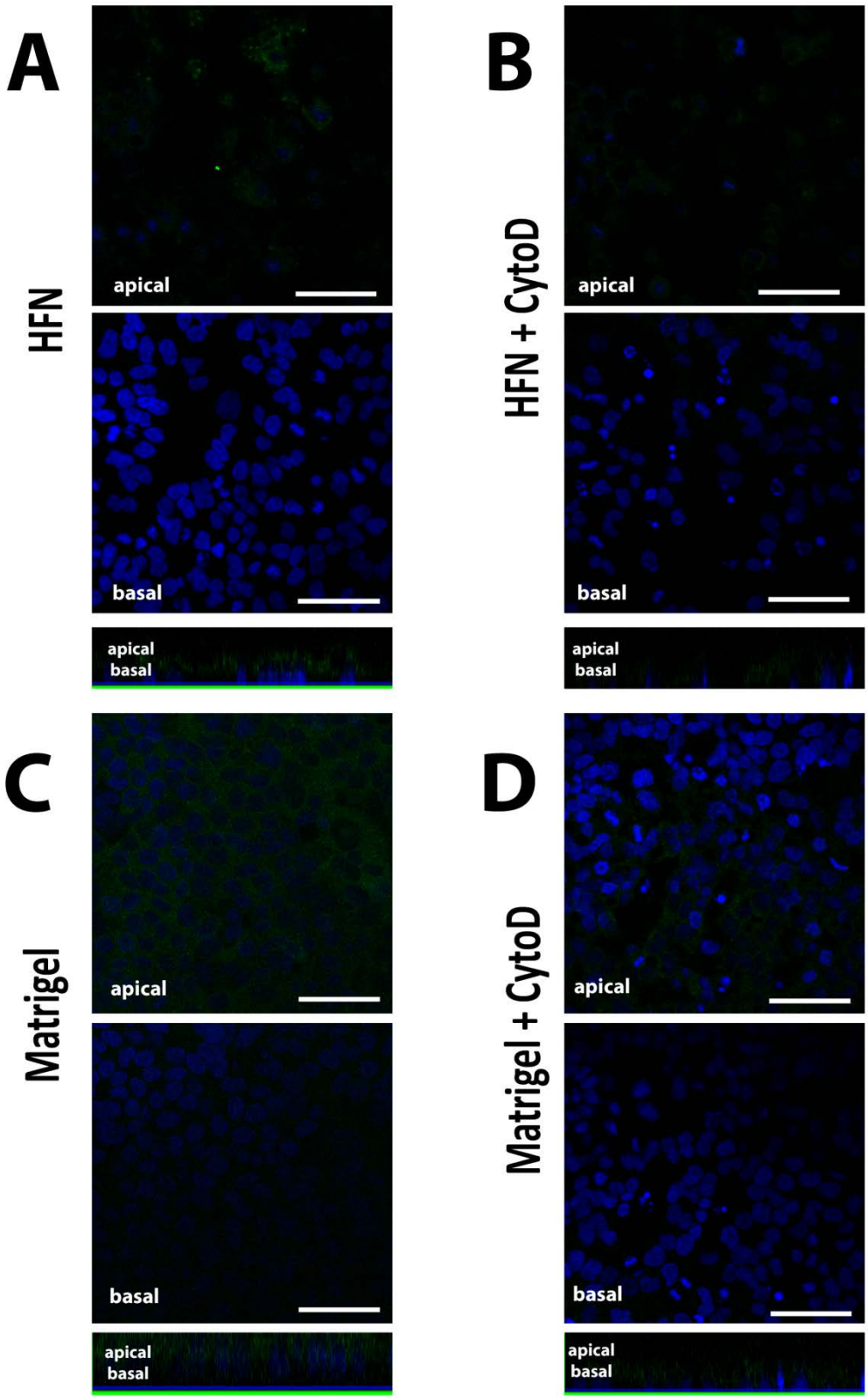
Talin
Talin - cleaved
42 kDa
β-actin
B
1
2
225 kDa
190 kDa
Talin
Talin - cleaved
42 kDa
β-actin
C
Talin 225 kDa HFN
IDV (arbitrary units)
HFN
HFN+cyto D
Talin 190 kDa HFN
*
IDV (arbitrary units)
HFN
HFN+cyto D
D
Talin 225 kDa Matrigel™
IDV (arbitrary units)
Matrigel™
Matrigel™+cyto D
Talin 190 kDa Matrigel™
IDV (arbitrary units)
Matrigel™
Matrigel™+cyto D
E
Talin 225 kDa HFN vs Matrigel™
IDV (arbitrary units)
HFN
Matrigel™
Talin 225 kDa HFN+cyto D vs Matrigel™ +cyto D
IDV (arbitrary units)
HFN+cyto D
Matrigel™+cyto D
F
Talin 190 kDa HFN vs matrigel
IDV (arbitrary units)
HFN
Matrigel™
Talin 190 kDa HFN+cyto D vs Matrigel™ +cyto D
*
IDV (arbitrary units)
HFN+cyto D
Matrigel™+cyto D

* = p<0.05

Figure 5.3 Confocal Z-series optical sections of an Ishikawa cell monolayer grown on an extracellular matrix and stained with paxillin antibody (green) and counterstained with dapi (blue). Localisation of paxillin is observed apically in an Ishikawa cell monolayer grown on fibronectin (A), showing the apical and the basal surface of Ishikawa cells. Ishikawa cells grown on fibronectin and treated with cytochalasin D (B) display a reduced apical localisation of paxillin. Paxillin is observed apically in Ishikawa cells grown on Matrigel™ (C) and this localisation is reduced when Ishikawa cells grown on Matrigel are treated with cytochalasin D (D).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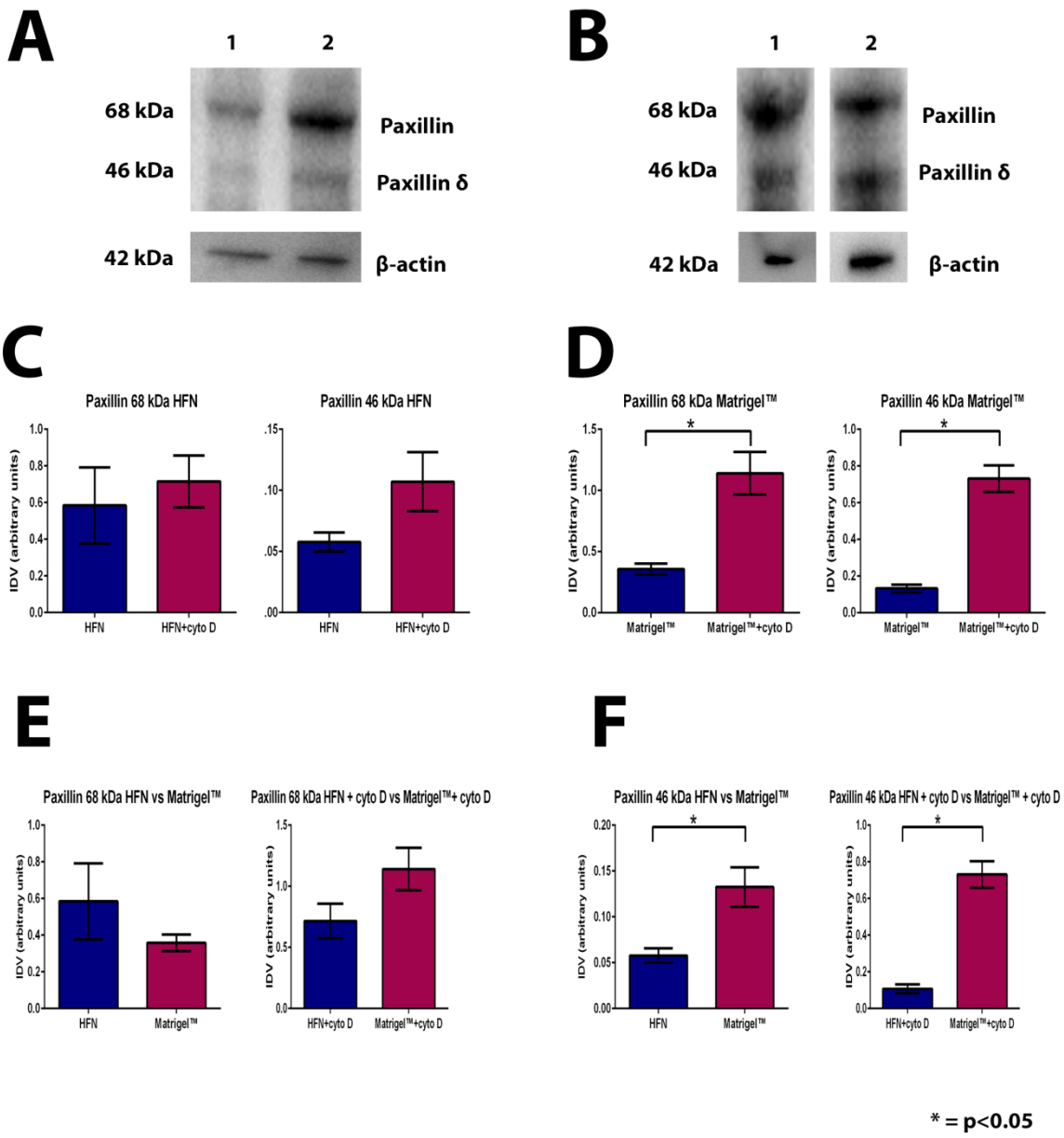
D
Matrigel + CytoD
apical
basal
apical
basal

Figure 5.4 Western blotting analysis of paxillin from Ishikawa cells grown on fibronectin (A) or Matrigel™ (B) in both untreated (1) and cytochalasin D treated (2) cells. Paxillin is observed at 68 kDa and also the isoform paxillin δ at 46 kDa. Densitometric analysis of western blotting and statistical analysis by Student's t-test showed no significant difference in protein abundance of paxillin at 68 kDa in Ishikawa cells grown on fibronectin when compared to untreated Ishikawa cells grown also on fibronectin (C). Comparison of both molecular weights of paxillin protein abundance between cytochalasin D treated and untreated cells grown on Matrigel™ was significantly greater in cytochalasin D treated Ishikawa cells (D). No significant difference of 68 kDa paxillin protein abundance between Ishikawa cells grown on FIBRONECTIN or Matrigel™ (E). Paxillin δ (46 kDa) protein abundance was greater in Ishikawa cells grown on Matrigel™ compared to those grown on fibronectin, and also in cytochalasin D treated Ishikawa cells grown on Matrigel™ compared to fibronectin (F). Integral density values (IDV) were calculated and are shown as ± SEM.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IDV (arbitrary units)
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HFN+cyto D
Paxillin 46 kDa HFN
IDV (arbitrary units)
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Paxillin 68 kDa Matrigel™
IDV (arbitrary units)
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IDV (arbitrary units)
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Matrigel™
Paxillin 46 kDa HFN + cyto D vs Matrigel™ + cyto D
IDV (arbitrary units)
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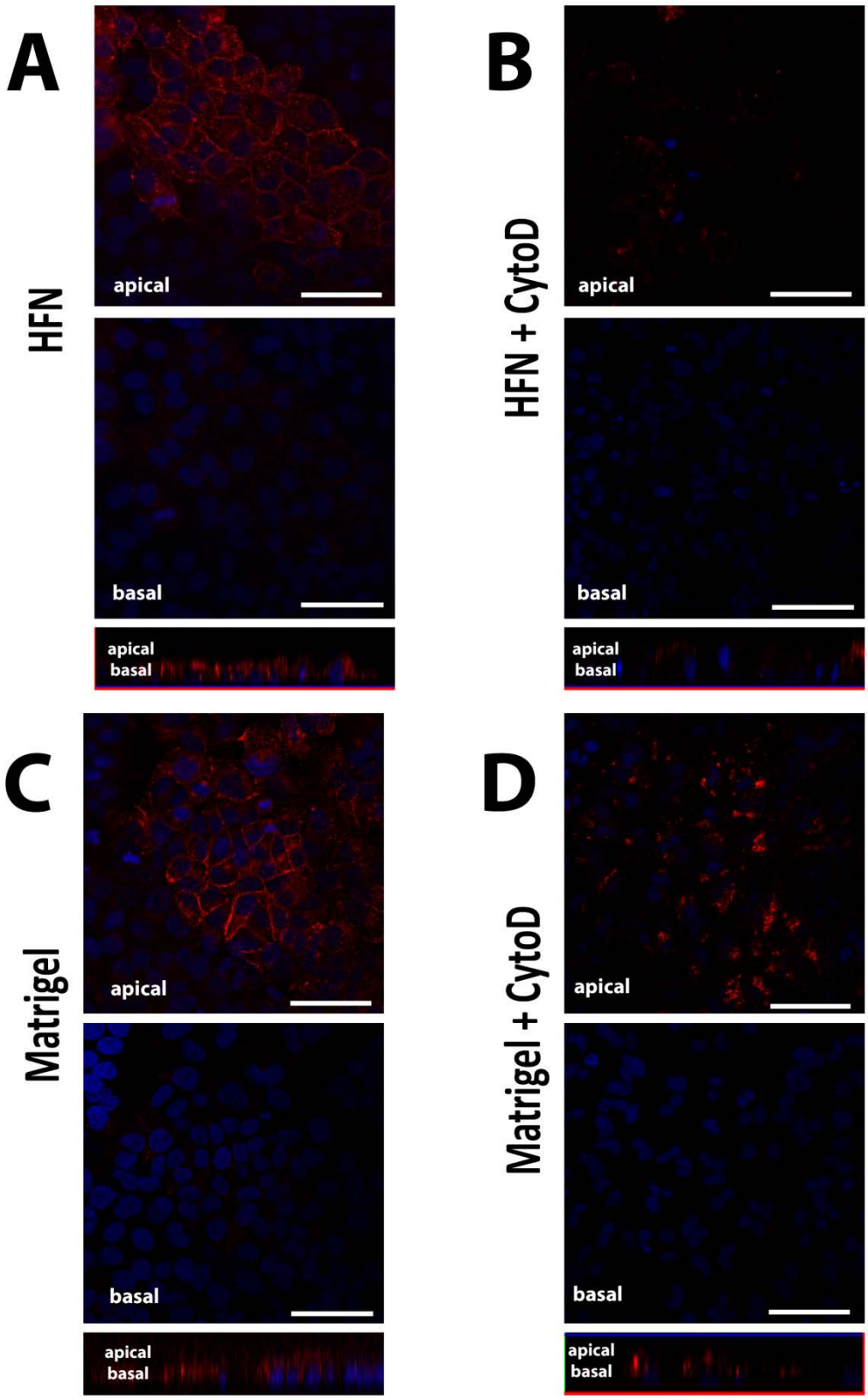
HFN+cyto D
Matrigel™+cyto D

