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Invited Review for Reproduction, special edition on 'Women Leaders in Reproductive Research.

My WOMBan's Life: Understanding Human Endometrial Function

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Short title: Understanding human endometrial function

Abstract

The focus of my life in science has been to improve reproductive health for women, with an emphasis on the endometrium, the most dynamic tissue in the human body: its remarkable cyclical remodelling is essential for establishment of pregnancy. The most notable events in a woman's endometrial cycle are menstruation and endometrial repair, regeneration of the endometrium during the proliferative phase, attainment of receptivity by the mid-secretory phase of the cycle and the embryo-maternal interactions that initiate peri-implantation events within the microenvironment of the uterine cavity. I have contributed to understanding the molecular and cellular changes underpinning these events, and how disturbance of them leads to menstrual disorders, infertility and endometrial diseases including abnormal uterine bleeding, endometriosis and endometrial cancer. My team have contributed to changes in clinical IVF practice, to a new diagnostic for endometrial receptivity in infertile women, and to enhancing endometrial repair. I have shared my world with many amazing younger scientists: it has indeed been a privileged journey.

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Introduction

My first introduction to the topic of reproductive processes was as a young research assistant working with Professor Henry Burger and the late Dr James R. Goding Sr, at the then Medical Research Centre at Prince Henry's Hospital in Melbourne, Australia. This was at the start of the 1970's when the technique of radioimmunoassay (RIA), first developed by Sol Berson and Rosalyn Yalow (Berson and Yalow 1968), was being applied to reproductive hormones. With Henry Burger, I developed the first RIA for ovine FSH which enabled recognition of its pulsatile release (Salamonsen, et al. 1973). I became fascinated by the physiology of reproduction, and later, after a substantial period out of the workforce for motherhood (as common in those days), I undertook a PhD under the supervision of Professor Jock Findlay at Prince Henry's Institute of Medical Research. He had an interest in embryo implantation and I took up a project to examine molecular mechanisms underpinning implantation in sheep. This work, which focussed on identifying endometrial protein changes in ovine tissue and uterine fluid under different steroidal stimulation and during very early pregnancy (Salamonsen LA 1986, Salamonsen, et al. 1985), stimulated and underpinned my subsequent long term interest in endometrial function and strong focus on improving female reproductive health.

Over the next 30 years my interests evolved, in part by identifying clinical needs for basic science that would underpin and modify evidence-based clinical practice to improve women's reproductive wellbeing and also in response to availability of funding. Another strong driver was the opportunity to perform more complex molecular and cellular analyses as new technologies became available.

My specific interests have focussed around the extensive remodelling that occurs within the endometrium, particularly the mechanisms underpinning menstruation and abnormal uterine bleeding and development of endometrial receptivity for successful embryo implantation, in addition to unravelling the microenvironment of implantation within the uterine cavity. Given that disturbances

of endometrial remodelling severely affect women's health, my team has also contributed to our understanding of infertility, endometriosis and endometrial cancer. Further, we have identified a number of targets for new women-centred non-steroidal contraceptives: regrettably, funding bodies and pharmaceutical companies in the western world are not yet accepting of post-coital contraception. Some of our discoveries have laid the groundwork for changes to clinical practice while new models we have developed for the study of 'human' problems which cannot be studied in vivo, have subsequently been adapted by others and enabled new discoveries. The remainder of this review focuses on some of the major contributions from my laboratory, placing them in the framework of current knowledge, and how they have progressed our understanding of endometrial function in women. A brief personal profile along with a list of some of my published research is presented in Box 1.