* = p<0.05

Figure 5.5 Confocal Z-series optical sections of an Ishikawa cell monolayer grown on an extracellular matrix and stained with caveolin-1 antibody (red) and counterstained with dapi (blue). Localisation of caveolin-1 is observed apically in an Ishikawa cell monolayer grown on fibronectin (A), showing the apical and the basal surface of Ishikawa cells. Ishikawa cells grown on fibronectin and treated with cytochalasin D (B) display a reduced apical localisation of caveolin-1. Caveolin-1 is observed apically in Ishikawa cells grown on Matrigel™ (C) and when Ishikawa cells grown on Matrigel are treated with cytochalasin D punctate staining of caveolin-1 is observed (D).


A
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B
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basal
apical
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D
Matrigel + CytoD
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basal
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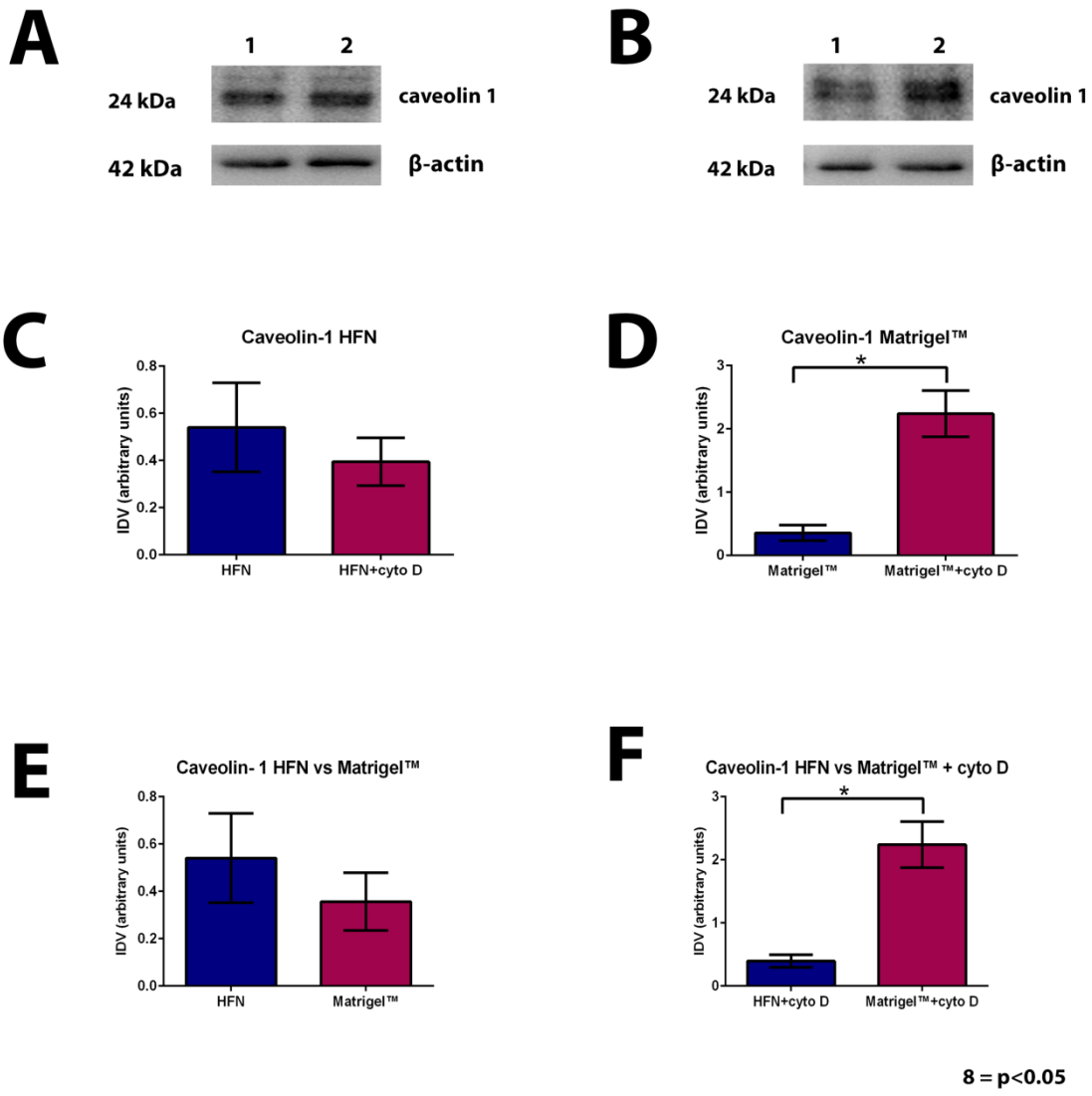
basal

Figure 5.6 Western blotting analysis of caveolin-1 from Ishikawa cells grown on fibronectin (A) or Matrigel™ (B) in both untreated (1) and cytochalasin D treated (2) cells. Densitometric analysis of western blotting and statistical analysis by Student's t-test showed no significant difference in protein abundance of Caveolin-1 (24 kDa) in Ishikawa cells grown on fibronectin when compared to untreated Ishikawa cells grown also on fibronectin (C). Caveolin-1 (24 kDa) protein abundance was greater in cytochalasin D treated Ishikawa cells grown on Matrigel™ compared with untreated cells grown on Matrigel™ (D). There was no significant difference of Caveolin-1 (24 kDa) protein abundance between Ishikawa cells grown on fibronectin or Matrigel™ (E). Caveolin-1 (24 kDa) protein abundance was greater in cytochalasin D treated Ishikawa cells grown on Matrigel™ compared to fibronectin (F). Integral density values (IDV) were calculated and are shown as ± SEM.


A
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24 kDa
caveolin 1
42 kDa
β-actin
B
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2
24 kDa
caveolin 1
42 kDa
β-actin
C
Caveolin-1 HFN
IDV (arbitrary units)
0.8
0.6
0.4
0.2
0.0
HFN
HFN+cyto D
D
Caveolin-1 Matrigel™
*
IDV (arbitrary units)
3
2
1
0
Matrigel™
Matrigel™+cyto D
E
Caveolin- 1 HFN vs Matrigel™
IDV (arbitrary units)
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F
Caveolin-1 HFN vs Matrigel™ + cyto D
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IDV (arbitrary units)
3
2
1
0
HFN+cyto D
Matrigel™+cyto D

8 = p<0.05

5.5 DISCUSSION

Cellular adhesion in UECs is an exquisitely controlled process, to both maintain an epithelial barrier and to also allow for attachment and invasion of the embryo during early pregnancy. Adhesion of UECs to the underlying ECM is mediated by focal adhesions providing a structural link between the ECM and the actin cytoskeleton (Burridge et al. 1988; Burridge and Chrzanowska-Wodnicka 1996; Dubash et al. 2009). Fibronectin has been implicated as a key ECM ligand that facilitates cell-ECM adhesion through interaction with integrins β1 and β3. Fibronectin has been shown to be present in the basement membrane of UECs and underlying stroma, as well as the trophoblast cells of the blastocyst where it interacts with apically localised integrin β3 in UECs. Fibronectin is also found in Ishikawa cells to facilitate adhesion (Kaneko et al. 2011b; Kaneko et al. 2009; Kaneko et al. 2008). However, there have been no studies on the effect of fibronectin in the expression of other proteins involved in the focal adhesion complex such as talin and paxillin.

This study investigated talin, paxillin, and also caveolin-1 in Ishikawa cells grown on Matrigel™ or human fibronectin with or without actin disruption with cytochalasin D. Talin, paxillin and caveolin 1 were localised apically in Ishikawa cells when grown on both Matrigel™ and fibronectin. When the actin cytoskeleton of Ishikawa cells was disrupted a decrease in the apical staining of paxillin and talin was observed, while punctate staining was observed for caveolin-1.

Talin and paxillin were observed to be localised apically in Ishikawa cells, which may correspond to the apical localisation of integrin β3 previously described in Ishikawa cells (Kaneko et al. 2011a). In this apical localisation talin and paxillin are able to link the actin cytoskeleton via integrins which in turn bind to ECM ligands, facilitating cell adhesion (Hynes 1992). Various integrins including integrin β3 have been characterised in the Ishikawa cell line (Castelbaum et al. 1997) and have also been shown to bind to ECM ligands including fibronectin, vitronectin, von Willebrand factor, thrombospondin and osteopontin all of which contain the Arg-Gly-Asp (RGD) integrin recognition sequence (Hynes 1992). Hence, ligands expressed in the ECM can influence the expression of these proteins in cells. The adhesive apical surface of Ishikawa cells facilitates its use as receptive endometrial

epithelial cell model (Hannan et al. 2010; Nishida et al. 1985), and molecules such as integrins, paxillin and talin seen in this study may contribute to this trait.

The expression of talin (225 kDa) protein abundance showed no significant difference when grown on Matrigel™ or fibronectin ECM, however, when actin was disrupted with cytochalasin D the protease-cleaved product of talin (190kDa) (Franco et al. 2004) was significantly greater when Ishikawa cells were grown on fibronectin. While calpain expression or activity has not been investigated in Ishikawa cells the presence and elevated protein abundance of the calpain-cleaved product of talin in Ishikawa cells grown on fibronectin and treated with cytochalasin D suggests that calpain and its activity may be elevated under these conditions, where it may facilitate focal adhesion protein turnover.

Paxillin protein (68 kDa) showed no significant difference when grown on Matrigel™ or fibronectin ECM substrates; while the paxillin δ isoform (46 kDa) showed a significant increase when grown on Matrigel™ and when treated with cytochalasin D. Paxillin has been shown to play an important role in cell migration, a process that requires remodelling of the actin cytoskeleton (Turner 2000a, b). Paxillin δ lacks the two main phosphorylation sites of paxillin at tyrosine residues 31 and 118 (Tumbarello et al. 2005) and its expression has been correlated with inhibition of tyrosine phosphorylation of paxillin. Actin disruption of cells influences the expression of paxillin in this manner (Hu et al. 2006); as shown by the significant increase in paxillin δ observed in cytochalasin D treated Ishikawa cells. Actin disruption of Ishikawa cells grown on Matrigel™ may affect focal adhesion turnover, and cell migration as seen by the increase in paxillin δ protein abundance under cytochalasin D treatment.

Caveolin-1 was localised apically in Ishikawa cells grown on both Matrigel™ and fibronectin. Interestingly, caveolin-1 is localised basally in rat luminal UECs and is associated with basally located morphological caveolae at the time of implantation (Chapter 3). However in some cell culture models morphological caveolae and caveolin-1 have been found apically (Frank et al. 2003; Hu et al. 2008; Inder et al. 2012; Mora et al. 2006). Caveolin-1 has been implicated in focal adhesion turnover, by its association with integrin β1 (Borza et al. 2010; del Pozo et al. 2005; Madawala et al. Chapter 3) and also FAK which has also been localised apically in Ishikawa cells (Kaneko et al. 2012).

Previous studies of caveolae in the plasma membrane have shown that they are connected to the actin cytoskeleton by the association between caveolin-1 and the actin cross linking protein filamin A (Stahlhut and van Deurs 2000). Actin disruption led to a decrease in apical staining of caveolin-1 in Ishikawa cells grown on fibronectin. However, Ishikawa cells grown on Matrigel™ showed punctate staining of caveolin-1 in the cytoplasm. The punctate staining observed may be due to the disruption of the association of caveolin-1 with filamin A resulting in the shift of caveolin-1 from the apical plasma membrane to another cellular compartment such as the golgi complex. The punctate staining of caveolin-1 in cytochalasin D treated Ishikawa cells grown on Matrigel™ correlates with the significantly greater amount of caveolin-1 protein in these cells compared to treat Ishikawa cells grown on fibronectin.

Cellular adhesion is a process that is both tightly controlled and multifaceted. This study has demonstrated the apical localisation of talin, paxillin, and caveolin-1 in Ishikawa cells when grown on a basement membrane or on fibronectin alone. However, the mechanisms by which these cells interact with the ECM at the basal plasma membrane require further study. The expression and localisation of the proteins investigated here in Ishikawa cells can be attributed to the adhesive qualities of the apical plasma membrane with the expression of focal adhesion proteins talin and paxillin being susceptible to actin disruption when grown on fibronectin. This suggests fibronectin influences the expression and localisation of these proteins and their association with the actin cytoskeleton in Ishikawa cells and hence the nature of these cells in culture.

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CHAPTER 6: Actin Crosslinking Protein Filamin A During Early Pregnancy In The Rat Uterus

6.1 ABSTRACT

During early pregnancy the endometrium undergoes a dramatic transformation in order for it to become receptive to blastocyst implantation. The actin cytoskeleton and plasma membrane of luminal uterine epithelial cells (UECs) and the underlying stromal cells undergo dramatic remodelling to facilitate these changes. Filamin A (FLNA), a protein that cross links actin filaments and also mediates the anchorage of membrane proteins to the actin cytoskeleton was investigated in the rat uterus at fertilisation (day 1) and implantation (day 6) to determine the role of FLNA in actin cytoskeletal remodelling of UECs and decidua during early pregnancy.

Localisation of FLNA in UECs at the time of fertilisation was cytoplasmic, and is distributed apically at the time of implantation, and under the influence of progesterone. FLNA was also concentrated to the first two to three stromal cell layers at the time of fertilisation and shifts to the primary decidualisation zone at the time of implantation. This shift in localisation was found to be dependent on the decidualisation reaction. Protein abundance of the FLNA 280 KDa monomer, and calpain cleaved fragment (240 KDa) did not change during early pregnancy in UECs.

There is a vast array of actin cytoskeletal remodelling during early pregnancy in UECs and in decidual cells. These results suggest that FLNA may be an important regulator of cytoskeletal remodelling of these cells to allow uterine receptivity and decidualisation necessary for successful implantation in the rat.

6.2 INTRODUCTION

Cells of the endometrium undergo dramatic changes during early pregnancy to allow blastocyst implantation. The transformation of non-receptive luminal uterine epithelial cells (UECs) to a receptive state involves the loss of apical microvilli, terminal web, integrin based focal adhesions (Kaneko et al. 2011b; Kaneko et al. 2009; Kaneko et al. 2008; Luxford and Murphy 1989; Luxford and Murphy 1992b), the appearance of uterodomes apically (Murphy 2000) and increase in basolateral membrane tortuosity in UECs (Shion and Murphy 1995) . All of these features constitute the plasma membrane transformation (Murphy 2004). Decidualisation of endometrial stromal cells (ESCs) involves a number of changes including transition from long thin fibroblast cells to large and rounded decidual cells along with the formation of adherens type junctional structures at sites of contact between adjacent decidual cells (Finn and Keen 1963; Finn and Lawn 1967). There is also an accumulation of glycogen, lipids and fibrillar material arranged into a filamentous meshwork in decidual cells at decidualisation (Kearns and Lala 1983). These changes that both UECs and ESCs undergo involve remodelling of the actin cytoskeleton, which is mediated by actin binding proteins (Loo et al. 1998; Shaw et al. 1998; Terry et al. 1996). A number of actin binding proteins have been previously investigated in the uterus during early pregnancy. Plectin and α-actinin are two actin binding proteins that both localise apically in UECs at the time of implantation (Terry et al. 1996), with α-actinin also present in decidual cells (Shaw et al. 1998). Proteomic analysis of the human endometrium during the secretory/proliferative phase has also identified the presence of Filamin A (DeSouza et al. 2005).

Filamin A (FLNA) is an F-actin cross linking protein, that has wide tissue expression during development (Feng and Walsh 2004). The FLNA molecule is comprised of two 280 kDa subunits that form a homodimer at the C-terminus. The actin binding domain of FLNA is located at the N-terminus, where orthogonal actin networks have been shown to be arranged *in vitro* (Boxer and Stossel 1976; Gorlin et al. 1990; Hartwig and Stossel 1975; Stossel et al. 2001). FLNA has been reported to interact with a number of proteins (van der Flier and Sonnenberg 2001) that are membrane receptors for cell signalling molecules such as integrins

which have been shown to be important for uterine receptivity with regards to blastocyst-epithelial attachment (Cunningham et al. 1992; Gorlin et al. 1990; Kaneko et al. 2011a; Kaneko et al. 2011b; Stossel et al. 2001).

The actin cytoskeleton plays an important role in the formation of a number of key cellular structures such as the terminal web, microvilli, and cellular junctions, which form the basis for cell polarity and plasma membrane stability (Li and Gundersen 2008). These structures undergo dynamic changes during early pregnancy (Luxford and Murphy 1989; Luxford and Murphy 1993; Murphy 2001) leading to uterine receptivity. As FLNA is an actin binding protein which may be involved in the remodelling of the actin cytoskeleton of UECs and ESCs during early pregnancy, this study investigated the localisation and expression of FLNA in the uterus of the rat during early pregnancy, and also determined its hormonal regulation, and decidualisation.

6.3 MATERIALS AND METHODS

6.3.1 Animals

6.3.1.1 Normal Pregnancy

Female virgin Wistar rats (10–12 weeks of age) were housed in plastic cages at 21°C with a 12-h light–dark cycle and were fed and watered *ad libitum*. Vaginal smearing was performed to determine the stage of the oestrous cycle, and those rats showing a vaginal smear characteristic of pro-oestrus were caged overnight with a male of proven fertility. The presence of sperm in the vaginal smear taken the following morning indicated mating and was designated day 1 of pregnancy. Uterine horns from days 1 and 6 were collected to correspond to the time of fertilisation, and time of implantation. Five rats were collected for each day of pregnancy. All experimental procedures were approved by The University of Sydney ethics committee.

6.3.1.2 Ovariectomy

Female virgin Wistar rats were bilaterally ovariectomised while anaesthetised with an intraperitoneal injection of xylazine (4 mg/kg; TROY laboratories Pty. Ltd., Smithfield, NSW, Australia) and ketamine (75 mg/kg; Parrell Laboratories (AUST) Pty. Ltd., Alexandria, NSW, Australia) and were allowed to recover for at least 3 weeks. Progesterone (Sigma, St Louis, MO, USA) and 17-β-oestradiol (Sigma) were dissolved in benzyl alcohol (Sigma) and peanut oil in 1:4 v/v and injected as previously described (Murphy and Rogers 1981). Five animals were randomly allocated to each of the following hormonal regimes. Group 1 (control) was injected with 0.1 ml of carrier (benzyl alcohol and peanut oil) alone for three consecutive days. Group 2 (PPP) received 5 mg progesterone dissolved in 0.1 ml of carrier for three consecutive days. Group 3 (EEE) received 0.5 μg 17-β-oestradiol in 0.1 ml of carrier for 3 consecutive days. Group 4 (PPPE) received 0.1 ml of 5 mg progesterone for 3 consecutive days and also 0.5 μg of 17-β-oestradiol dissolved in 0.1 mL on the opposite side of the neck on the third day. PPPE is the minimal requirement for blastocyst implantation (Murphy and Rogers 1981; Psychoyos 1973) Injections were administered in the morning and animals were killed 24 h after the last injection.

6.3.1.3 Pseudo-pregnancy and Artificial Decidualisation

Rats in pro-oestrus were caged overnight with a vasectomised male. The presence of a vaginal plug the following morning indicated mating and was designated day 1 of pseudo pregnancy. Vaginal smears were taken each day of pseudo pregnancy (n=5) to confirm that a pseudo pregnant state remained. Uterine horns from day 6 of pseudo pregnancy were collected to correspond to the time of implantation. Artificial decidualisation was induced on day 5 of pseudo pregnancy by making a small unilateral incision on the left side to expose the tubal end of the uterine horn. An intraluminal injection of 0.1 mL of peanut oil was administered to the left horn only with the right horn left intact. On day 6 of pseudo pregnancy a 0.25 mL 1% pontamine sky blue dye was administered by tail vein injection, under isofluorane anaesthesia. Observation of a pontamine sky blue reaction (PSB) indicated successful induction of decidualisation (Finn and Keen 1963; Finn and McLaren 1967). Five rats were collected for both pseudo pregnancy and also artificially induced decidualisation.

All animals in pregnancy, pseudo pregnancy, artificially induced decidualisation and hormone treated groups were killed with an intraperitoneal injection of sodium pentobarbitone (Nembutal; Merial Australia, Parramatta, NSW, Australia). Uterine horns were excised and randomly allocated for either immunofluorescence microscopy (IF), or isolation of uterine epithelial cells for western blotting analysis.

6.3.2 Immunofluorescence microscopy

Uterine tissue from all animals was embedded in O.C.T compound (Tissue-Tek; Sakura Firetek, Torrance, CA, USA), snap frozen in supercooled isopentane (BDH Laboratory supplies, Poole Dorset, England, UK) and stored under liquid nitrogen until use. Sections, 7 μm, were cut using a Leica LM 3050 cryostat (Leica, Heerbrugg, Switzerland). Tissue sections were air dried on gelatine-chrome alum-coated glass slides at room temperature and fixed with 4% paraformaldehyde for 10 min at room temperature, washed with PBS 3 x 5 min, and blocked in 1% bovine serum albumin (BSA; Sigma) in PBS for 30 min. All primary and secondary antibodies were diluted with this blocking solution. Sections were incubated with primary antibody of mouse monoclonal Filamin 1 (E-3) antibody (0.8 μg/mL;

Santa Cruz Biotechnology, Dallas, Texas, USA: sc-17749), overnight at 4 ˚C. Sections were washed in PBS 3 × 5 min, and incubated with fluorescein isothiocyanate (FITC)-conjugated AffiniPure goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at a concentration of 2.5 μg/mL for 1 h in the dark followed by 3 × 5 min PBS wash. Sections were then mounted with vectashield containing DAPI (Vector, Burlingame, CA, USA), cover slipped and examined with a Zeiss Deconvolution microscope (Carl Zeiss Inc., Jena, Germany). Immunofluorescence micrographs were taken using a Zeiss AxioCamHR digital monochrome CCD camera (Carl Zeiss Inc., Jena, Germany) and Zeiss AxioVision version 4.0 image-acquisition software. Non-immune controls were carried out with experimental runs where sections were incubated with mouse IgG purified immunoglobulin (0.8 μg/mL; Sigma) in place of the primary antibody.

6.3.3 Isolation of uterine luminal epithelial cells

Uterine luminal epithelial cells were isolated from each animal as previously described (Kaneko et al. 2008). The uterine horn was opened longitudinally and surface luminal epithelial cells were scraped off using sterile surgical blades (Livingstone International, Rosebery, NSW, Australia) and immediately placed into lysis buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.1% SDS, 0.5% Deoxycholic acid, 1% Igepal and protease inhibitor cocktail; 10% phosphatase inhibitor; Sigma Mammalian Cell lysis kit, Sigma). The cell lysate was passed through a 23 gauge needle using a 1 ml syringe (Livingstone, International, Rosebery, NSW, Australia) and briefly centrifuged at 8,000g at 4 °C. The supernatant was collected and frozen immediately in liquid nitrogen and stored at −80°C until use.

6.3.4 Western blotting analysis

Protein concentrations were determined using the BCA protein assay (Micro BCA™ Protein assay kit; Quantum Scientific, Murarrie, QLD, Australia) and POLAR Star Galaxy microplate reader (BMG LabTech, Durham, NC, USA) according to the manufacturer's instructions. Protein samples (20 μg) and sample buffer (8% glycerol, 50 mM Tris–HCl, pH 6.8, 1.6% SDS, 0.024% bromophenol blue, 4% β-2-mercaptoethanol) were boiled at 95°C for 5 minutes prior to loading onto a 4-20%

precast SDS-polyacrylamide gel (Bio-Rad Inc. Hercules, CA, USA). Gels were subjected to electrophoresis at 200 V for 40 minutes and proteins transferred to a polyvinylidene dilfluoride (PVDF) membrane (Immunobilon™ transfer membrane; Millipore, Bedford, MA, USA) at 100 V for 1 h 15 min. Membranes were blocked by incubating in a solution of 5% skim milk powder in TBS-t (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature with constant agitation, followed by primary antibody incubation with mouse monoclonal anti-Filamin -1 antibody (0.13 µg/mL, Santa Cruz: sc-17749) overnight at 4°C. All antibodies were diluted with 1% skim milk powder in TBS-t. The membranes were washed 3 × 10 min in TBS-t and subsequently incubated for 2 h with sheep anti mouse IgG antibody (0.4 µg/mL; Amersham, UK) at room temperature with constant agitation. The proteins were detected by enhanced chemiluminescence (Immobilon Western Chemiluminescent HRP, Merk Millipore Billerica, MA, USA) and unsaturated images were captured using the Bio-Rad ChemiDoc MP Imaging System (Bio-Rad Inc. Hercules, CA, USA). Membranes were subsequently rinsed in TBS-t and antibodies removed by heating at 60°C for 45 min in stripping buffer (62.5 mM Tris–HCl, pH 6.7, 2% SDS and 100 mM β-2-mercaptoethanol) and reprobed with mouse monoclonal anti-β-actin antibody (0.4 µg/mL; Sigma, NSW, Australia) to ensure equal loading.

6.3.5 Statistical analysis

FLNA protein abundance was quantified by densitometric analysis. The intensity of the bands detected from western blotting analysis was quantified using the two-dimensional spot density from the Bio-Rad ChemiDoc MP Imaging System (Bio-Rad Inc. Hercules, CA, USA). The integrated density value (IDV) was calculated using the Image Lab 4.1 (Bio-Rad Inc. Hercules, CA, USA) software and FLNA bands were normalised to those of actin. Statistical analysis was performed by student's t-test for (day 1, day 6) early pregnancy and one-way ANOVA for comparison FLNA protein abundance in hormone treatment groups (P, E,PE, C). The mean ± SEM was calculated and $p < 0.05$ was considered to be significant.