Mechanisms of menstruation and endometrial repair

Menstruation is the process whereby most of the functionalis layer of the endometrium is shed, accompanied by bleeding from the fragmented blood vessels, at the end of each non-conception menstrual cycle (reviewed: (Salamonsen 2018). It occurs in only a limited number of species including women, old world primates, some bats and the spiny mouse (Bellofiore, et al. 2018) and is a response to the rapid fall in levels of progesterone and estradiol-17β that accompany the demise of the corpus luteum. Current knowledge indicates that menstruation is limited to these species, since they are the only ones in which the endometrial stroma undergoes the process of differentiation known as decidualization, during the secretory phase, even in the absence of an embryo. Since this is irreversible, the endometrium must be shed and replaced to provide an opportunity for implantation in the next cycle. Menstrual shedding, as observed by scanning electron microscopy (Ferenczy and Richart 1973, Ludwig and Spornitz 1991), occurs at focal points, with rapid re-epithelialisation of the shed surface occurring even as shedding is initiated at adjacent sites. The first day of bleeding is by definition, day 1 of the next menstrual cycle.

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86 Menstrual breakdown

87 The withdrawal of steroidal control of the endometrium initiates a sequence of events leading to
88 menstrual breakdown (Figure 1) and these represent a highly controlled inflammatory process (Finn
89 1986). Progesterone is an inhibitor of inflammation, as evidenced in mice lacking the progesterone
90 receptor (PR) in which the uterus is highly inflamed (Lydon, et al. 1995). The initial response to
91 falling progesterone in a non-conception cycle (when the corpus luteum is not rescued by hCG),
92 occurs within the decidualized stromal cells, which express the PR and respond to progesterone
93 withdrawal by intracellular processes known for their role in inflammation. These include decreased
94 cytoplasmic I-kappaB and a progressive increase in NF-kappaB accumulation in the nucleus. In
95 parallel a host of pro-inflammatory mediators, including chemokines and cytokines, are released: this
96 can be abrogated by an inhibitor of NF-kappaB (Evans, et al. 2011) (Evans and Salamonsen 2012).

97 Leukocytes are present in the endometrium in only small numbers during the proliferative phase of
98 the cycle, but uterine natural killer cells (uNK, increase in the mid-secretory phase with their
99 proliferation and differentiation occuring in response to IL15 induced by progesterone acting on the
100 stromal cells (Kitaya et al, PMID:15713701) ; these are associated with endometrial receptivity.
101 However, during the late secretory phase, in response to progesterone withdrawal and the
102 chemokines and cytokines released into the tissue from the decidualized cells, there is a massive
103 influx of inflammatory cells including macrophages (Figure 1A), but predominantly granulocytes
104 (neutrophils, mast cells, eosinophils, basophils), which become activated locally (Figure 1B). These
105 release factors such as degradative enzymes and cytokines stored in their intracellular granules, but
106 also chemo-attractants for uNK and monocytes/macrophages (Salamonsen and Woolley 1999).
107 These inflammatory cells comprise up to 50% of the total cells in peri-menstrual endometrium and
108 set up a cascade of events that lead to tissue destruction. Since uNK cells predominate prior to the
109 menstrual cascade, their major role is most likely during pregnancy when they are important
110 components of pregnancy decidua that orchestrate vascular adaption and trophoblast invasion(Hanna,

et al. 2006) (Xiong, et al. 2013). However, there is evidence that within the late secretory phase of the cycle, once decidualization has commenced, the uNK cells selectively target and clear senescent decidual cells through granule exocytosis (Brighton, et al. 2017) .