6.4 RESULTS

FLNA is localised apically in luminal uterine cells only at the time of implantation.

Immunofluorescence microscopy showed FLNA to be concentrated at the first 2-3 ESC layers underlying the uterine epithelium on day 1 of pregnancy (arrow head) along with a diffuse cytoplasmic localisation in UECs **(figure 6.1a, b)**. On day 6 of pregnancy FLNA was localised apically in UECs **(figure 6.1c, d)** and also redistributed to the primary decidualisation zone of the endometrium **(figure 6.1c)**.

Western Blotting analysis of isolated UECs from day 1 and day 6 of pregnancy showed no significant difference in protein abundance of the two bands of FLNA observed at 280 kDa and 240 kDa **(figure 6.1e)**.

Apical localisation of FLNA in UECs is dependent on progesterone

The hormonal control of FLNA localisation was determined using ovariectomised rats treated with various hormonal regimes. FLNA is localised to the apical plasma membrane of UECs in rats treated with PPP, and PPPE hormonal regimes **(figure 6.2a, c)**. In rats under EEE treatment, FLNA was strongly localised to the underlying stromal cells **(figure 6.2b)**, while the PPP and PPPE treatments showed an even distribution of FLNA throughout the endometrium. Minimal staining in UECs was observed in vehicle control **(figure 6.2d)**.

Hormonal treatment did not affect the protein abundance of 280 kDa band of FLNA **(figure 6.2e)**. Expression of a 240 kDa band was also observed under the influence of exogenous ovarian hormones **(figure 6.2e)** but there was no statistical difference in its abundance.

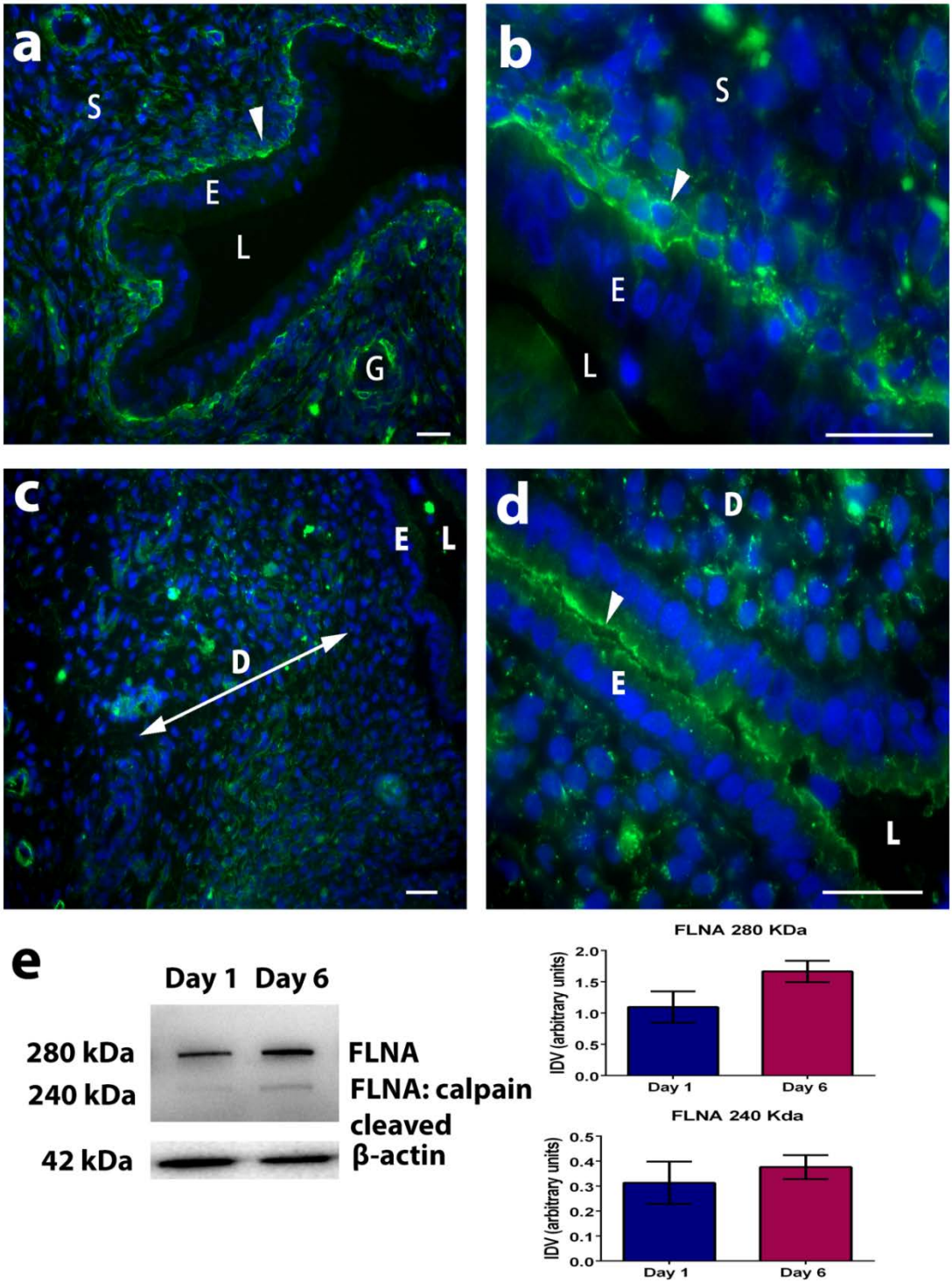
FLNA localisation in endometrial stroma is altered with decidualisation

FLNA localisation in the endometrium was found to be evenly distributed under PPP and PPPE hormone regimes, but did not mimic what was observed in the stroma of day 6 pregnant rats. Artificial decidualisation was induced in the left uterine horn on day 5 of pseudo pregnancy **(figure 6.3b)**. Day 6 pseudo-pregnant

uteri showed an apical localisation of FLNA in UECs (arrow head) but an even distribution throughout the underlying stroma. Artificially induced decidualisation of the left horn on day 5 of pseudo pregnancy showed a shift in the localisation of FLNA to the primary decidualisation zone of the endometrium by day 6 **(figure 6.3c, e)**. The right control horn showed the same localisation of FLNA observed in pseudo pregnancy **(figure 6.3d, f)**. Non immune controls were carried out with all experimental runs, and no staining was observed. Day 6 is shown as a representative example **(figure 6.3a)**.

Figure 6.1 Immunofluorescence microscopy and western blotting of FLNA in the uterus during early pregnancy

Immunofluorescence micrographs of endometrium stained with monoclonal FLNA antibody (green) and counterstained with dapi (blue). On day 1 of pregnancy (a, b) FLNA displays cytoplasmic localisation in UECs, and is also localised to the first 2-3 stromal cells underlying UECs (arrowhead). On day 6 of pregnancy (c, d) FLNA is localised apically in UECs (arrow head) and is distributed to the primary decidual zone of the endometrium (double headed arrow). Western blotting of isolated UECs from day 1 and day 6 of pregnancy (e), β-actin was used as a loading control. Densitometric analysis of western blotting and statistical analysis by students t-test of FLNA (280 kDA) protein abundance did not change between the two days of pregnancy studied (n=5). A calpain cleaved product of FLNA (240 kDa) was observed on both days of pregnancy investigated, this band was not found to change significantly between day 1 and day 6 of pregnancy. E, luminal epithelial cells; L, lumen; S, stroma; G, gland; D, decidua; double headed arrow, primary decidual zone.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D
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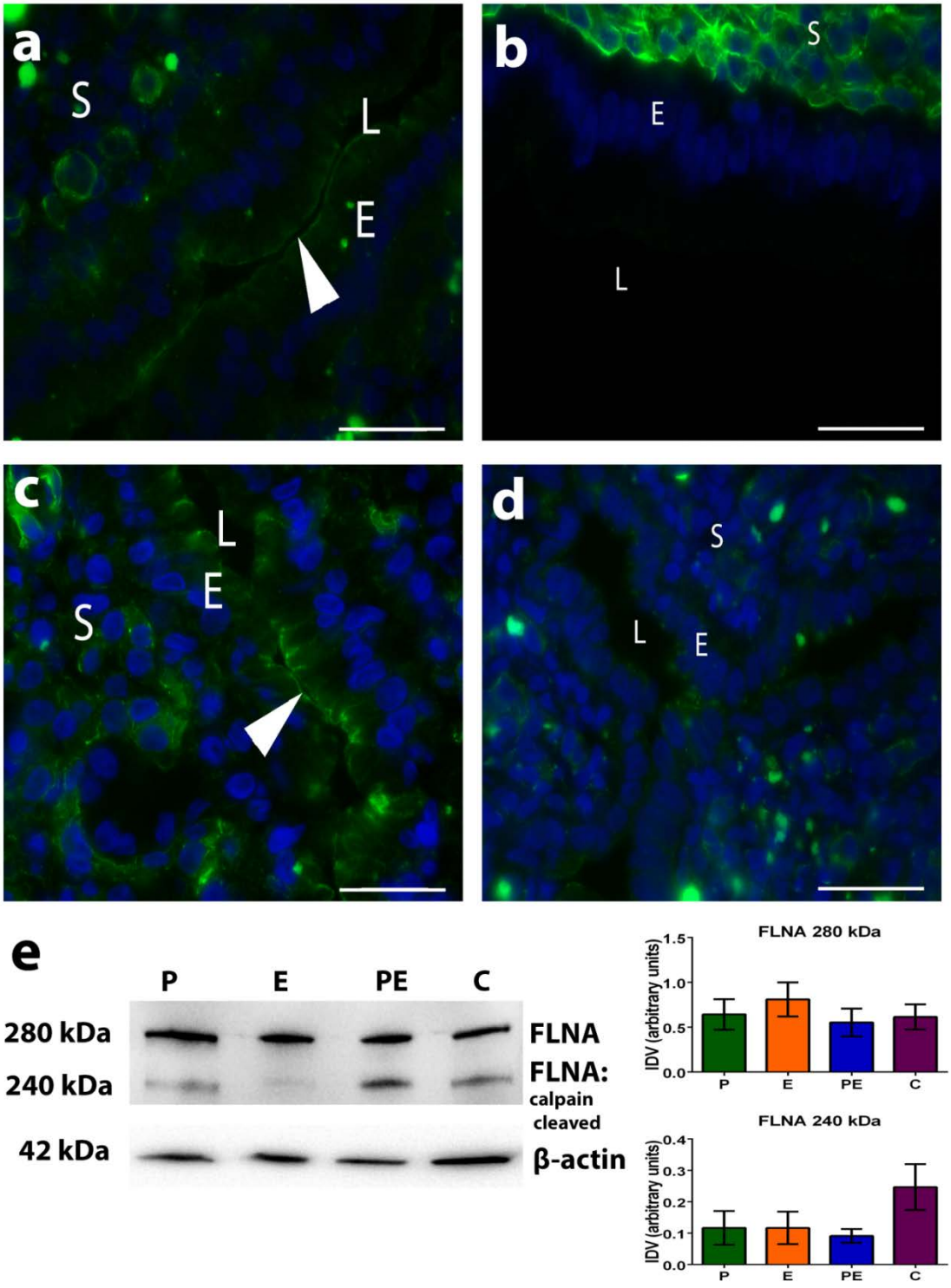
Day 1 Day 6
280 kDa
240 kDa
42 kDa
FLNA
FLNA: calpain cleaved
β-actin
FLNA 280 KDa
IDV (arbitrary units)
2.0
1.5
1.0
0.5
0.0
Day 1
Day 6
FLNA 240 Kda
IDV (arbitrary units)
0.5
0.4
0.3
0.2
0.1
0.0
Day 1
Day 6

Figure 6.2 Immunofluorescence microscopy and western blotting of FLNA in ovariectomised rats

Immunofluorescence micrographs of endometrium stained with monoclonal FLNA antibody (green) and counterstained with dapi (blue). FLNA apical localisation in UECs from uteri of ovariectomised rats treated progesterone only (a) and also with combination of progesterone and oestrogen (c). FLNA was localised to the stromal cells that are directly beneath the UECs under oestrogen only treatment (b). FLNA displayed a diffuse cytoplasmic staining in UECs of the vehicle control (d). Western blotting analysis of isolated UECs from ovariectomised rats (n=5) (e) treated with progesterone alone (P), oestrogen alone (E), oestrogen and progesterone (PE), and vehicle alone (C). Densitometric analysis of western blotting and statistical analysis by one-way ANOVA showed no significant difference of FLNA protein abundance between hormone regimes. Integral density values (IDV) were calculated and are shown as ± SEM. E, luminal epithelial cells; L, lumen; S, stroma.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280 kDa
240 kDa
42 kDa
FLNA
FLNA: calpain cleaved
β-actin
FLNA 280 kDa
IDV (arbitrary units)
1.5
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0.5
0.0
P
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FLNA 240 kDa
IDV (arbitrary units)
0.4
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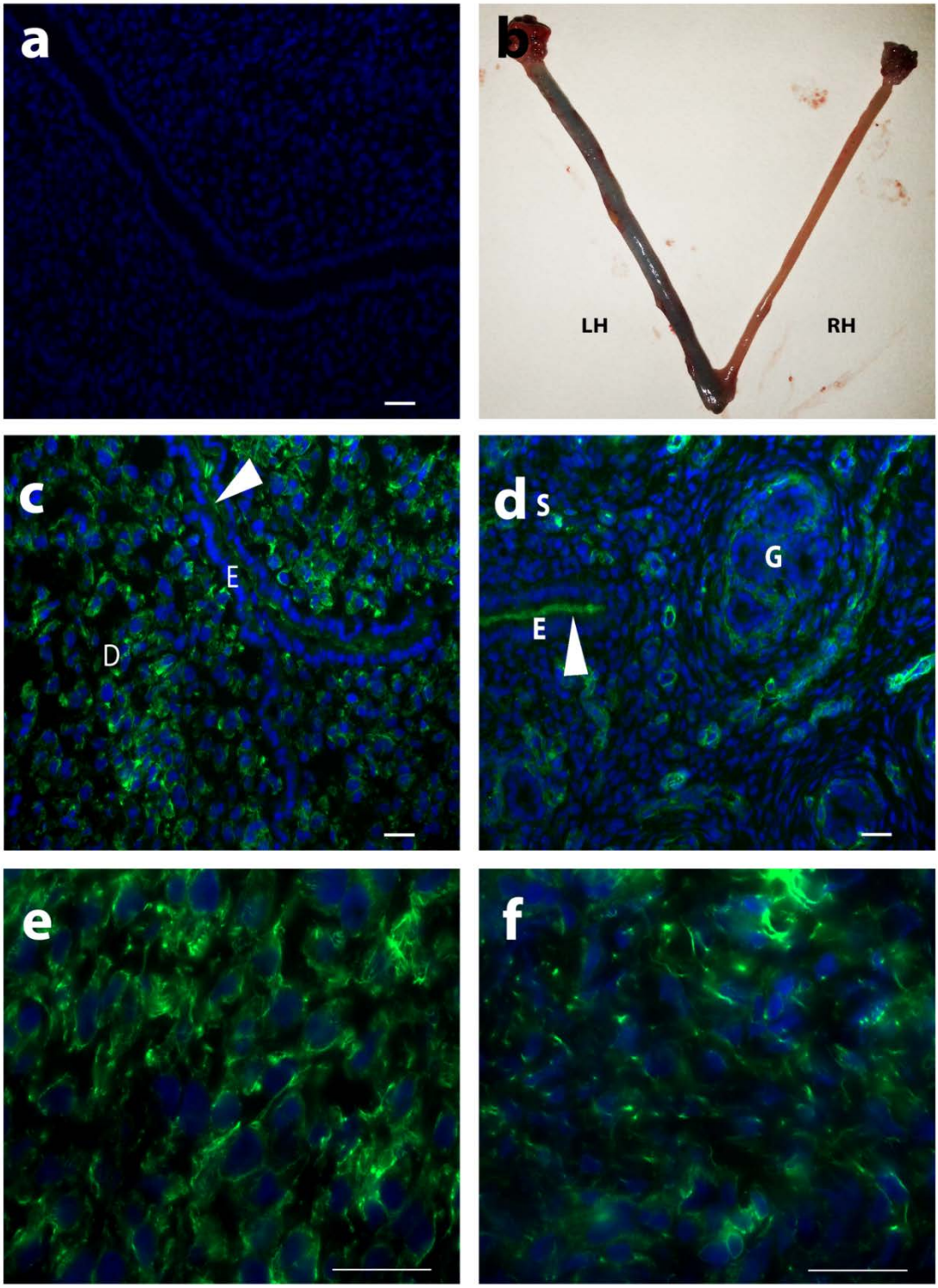
Figure 6.3 FLNA localisation in artificially induced decidualised uteri.

Representative random mouse IgG control immunofluorescence micrograph (a). A photograph of day 6 pseudo pregnant uterine horns, where oil was administered intraluminally to the left horn only displays a strong pontamine sky blue (PSB) reaction staining, indicative of successful inducement of decidualisation (n=5) (b). Immunofluorescence of endometrium stained with monoclonal FLNA antibody (green) and counterstained with dapi (blue). FLNA is localised to the apical plasma membrane in the left horn that decidualisation is induced (c, e) on day 6 of pseudo pregnancy and also in the control day 6 pseudo pregnant horn (d) (arrow head). FLNA is localised to the primary decidualisation zone of the endometrium in the left horn of the uterus that decidualisation was induced with oil. (c, e). FLNA was evenly distributed throughout the endometrium in the control day 6 pseudo pregnant right horn (d, f).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6.5 DISCUSSION

Remodelling of the actin cytoskeleton of UECs and ESCs during the transition between a non-receptive to receptive state in early pregnancy is mediated by actin binding proteins (Luxford and Murphy 1992b; Terry et al. 1996). This study investigated FLNA, an actin binding protein that crosslinks F-actin into an orthogonal meshwork (Davies et al. 1978; Feng and Walsh 2004). At the time of fertilisation FLNA displayed a diffuse localisation in UECs, and was concentrated to the underlying two to three layers of stromal fibroblasts. FLNA redistributed apically in UECs at the time of implantation and also under the influence of progesterone and was localised to the primary decidualisation zone of the endometrium.

Actin filaments at the apical portion of UECs have been shown in transmission electron microscopy studies to be aligned at the time of fertilisation, and are then replaced by a diffuse network of actin filaments at the time of implantation (Luxford and Murphy 1989; Luxford and Murphy 1992b; Luxford and Murphy 1993), with these changes found to be hormonally regulated (Luxford and Murphy 1992a). The apical localisation of FLNA seen in this study at the time of implantation and in response to progesterone may be involved in crosslinking F-actin filaments found apically, and may also form part of the actin meshwork seen in the broad surface protrusions (Adams et al. 2002; Murphy 2000) known as pinopods at this time.

Membrane association of FLNA is attained by its association with transmembrane proteins such as β integrins (Loo et al. 1998; Muriel et al. 2011; van der Flier and Sonnenberg 2001) where FLNA serves to link these proteins to the actin cytoskeleton (Stossel et al. 2001). FLNA contributes to the maintenance of plasma membrane stability in M2 melanoma cells, which, when deficient in FLNA show defects in membrane stability and also migration, and upon reintroduction of FLNA both these characteristics are restored (Cunningham et al. 1992; Planaguma et al. 2012). UECs at the time of implantation are under mechanical stress (Bazer et al. 2009), where a number of junctions and integral membrane proteins such as integrins are lost or are redistributed (Kaneko et al. 2011b; Kaneko et al. 2008; Nicholson et al. 2010). In addition, the apical and basal plasma membranes differ

in their rigidity at the time of implantation (Esue et al. 2009); so FLNA may contribute to the maintenance of plasma membrane stability at this time by facilitating the association with integral membrane bound proteins localised apically with the actin cytoskeleton such as integrin β3 (Kaneko et al. 2011b). Previous work has shown apical localisation of integrin β3 at the time of implantation and also the importance of integrin β3 in initial embryo attachment (Kaneko et al. 2011b; Kaneko et al. 2012), thus apical localisation of FLNA at the time of implantation may provide a scaffolding for integrin β3 where it links integrin β3 to the actin cytoskeleton.

Despite the clear change in localisation of FLNA in UECs during early pregnancy Western blotting analysis did not demonstrate a significant difference in protein abundance of FLNA between the time of fertilisation and implantation. FLNA at the time of fertilisation may be present in the cytoplasm where it is not concentrated enough to be detected by the immunofluorescence protocol. FLNA is a known substrate of calpain 2, and evidence of both the full length FLNA (280 kDa) and also the calpain cleaved fragment of FLNA (240 kDa) (Davies et al. 1978; Gorlin et al. 1990) at both the time of fertilisation and implantation suggest a role for calpain 2 in cleaving not only focal adhesion proteins (Beckerle et al. 1987; Franco et al. 2004) but also FLNA which is an actin binding protein. Calpain activity has been previously reported in UECs during early pregnancy to be increased at the time of implantation (Kaneko et al. 2013). It is interesting that the actin cytoskeleton undergoes a dramatic remodelling in a short time frame and the protein abundance of an actin binding protein such as FLNA is also unchanged. It may be that actin binding proteins such as FLNA are present as a pool in the cytoplasm to elicit their function quickly without the need for new protein synthesis (Thomas et al. 1981).

FLNA was observed to be localised to the primary decidualisation zone at the time of implantation. This localisation was found to be dependent upon the decidualisation reaction. The ESCs also undergo dramatic remodelling during the transition from a fibroblast phenotype to a decidual cell. These cells are essential for the continued growth and development of the embryo (Abrahamsohn and Zorn 1993) and not only function in the development and growth of the embryo but also

restrict excessive invasion of the embryo through the uterine wall (Carson et al. 1992). The histological changes that these cells undergo include dilation of the endoplasmic reticulum, extensive areas of cell-cell contact with the formation of junctional complexes and also the accumulation of glycogen, lipids, and an increase actin filaments arranged in a filamentous meshwork (Kearns and Lala 1983). The primary decidualisation zone is comprised of large decidual cells (LDC), the ESC precursors of which were originally located immediately underlying the basal lamina of the uterine epithelium (Mikhailov 2003; Parr et al. 1986). The localisation of FLNA observed in the 2-3 layers of ESCs immediately underlying the luminal epithelium that will later form the LDCs of the primary decidual zone suggests that FLNA is associated with the remodelling of the actin cytoskeleton of these cells during their transition from a fibroblast to a decidual cell. Other actin binding proteins such as α-actinin, CLP36, and moesin also shift in their distribution in response to decidualisation (Miehe et al. ; Shaw et al. 1998; Venuto et al. 2008), these proteins together with the localisation and expression of FLNA in these ESCs may function together. FLNA and α-actinin cross link actin filaments which provides a stiff actin network (Esue et al. 2009), this may play a role in regulating embryo invasion.

In summary this work has shown redistribution of the actin crosslinking protein FLNA apically in UECs and in the primary decidualisation zone at the time of implantation. We suggest that the presence of FLNA contributes to the molecular and morphological changes that both UECS and ESCs undergo in order for them to be receptive to implantation by contributing to the remodelling of the actin cytoskeleton and hence the maintenance of plasma membrane stability of UECs and ESCs during early pregnancy.

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CHAPTER 7: AFADIN AN ACTIN FILAMENT BINDING PROTEIN IS LOCALISED AT APICAL CELL-CELL JUNCTIONS IN UTERINE EPITHELIAL CELLS DURING EARLY PREGNANCY

7.1 ABSTRACT

Luminal uterine epithelial cells undergo many changes to become receptive to blastocyst implantation. These changes include the loss of microvilli, terminal web and deepening of the tight junction and loss of basal focal adhesions and morphological adherens junction (*zonula adherens*). Despite these changes UECs maintain a polarised epithelial phenotype. Afadin is an actin binding protein that associates with the nectin-afadin-ponsin protein complex that is found at cell-cell adherens junctions. Afadin has been reported to associate with both the adherens junction and also the tight junction in other cells. This study investigated the morphological changes of the adherens junction and the localisation and expression of afadin during early pregnancy in the rat to explore and its role in the maintenance of epithelial polarity.

Afadin was found to localise to apical junctions between UECs at both the time of fertilisation and implantation, and co-localise with ZO-1 at both these times. Afadin protein abundance is increased at the time of implantation in isolated uterine epithelial cells. The co-localisation of afadin with ZO-1 and protein expression of afadin at the time of implantation suggests that it is associated not only with the adherens junction at the time of fertilisation but also the tight junction at both the time of fertilisation and implantation.

7.2 INTRODUCTION

Polarised epithelial cells are characterised by the organisation of the plasma membrane lipids and proteins into distinct domains (Rodriguez-Boulan and Nelson 1989). These domains, both structural and functional consist of an apical plasma membrane that faces the lumen, a lateral plasma membrane where junctional complexes are found, and the basal plasma membrane. All these domains undergo many changes during the transition from a non-receptive uterine epithelial cell to one that is receptive to blastocyst implantation, which are known collectively as the plasma membrane transformation. These changes include deepening of tight junctions and loss of the adherens junction, and desmosomes at the time of implantation (Murphy and Shaw 1994). Despite the loss of these junctional structures the uterine epithelium still retains its polarised epithelial phenotype.

The adherens junction (*zonula adherens*) is a lateral junction that links the actin cytoskeleton of adjacent UECs and functions to maintain cell to cell contact. This junction provides adhesion between cells to resist mechanical stresses (Hartsock and Nelson 2008). The adherens junction is comprised of two adhesion complexes which are the E Cadherin-Catenin complex and the Nectin-Afadin-Ponsin (NAP) complex (Asakura et al. 1999). At cell-cell adherens junctions cadherins interact with each other at the extracellular surface in a Ca^{2+} dependent manner and also with the actin cytoskeleton at the cytoplasmic interface through the F-actin binding peripheral membrane proteins α-actinin, α/β-catenin, and vinculin (Asakura et al. 1999; Li et al. 2002).

The tight junction (*zonula occludens*) of polarised epithelial cells is responsible for the formation of an apical seal between adjacent epithelial cells (Fanning et al. 1999). Tight junctions control the diffusion of proteins and molecules between apical and basolateral cell membranes as well as regulate ion, water and molecular transport through the paracellular pathway between the cells (Tsukita et al. 2009). Claudin proteins, occludin and tricellulin are key proteins of the tight junctional complex. ZO-1 is a peripheral membrane protein that interacts with the tight junctional complex, but is also capable of binding to proteins associated with the adherens junction (Tsukita et al. 2009; Furuse et al. 1994). Previous studies of

ZO-1 during early pregnancy have demonstrated junctional localisation of ZO-1 in UECs throughout early pregnancy in the rat (Orchard and Murphy 2002).