Extracellular matrix breakdown

Matrix metalloproteinases (MMP) are the family of enzymes primarily responsible for breakdown of extracellular matrix: different enzymes have specific substrate specificities. The first indication of the in vivo association of MMPs with menstruation, using Northern blot on endometrial samples taken from across the cycle, showed clearly that MMP1 and MMP3 mRNA were highly expressed only immediately before and during menstruation (Hampton and Salamonsen 1994) and, subsequently, we and others identified a range of MMP mRNA and protein capable of fully breaking down the endometrial ECM in peri-menstrual endometrium (Rodgers, et al. 1994) (Jeziorska, et al. 1996, Marbaix, et al. 1996). MMP expression in human endometrium is regulated both by withdrawal of progesterone (Marbaix, et al. 1992, Zhang, et al. 2000) and also by locally produced cytokines including IL1β and TNFα (Rawdanowicz, et al. 1994). MMPs are released as latent forms requiring extracellular cleavage for activity: in vivo they are balanced by natural inhibitors (TIMPs) that bind the active forms with a 1:1 stoichiometry. This provides stability of tissues as it is only when active MMPs are present in excess of the TIMPs that ECM degradation can occur. MMP activators include other MMPs (providing a cascade of activity), enzymes from leukocytes including mast cell chymase and tryptase and cytokines (Salamonsen and Lathbury 2000) (Figure 1B, (Zhang, et al. 1998). Using in situ zymography, focal sites of MMP activity were demonstrated in menstrual endometrium, supporting that MMPs that are likely primarily responsible for tissue degradation at menstruation while the focal activation highlights the mechanism underpinning the piecemeal nature of menstrual tissue breakdown (Figure 1C; (Zhang and Salamonsen 2002). New mechanisms by which MMPs are so tightly regulated are emerging,

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and include inhibition of endocytic clearance by the low density lipoprotein receptor-related protein-1 (LRP-1) by ectodomain shedding (Gaide Chevronnay, et al. 2012).

Mouse models of menstruation

Since it is possible to undertake only 'snapshots in time' of endometrial tissue in women, a good animal model for menstruation is needed. Colin Finn (Finn and Pope 1984) first developed a mouse model for menstruation, providing proof that both progesterone withdrawal and artificial decidualization (which occurs only in response to an embryo in mice), were essential for menstrual breakdown. This was refined by us in the early 2000's (Brasted, et al. 2003) and has since been modified by others (Armstrong, et al. 2017, Cousins, et al. 2014, Menning, et al. 2012, Xu, et al. 2013) providing insights into molecular and cellular mechanisms of breakdown and repair detailed below.

Recently, the spiny mouse, a native to the deserts of Africa and the Middle East, has been shown to undergo menstruation very similar to that in women. It demonstrates similar variation in degree of menstrual bleeding with some females having noticeable heavy periods along with inflammation, breakdown and repair processes as in women. This menstruation occurs for approximately 72 hours every nine days (Bellofiore, et al. 2018), making this a very useful laboratory model for study of the human condition.

Endometrial repair

Redevelopment of Finn's mouse model of menstruation, particularly enabled molecular evaluation of endometrial repair, which is essential to stop menstrual bleeding and which, uniquely, is scar free. As previously shown in menstruating women by scanning electron microscopy, endometrial repair in the mouse model occurs simultaneously at focal points, adjacent to those which are still undergoing breakdown. Importantly, initial repair (re-epithelialisation) can take place in the

159 complete absence of estrogen (Kaitu'u-Lino, et al. 2007a) but is hampered by androgen (Cousins, et
160 al. 2016). Furthermore, activins stimulate repair (Kaitu'u-Lino, et al. 2009) and neutrophil influx is
161 essential (Kaitu'u-Lino, et al. 2007b). In a similar mouse model, physiological endometrial hypoxia
162 and decreased HIF-1α, occur during bleeding while maintenance of the mice under hyperoxia
163 during menses both decreases HIF-1α induction and delays repair (Maybin, et al. 2018). In a unique
164 approach, Jemma Evans proposed that as repair occurs in the presence of menstrual effluent, this
165 fluid would contain repair factors. Application of menstrual fluid to in vitro endometrial wound
166 models dramatically enhanced re-epithelialization. Differential proteomic analysis of menstrual
167 serum versus blood serum (collected at the same time) identified both unique and upregulated
168 proteins in menstrual serum. These included proteases, anti-proteases, antimicrobials and factors
169 protective against stress, along with others that could be predicted to facilitate post-menstrual repair.
170 Indeed, a number of the identified proteins were functionally active in promoting endometrial repair
171 in vitro (Evans, et al. 2018).