Afadin is an F-actin binding protein that consists of two splice variants I-afadin and s-afadin (Mandai et al. 1997). I-Afadin is ubiquitously expressed, unlike s-afadin which is abundantly expressed in neural tissue (Ikeda et al. 1999). Afadin is restricted to the adherens junction in luminal epithelial cells of the small intestine. As junctional proteins such as ZO-1 have been localised at cell junctions of UECs during early pregnancy despite the loss of a morphological adherens junction at the time of implantation this study investigated the morphology of the adherens junction during early pregnancy and investigated the localisation and expression of afadin in the rat uterus during early pregnancy to explore its potential role in the maintenance of a polarised epithelium.

7.3 MATERIALS AND METHODS

7.3.1 Animals

Female virgin Wistar rats (10–12 weeks of age) were housed in plastic cages at 21°C with a 12-h light–dark cycle and were fed and watered *ad libitum*. Vaginal smearing was performed to determine stage of the oestrous cycle, and those rats showing a vaginal smear characteristic of pro-oestrus were caged overnight with a male of proven fertility. The presence of sperm in the vaginal smear taken the following morning indicated mating and was designated day 1 of pregnancy. Uterine horns from days 1 and 6 were collected to correspond to the time of fertilisation and implantaton. Five rats were collected for each day of pregnancy. All experimental procedures were approved by The University of Sydney ethics committee.

7.3.2 Immunofluorescence microscopy

Uterine tissue from normal pregnant and ovariectomised rats treated with ovarian hormones, were embedded in O.C.T compound (Tissue-Tek; Sakura Firetek, Torrance, CA, USA), snap frozen in supercooled isopentane (BDH Laboratory supplies, Poole Dorset, England, UK) and stored under liquid nitrogen until use. Sections, 7 μm, were cut using a Leica LM 3050 cryostat (Leica, Heerbrugg, Switzerland). Tissue sections were air dried on gelatine-chrome alum-coated glass slides at room temperature and fixed with 4% PFA for 10 minutes at room temperature, washed in PBS 3 x 5 min, and subsequently blocked in 1% bovine serum albumin (BSA; Sigma) in PBS for 30 min. All primary and secondary antibodies were diluted with this blocking solution. Sections were incubated with primary antibodies of mouse monoclonal anti afadin (1 μg/mL, Abcam, Cambridge, England: ab 90809) and rabbit polyclonal anti ZO-1 (0.5 μL/mL, Life Technologies, Carlsbad, California, USA; 402200) overnight at 4 °C. Sections were washed in PBS 3 × 5 min, and incubated with secondary antibody fluorescein isothiocyanate (FITC)-conjugated AffiniPure goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or anti-rabbit IgG $F(ab')_2$ fragment-cy3 secondary antibody (0.5 mg/ml; Sigma, Sydney, NSW, Australia) for 1 h in the dark followed by 3 × 5 min PBS wash. Sections were then mounted with vectashield containing DAPI (Vector, Burlingame, CA, USA), cover

slipped and examined with a Zeiss Deconvolution microscope (Carl Zeiss Inc., Jena, Germany). Immunofluorescence micrographs were taken using a Zeiss AxioCamHR digital monochrome CCD camera (Carl Zeiss Inc., Jena, Germany) and Zeiss Zen 2011 image-acquisition software. Non-immune controls were carried out with experimental runs where sections were incubated with mouse and rabbit IgG purified immunoglobulin (0.8 μg/mL; Sigma) in place of the primary antibody.

7.3.3 Transmission Electron Microscopy

Uteri were excised and cut into 5mm pieces then immediately immersed in freshly prepared Karnovsky's fixative solution (pH 7.4) comprised of 2.5% glutaraldehyde (ProSciTech, Queensland, Australia), 2% paraformaldehyde (ProSciTech, Queensland, Australia) in 0.1M Sorenson's phosphate buffer (PB, pH 7.4) for 45 mins at room temperature. Tissue was then cut into 0.5-1mm slices on dental wax under a droplet of fixative using a double-edged razor blade, then returned to fresh fixative for a further 45 mins. The tissue was washed in PB then postfixed for 1hr in a solution of 1% osmium tetroxide (OsO_4) and 0.8% potassium ferricyanide in PB, to enhance the contrast of the plasma membrane (Karnovsky 1971). This solution was rinsed off with PB, before a 2% OsO_4 solution (in PB) was added for 10 min to remove any unreacted potassium ferricyanide, which can cause infiltration problems (Hoshino et al. 1976). The tissue was then washed extensively with MilliQ water before being dehydrated in a graded series of alcohols and infiltrated with Spur's resin (SPI supplies, Leicestershire, England, UK). Tissue slices were embedded in fresh Spur's resin in BEEM® capsules (ProSciTech, Australia) and polymerised at 60°C for 24hrs. Two randomly-selected blocks per animal were cut using a Leica Ultracut T ultra-microtome (Leica, Heerbrugg, Switzerland) and 60-70 nm sections were mounted onto 400-mesh copper grids. Sections were post-stained with a saturated solution of uranyl acetate in 50% ethanol for 45mins, followed by Reynold's lead citrate for 10mins. These sections were viewed with a Jeol 1011 transmission electron microscope (Jeol Ltd., Tokyo, Japan) at 80kV and imaged with a Gatan SC200 Orius CCD Camera (Gatan Inc., California, USA).

7.3.4 Isolation of uterine luminal epithelial cells

Uterine luminal epithelial cells were isolated from each animal as previously described (Kaneko et al. 2008). The uterine horn was opened longitudinally and surface luminal epithelial cells were scraped off using sterile surgical blades (Livingstone International, Rosebery, NSW, Australia) and immediately placed into lysis buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.1% SDS, 0.5% Deoxycholic acid, 1% Igepal and protease inhibitor cocktail; Sigma Mammalian Cell lysis kit, Sigma). The cell lysate was passed through a 23 gauge needle using a 1 ml syringe (Livingstone, International, Rosebery, NSW, Australia) and briefly centrifuged at 8,000g at 4 °C. The supernatant was collected and frozen immediately in liquid nitrogen and stored at −80°C until use.

7.3.5 Western blotting analysis

Protein concentrations were determined using the BCA protein assay (Micro BCA™ Protein assay kit; Quantum Scientific, Murarrie, QLD, Australia) and POLAR Star Galaxy microplate reader (BMG LabTech, Durham, NC, USA) according to the manufacturer's instructions. Protein samples (20 µg) and sample buffer (8% glycerol, 50 mM Tris–HCl, pH 6.8, 1.6% SDS, 0.024% bromophenol blue, 4% β-2-mercaptoethanol) were boiled at 95°C for 5 minutes prior to loading onto a 10% SDS-polyacrylamide gel. Gels were subjected to electrophoresis at 200 V for 40 minutes and proteins transferred to a polyvinylidene dilfluoride (PVDF) membrane (Immunobilon™ transfer membrane; Millipore, Bedford, MA, USA) at 100 V for 1 hour. Membranes were blocked by incubating in a solution of 5% skim milk powder in TBS-t (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature with constant agitation, followed by primary antibody incubation with mouse monoclonal anti afadin (0.005µg/mL, Abcam, Cambridge, England: ab 90809) overnight at 4°C. Membranes were washed 3 × 10 min in TBST and subsequently incubated for 2 h with sheep anti mouse (0.4 ug/mL, Amersham, GE healthcare, Buckinghamshire, UK) at room temperature with constant agitation. All antibodies were diluted with 1% skim milk powder in TBST. The proteins were detected by enhanced chemiluminescence (ECL Plus Western Blotting Detection System; Amersham, GE Healthcare, NSW, Australia) and unsaturated images were captured using the Alpha Innotech Digital Imaging System (Alpha Innotech, San

Leandro, CA, USA). Membranes were subsequently rinsed in TBST and antibodies removed by heating at 60°C for 45 min in stripping buffer (62.5 mM Tris–HCl, pH 6.7, 2% SDS and 100 mM β-2-mercaptoethanol) and reprobed with mouse monoclonal anti-β-actin antibody (0.4 μg/mL; Sigma, NSW, Australia) to ensure equal loading.

7.3.6 Statistical analysis

The intensity of the bands detected from western blotting analysis was quantified using the two-dimensional spot density from the Alpha Innotech Digital System (Alpha Innotech). The integrated density value (IDV) was calculated using the AlphaEaseFC software. Statistical analysis was performed by student's t-test for early pregnancy a $p < 0.05$ was considered to be significant.

7.4 RESULTS

Morphological adherens junction is absent at the time of implantation

Transmission electron microscopy of luminal uterine epithelial cells on day 1 of pregnancy shows the presence of morphological tight junction, adherens junction and desmosomes at the apical portion of the lateral plasma membrane of UECs **(figure 7.1a).** By day 6 of pregnancy a morphological adherens junction was absent at the apical portion of the lateral plasma membrane **(figure 7.1b).**

Afadin is localised to sites of cell to cell contact and co-localises with ZO-1 during early pregnancy.

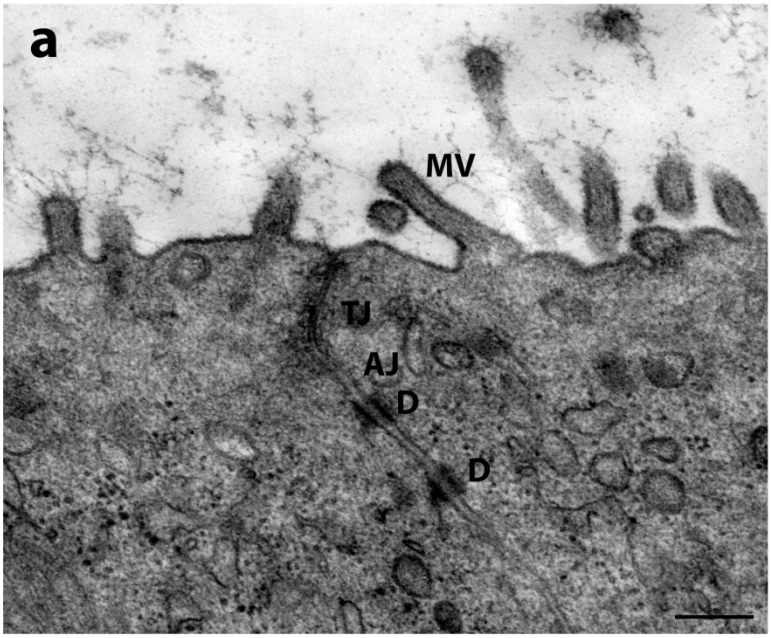
On day 1 **(figure 7.2a-c)** and day 6 **(figure 7.2d-f)** of pregnancy afadin (red) is localised apically in UECs at sites of cell-to-cell contact (arrow head). Afadin co-localises (yellow) with ZO-1 (green) a tight junctional marker on both days of pregnancy investigated.

Afadin protein abundance is significantly increased at the time of implantation

Afadin was observed at 205 kDa as a doublet which is the reported molecular weight for the I-afadin isoform and also a degradation product at 65 kDa (Tanaka-Okamoto et al. 2011) on both days of pregnancy **(figure 7.3A).** Densitometric analysis of I-afadin isoform (205 kDa) protein abundance showed a significant increase on day 6 of pregnancy **(figure 7.3B).**

Figure 7.1 Transmission electron micrographs of uterine epithelial cells

Transmission electron micrographs of the apical portion of the lateral plasma membrane of luminal UECs on day 1 (a) and day 6 (b) of pregnancy. Morphological adherens junction is observed on day 1 of pregnancy and absent on day 6. MV, microvilli; TJ, tight junction (*zonula occludens*); AJ, adherens junction (*zonula adherens*); D, desmosome; MV, microvilli. Scale bar = 200 nm


a
MV
TJ
AJ
D
D

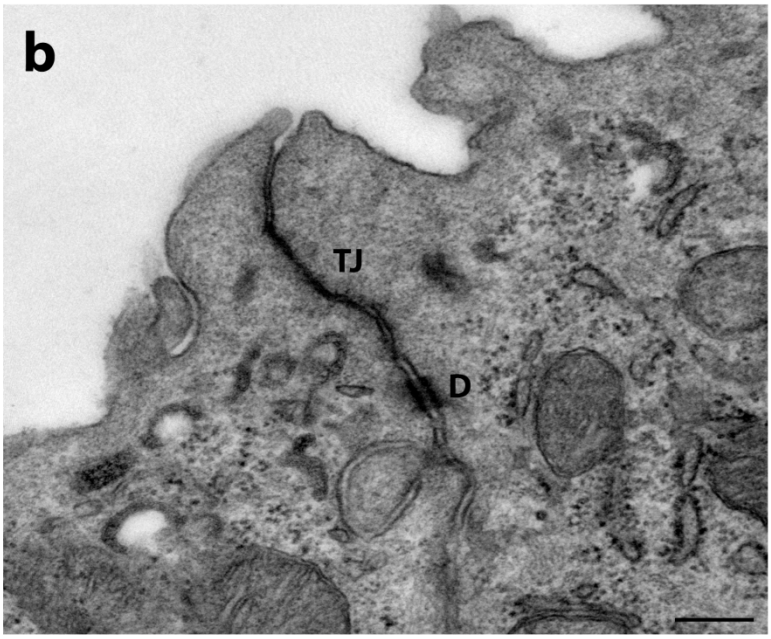

b
TJ
D

Figure 7. 2 Co-localisation of afadin and ZO-1 in UEC during early pregnancy

Immunofluorescence micrographs of the endometrium stained with monoclonal afadin antibody (green) , polyclonal ZO-1 antibody (red) and counterstained with dapi (blue). On day 1 (a-c) and day 6 (d-f) of pregnancy both afadin and ZO-1 are co-localised to the apical lateral junctions of luminal UECs. E, luminal epithelial cells; L, lumen; S, stroma. Scale bar = 30 μm.

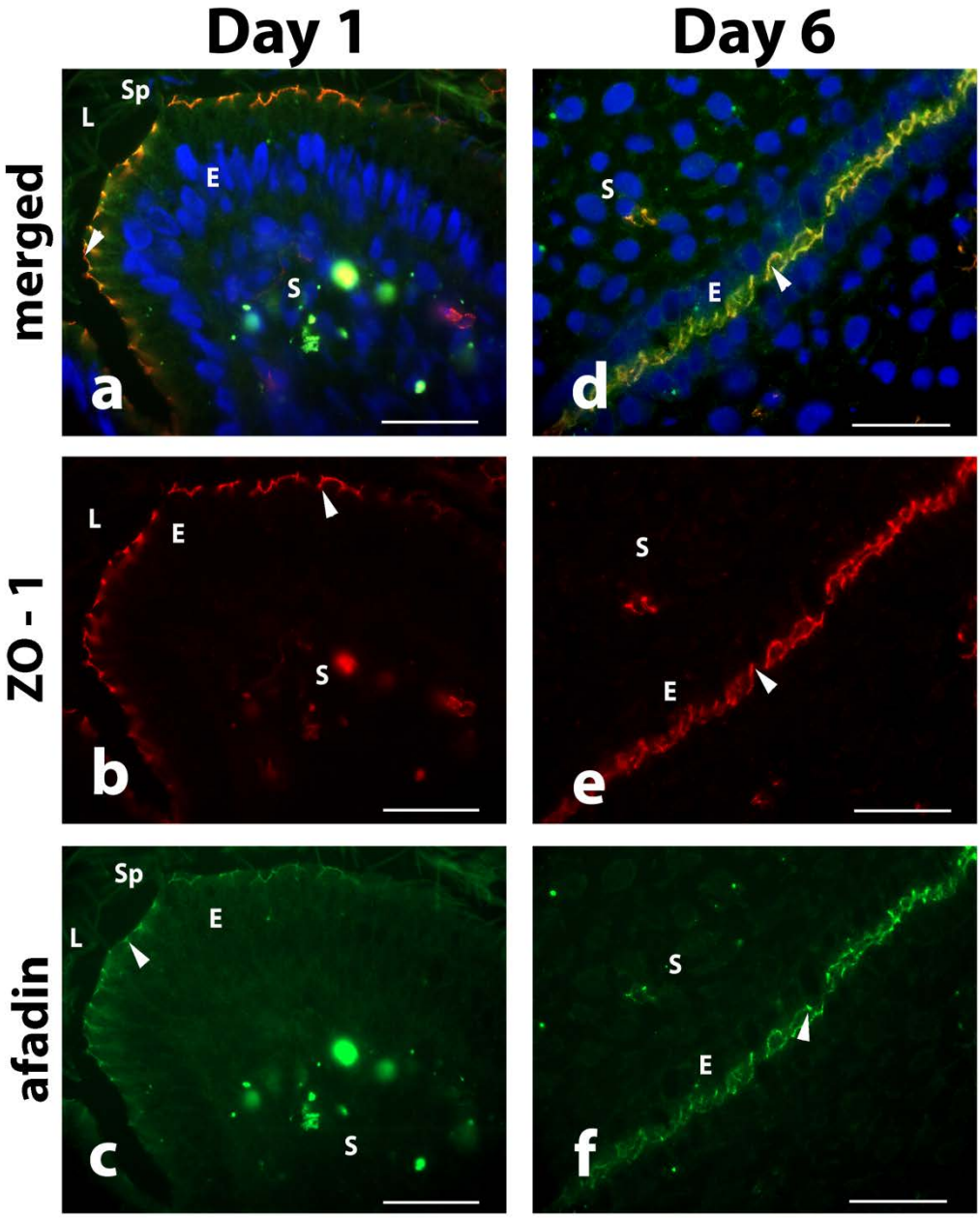
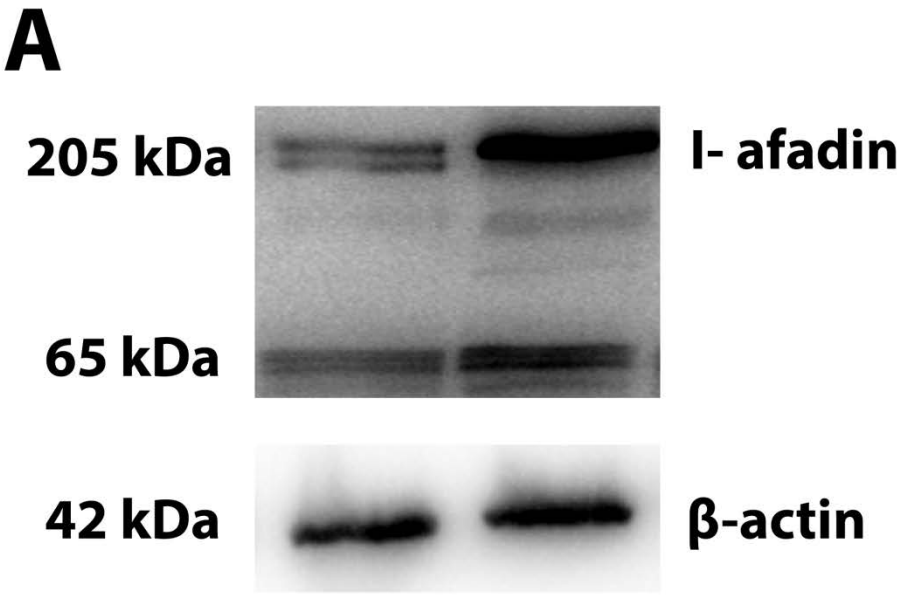
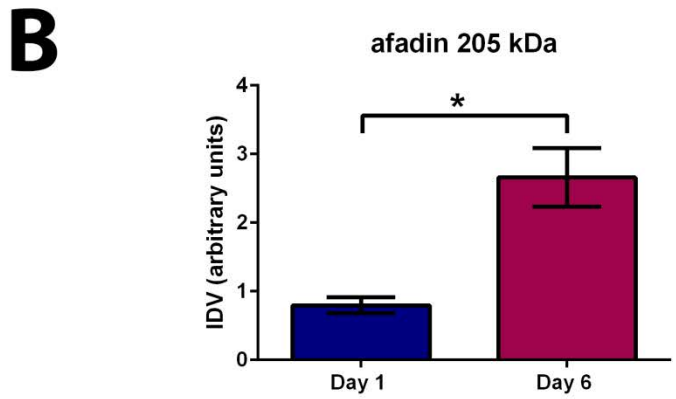

Day 1
Day 6
merged
ZO - 1
afadin
Sp
L
E
S
a
b
c
d
e
f

Figure 7.3 Western blotting of afadin in isolated UECs

Western blotting analysis of afadin (205 kDa) (A) from isolated UECs on day 1 and day 6 of pregnancy. Densitometric analysis of western blotting (B) and statistical analysis by Student's t-test showed a significant increase in afadin protein abundance between day 1 and day6 of pregnancy. Integral density values (IDV) were calculated and are shown as ± SEM.


A
205 kDa
l- afadin
65 kDa
42 kDa
β-actin


B
afadin 205 kDa
IDV (arbitrary units)
0
1
2
3
4
*
Day 1
Day 6

7.5 DISCUSSION

Uterine epithelial cells maintain a polarised state despite losing a number of membrane junctional structures and associated cytoskeletal components of these junctions (Luxford and Murphy 1992, 1993; Murphy and Shaw 1994; Murphy 2001) during the transition to an epithelium that is receptive to blastocyst implantation. Intercellular adhesion in polarised cells is mediated by the tight junctional (*zonula occludens*), adherens junctional (*zonula adherens*), and desmosome (*macula adherens*) complexes (Farquhar and Palade 1963). These complexes are important in the separation of cellular domains of a polarised epithelium. Afadin is a protein that has been found to be concentrated at cadherin based cell to cell adherens junctions (Mandai et al. 1997; Ikeda et al. 1999). The present study investigated the localisation and expression of afadin and also the morphological changes of the adherens junction at the time of fertilisation and implantation. The morphological adherens junction was observed to be absent at the time of implantation while afadin was observed to localise to apical cell-cell junctions, and co-localise with ZO-1 a tight junctional marker at both the time of fertilisation and implantation. The protein abundance of afadin was observed to increase at the time of implantation.

The localisation of afadin at the time of fertilisation is consistent with the observation of an intact adherens junction at this time. Interestingly, afadin co-localised with ZO-1 at both the time of fertilisation and implantation suggesting afadin is not only localised to the adherens junction but to the tight junction as well in rat uterine luminal epithelial cells. While afadin is a component of the NAP complex which is found at sites of cell-cell adherens junctions, and has been found to be restricted to the adherens junction in luminal epithelial cells of the small intestine (Mandai et al. 1997; Ikeda et al. 1999; Tanaka-Okamoto et al. 2011), afadin has also been observed to localise at both the tight junction and also the adherens junction in the polarised epithelial MDCK cell line (Asakura et al. 1999; Reinsch and Karsenti 1994).

The tight junction of UECs has been investigated extensively in UECs during early pregnancy where it serves to isolate the cytoplasmic and luminal environments. Transmission electron microscopy (TEM) studies of this junction have demonstrated an increase in the depth of the tight junction at the time of

implantation (Murphy et al. 1981, 1982; Murphy 2000), and loss of a morphological adherens junction at this time. These observations taken together with the observed co-localisation of afadin and ZO-1 in UECs and the increase in afadin protein at the time of implantation protein suggest that afadin is localised to the tight junction at this time and may contribute to the maintenance of both epithelial polarity and integrity.

In summary this study has demonstrated the loss of the morphological adherens junction. We have also shown the junctional localisation of afadin to coincide with the localisation of ZO-1 as well as the abundance of afadin protein to be increased at the time of implantation which correlates with the deepening of this junction at this time. We suggest that afadin not only facilitates cell-cell adherens junctions at the time of fertilisation but is also associated with the tight junction at both the time of fertilisation and implantation; and hence contributes to the maintenance of a polarised epithelium.

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CHAPTER 8: GENERAL DISCUSSION

8.1 GENERAL DISCUSSION

This thesis investigated several aspects of cell polarity including membrane lipid and cholesterol, actin cytoskeleton, and junctional complexes with the aim to understand the mechanisms in which uterine epithelial cells employ to maintain epithelial polarity at the time of implantation. The studies conducted suggest that a polarised lipid distribution in UECs during early pregnancy is a mechanism for UECs to maintain epithelial polarity as the location of, and abundance of, plasma membrane lipid and cholesterol affects the distribution and location of certain transmembrane proteins. The actin cytoskeleton is another cellular structure that is employed to maintain epithelial polarity where bundling of actin filaments by actin binding proteins throughout the cell affects the plasma membrane of the UECs, contributing not only to morphological structures such as the terminal web and associated microvilli, but also plasma membrane rigidity. Junctional structures of UECs also contribute to epithelial polarity as they maintain a partition between apical and basal compartments of UECs during pregnancy.

The plasma membranes of UECs outside the 'window of receptivity' are refractive to blastocyst implantation. At this time UECs have apical microvilli, a glycocalyx and apical junctions (Murphy and Shaw 1994; Murphy 2004). The basal plasma membrane of UECs at this time has many adhesive molecules such as integrins which are associated with focal adhesion molecules talin and paxillin (Kaneko et al. 2008; Kaneko et al. 2011a) as well as the flotillin raft marker flotillin-2 (Lecce et al. 2013).

At the time of implantation UECs are receptive to blastocyst implantation and have lost apical microvilli, and the associated terminal web (Luxford and Murphy 1992). Apical surface protrusions known as pinopods in rats and uterodomes in humans are observed (Adams et al. 2002; Martel et al. 1991; Singh et al. 1996; Murphy 2000). There is also an increase in basolateral membrane tortuosity (Shion and Murphy 1995). These are accompanied by an increase in apical plasma membrane cholesterol (Murphy and Martin 1985) as well as up-regulation and redistribution of a number of adhesion molecules such as integrins, and selectins to the apical plasma membrane (Aplin 1997; Singh and Aplin 2009; Kaneko et al. 2011b; Kaneko et al. 2011a) which serve to mediate adhesion of the blastocyst to UECs.

From the current studies, caveolin proteins, markers of another specialised lipid raft known as caveolae was observed to be cytoplasmically distributed in UECs at the time of fertilisation and redistributed to the basal plasma membrane at the time of implantation. At this time, morphological caveolae were also identified and observed to increase. Plasma membrane cholesterol has only been investigated in the apical plasma membrane of UECs during early pregnancy, where it has been reported to increase at the time of implantation. Caveolae are specialised lipid raft domains that are rich in cholesterol and glycosphingolipids which increase in number at the basal plasma membrane and are associated with the localisation and increase in caveolin-1 protein at the time of implantation. Caveolae and flotillin lipid rafts are two separate lipid raft domains (Lang et al. 1998; Volonté et al. 1999; Bauer and Pelkmans 2006); as such, flotillin localises apically in UECs (Lecce et al. 2013) whereas caveolae and associated caveolin proteins are localised basally at the time of implantation. Thus, the observations in this thesis provide evidence for a polarised distribution of lipid rafts between apical and basal surfaces to be a mechanism for UECs to maintain epithelial polarity. Furthermore, different proteins are dependent on the location and abundance of plasmalemmal lipid and cholesterol and hence the specific location of different lipid raft domains will concentrate particular proteins to particular membrane domains, thus influencing proteins and cellular functions.

The generation of membrane curvature at the basolateral membrane is another aspect of cell polarity that is altered in UECs at the time of implantation. Previous studies found an increase in the tortuosity of the basal plasma membrane at the time of implantation (Shion and Murphy 1995). Caveolin-1 was found to associate with PTRF at the time of implantation (Chapter 4). PTRF is a protein that associates with caveolar membranes and is known along with SDPR to generate membrane curvature. SDPR however was absent from UECs at the time of implantation (Chapter 4). The elevated levels of lipid and cholesterol in the apical plasma membrane (Murphy and Martin 1985) leads to a rigid plasma membrane, coupled with the absence of caveolae and caveolin protein at the apical membrane at the time of implantation (Chapter 3) supports this hypothesis. Conversely, the increase in the flexibility of the basal plasma membrane as seen by the increase in membrane tortuosity and in the number of morphological caveolae as well as the

association between caveolin-1 and PTRF at this time (Chapter 3 and 4) suggests that these proteins together contribute to the differences in apical and basal plasma membranes. Thus, basal lipid abundance may be mechanism for the maintenance of epithelial polarity at the time of implantation.

The actin cytoskeleton also plays a role in the maintenance of epithelial polarity. As has been mentioned earlier, both apical and basal plasma membranes differ in their membrane rigidity, with lipid and cholesterol abundance not bring the sole contributors to this (Esue et al. 2009) (Figure 8.1). Bundling of F-actin fibres at membrane surfaces by actin binding proteins such as filamin A (FLNA) may provide the structural integrity within the cell as well as at the plasma membrane. FLNA was observed to localise apically in UECs at the time of implantation. Also at the time of implantation pinopods (apical morphological protrusions) have been observed along with filamentous actin beneath the apical plasma membrane surface. The apical localisation of FLNA in UECs may not only facilitate the organisation of F-actin, but also apical membrane rigidity and stability by associating integral membrane-bound proteins localised apically with the actin cytoskeleton such as integrin β3 (Kaneko et al. 2011b) where it may indirectly serve to facilitate blastocyst adhesion.

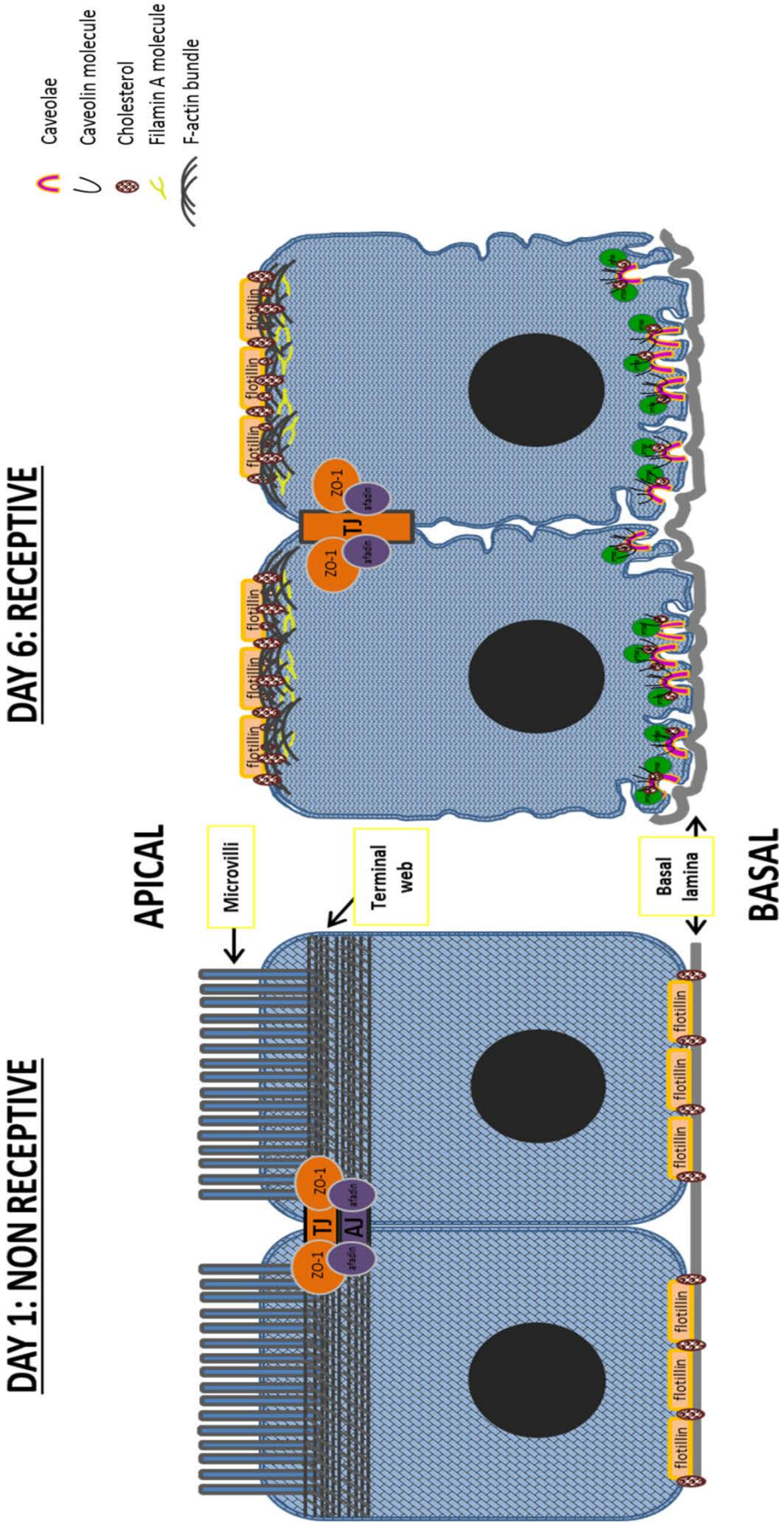
The collective alterations in the composition of apical and basal plasma membranes of UECs especially at the time of implantation suggest a rigid apical plasma membrane is due to elevated membrane cholesterol, and also FLNA associated actin networks; whereas the basal plasma membrane has a different lipid composition which provides a less rigid membrane, thereby facilitating the formation of caveolae and general membrane curvature. As such caveolin, cavin proteins PTRF and SDPR and also FLNA contribute to the polarised phenotype of UECs observed during early pregnancy (Figure 8.1).

The junctional structures of UECs also undergo many alterations during early pregnancy. The lateral plasma membrane junctions were also investigated with particular focus on the adherens junction which has been shown to be absent morphologically (Chapter 7) using afadin as an adherens junction marker. The tight junction of UECS has been investigated extensively in UECs during early pregnancy where transmission electron microscopy (TEM) studies of this junction have demonstrated an increase in its depth at the time of implantation (Murphy et

al. 1981, 1982), ZO-1 is not purely restricted to the tight junction (Tsukita et al. 2009) and co-localised with afadin at both the time of fertilisation and implantation (Chapter 7). These observations taken together with the increase in afadin protein at the time of implantation protein suggest that afadin is localised to the tight junction at this time and contributes to maintaining the separation between apical and basolateral plasma membrane domains hence, epithelial polarity (Figure 8.1).

The focal adhesion complex is a junction that undergoes disassembly at the time of implantation (Kaneko et al. 2008) and the association of caveolin 1 with integrin β1 in focal adhesion turnover (Chapter 3) is a mechanism that aids in blastocyst implantation. Membrane integrity is maintained at the time of implantation by the association of afadin at the tight junction. Together these mechanisms allow for the maintenance of membrane integrity and also epithelial polarity during early pregnancy

Figure 8.1 Schematic diagram of molecules involved in epithelial polarity and plasma membrane rigidity


Caveolae
Caveolin molecule
Cholesterol
Filamin A molecule
F-actin bundle
DAY 6: RECEPTIVE
DAY 1: NON RECEPTIVE
APICAL
BASAL
Microvilli
Terminal web
Basal lamina
flotillin
ZO-1
TJ
AJ
afadin

At the basal plasma membrane focal adhesions and their associated proteins are down regulated facilitating focal adhesion disassembly (Kaneko et al. 2008; Kaneko et al. 2011b). In the studies reported in this thesis, caveolin-1 and integrin β1 were shown to associate at the time of fertilisation (Chapter 3). This association implicates caveolin-1 in integrin turnover and subsequent focal adhesion disassembly during early pregnancy. The use of cell culture models of a receptive endometrium was investigated with regards to the influence of the ECM on epithelial polarity. Focal adhesion proteins talin and paxillin, as well as caveolin-1 were investigated in Ishikawa cells (an established receptive endometrial epithelial cell line) as both focal adhesion proteins and also caveolin-1 were observed to be localised basally in vivo. Talin and paxillin as well as caveolin-1 were observed to be localised apically in these cells despite the presence of ECM at the basal surface (Chapter 5). The observations on the localisation of focal adhesion molecules talin and paxillin (Chapter 5) were particularly interesting, as they did not mirror what is observed in receptive UECs in vivo. This may be due to adhesive qualities of the apical surface of Ishikawa cells where these cells have been reported to express adhesive molecules such integrin β3 (Kaneko et al. 2011a) and basigin (Iacono et al. 2007) apically. The apical integrin localisation in Ishikawa cells do however contribute to the observations of talin and paxillin localisation as these proteins cluster together to form the focal adhesion complex as has been observed in vivo (Burridge et al. 1988; Katoh et al. 1995; Burridge and Chrzanowska-Wodnicka 1996; Critchley et al. 1999).

8.2 Conclusion and Future Directions

In conclusion, these studies demonstrate each of the molecules investigated contribute to the maintenance of a polarised epithelial phenotype. This is the first study to identify and quantify an increase in morphological caveolae at the basal plasma membrane in UECs at the time of implantation. In the uterus, lipid rafts and their associated markers are responsive to ovarian hormonal changes and demonstrate a polarised distribution of lipid rafts and their associated markers. This is one of the few studies that have looked at these molecules associated with lipid rafts and actin remodelling as potential mediators of epithelial polarity.

It is clear that organisation of both molecules found in the plasma membrane and those associated with the actin cytoskeleton together serve to regulate not only epithelial polarity but also mediate remodelling of the actin cytoskeleton as observed in stromal to decidual cell transformation with FLNA. The alterations in the composition of apical and basal plasma membranes of UECs especially at the time of implantation suggest a rigid apical plasma membrane due to elevated membrane cholesterol, and also FLNA associated actin networks; whereas the basal plasma membrane has a different lipid composition which provides a less rigid membrane, thereby facilitating the formation of caveolae and general membrane curvature. Measurement of this rigidity would be beneficial to elucidate the components of the plasma membrane that give rise to such a polarised plasma membrane. Manipulation of the lipid and cholesterol content of the plasma membrane of UECs by chemical agents such as methyl β cyclodextrin D and also actin cytoskeleton disruption by disruptive agents such as cytochalasin D and latrunculin and measuring membrane rigidity with atomic force microscopy will provide further information on the components of the cell that contributes to the polarity of UECs during early pregnancy.

In the uterus it is well known that a significant amount of cross talk between blastocysts and uterine tissue is achieved through cytokines and growth factor secretions. Up regulation of a number of molecules is stimulated by inflammatory cytokines which include, but are not limited to interleukins, tumour necrosis factors and interferons (Singh and Aplin 2009). Caveolin was investigated in this thesis as a potential mediator of epithelial polarity and membrane curvature, it has also however been associated with the suppression of cytokine signalling in mammary epithelium (Jasmin et al. 2006; Park et al. 2002). It would be of interest to identify whether expression of caveolin-1 is altered when cytokines are administered locally without the involvement of ovarian hormones to further elucidate a role for caveolin-1 in cytokine signalling during the window of receptivity of UECs.

This thesis also investigated focal adhesion molecules talin and paxillin in Ishikawa cells. These proteins have previously been characterised with regards to their localisation and expression in UECs *in vivo* (Kaneko et al. 2008; Kaneko et al. 2009). From the present study it was found that these proteins were not only

expressed in Ishikawa cells but were unexpectedly localised apically in these cells grown on an extracellular matrix as opposed to being localised basally as has been observed *in* vivo. Epithelial polarity is an important characteristic that is maintained *in vivo* during pregnancy and has implications for the localisation of and expression of key proteins involved in uterine receptivity. From the studies of focal adhesion proteins, talin and paxillin in Ishikawa cells (Chapter 5) it can be said that while cell culture has its benefits, it also has limitations with regards to correlations made between what occurs *in vitro* to what occurs *in-situ*, with epithelial polarity being a potential limiting factor in the use of cell culture models of a receptive endometrium. It would therefore be of interest to identify other cell culture models (such as ECC-1, RL-95 cell lines) which have a polarised distribution that is consistent with what is observed *in vivo*.

* * *

In conclusion, the molecules studied are now known to be involved in the maintenance of uterine polarity and also contribute to uterine receptivity. The results of the work presented here contribute to our understanding of the process of uterine receptivity and increase our knowledge of the events that occur during early pregnancy.

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