172 Abnormal uterine bleeding

173 Abnormal uterine bleeding (AUB) is a major issue for women and their families. In women using
174 long-acting progestin only contraceptives (such as the implantable Implanon and the impregnated
175 IUD, Mirena), AUB primarily takes the form of irregular spotting. However, such irregularity is the
176 major reason for women discontinuing these very effective contraceptives. With support from the
177 World Health Organisation, we and others, investigated the mechanisms underpinning this bleeding.
178 One cause identified was inadequate control of MMP actions by different mechanisms: local
179 disturbance of TIMPs and excessive leukocyte activation (Vincent, et al. 1999, Vincent, et al. 2000).
180 Regrettably, clinical trials of treatments for frequent and/or controlled bleeding in women using
181 Implanon, based on this knowledge (Weisberg, et al. 2006, 2009), showed that while mifepristone
182 combined with ethinyl estradiol or doxycycline (which inhibits MMP action) was effective in
183 stopping a specific bleeding episode, it showed no improvement in subsequent bleeding episodes.

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In users of hormone replacement therapy, irregular bleeding is also associated with a distinct pattern of MMP and TIMP production, but this differs from that seen in normal menstrual bleeding and from that seen in contraceptive-related breakthrough bleeding. Again, evidence supports that the balance between MMP and TIMP in the endometrium contributes to vascular breakdown with HT but by a different mechanism than that seen in normal menstruation or in breakthrough bleeding (Hickey, et al. 2006).

There is still a clear need for treatments for abnormal uterine bleeding. It is to be hoped that others will continue this quest using new knowledge and models,.

Endometrial receptivity for implantation

Cyclical remodelling is the major feature of the endometrium in most, if not all, species but is most extreme in women. It is driven primarily by the cyclical production of the ovarian hormones estradiol-17β and progesterone and serves to prepare the endometrium for implantation of a blastocyst in a conception cycle. However, the endometrium is 'receptive' only for about 4 days (Navot, et al. 1991, Wilcox, et al. 1999) in the mid-secretory phase of each menstrual cycle. At this time, all the cell types in the endometrium, particularly the epithelial cells and the stromal fibroblasts have differentiated in response to the rising progesterone following ovulation. A receptive endometrium is essential for implantation when a hatched blastocyst attaches to and penetrates the luminal epithelium to begin its invasion through the decidualizing stroma (Figure 2). It is now clear that at least some of the unexplained infertility in women is a result of failure to attain receptive endometrium. We have sought to identify the critical signalling molecules that lead to receptivity and implantation.

Our first discovery studies for endometrial factors involved in implantation utilized the then new technique of RNA differential display, comparing expression levels in implantation versus inter-

implantation sites in mice on day 4.5 of pregnancy, just when the blastocyst was first in contact with the endometrium. Five of the transcripts identified encoded proteins which were further investigated: (Nie, et al. 2000b), MNSFβ (Nie, et al. 2000a), the High temperature resistant protein A3 (HtrA3; its first identification) (Nie, et al. 2003a), pro-protein convertase 5/6 (Nie, et al. 2003b), splicing factor SC35 (Nie, et al. 2002) and Calbindin d9k (Nie, et al. 2000b). Knockdown or inhibition of four of these demonstrated that they were each essential for implantation in mice: all but calbindin d9k were similarly expressed in receptive human endometrium: due to evolution, in women calbindin 28k most likely performs the same role (Luu, et al. 2004). PC6 and HtrA3 are both proteases and identification of their specific substrates has provided insight into pathways essential for implantation, potential targets for contraception, and a potential use in identifying receptive endometrium (see below).

A key role for cytokines in implantation was first demonstrated in leukaemia inhibitory factor (LIF) null mice which exhibited failure of implantation (Stewart, et al. 1992). Interestingly, extension of this work to human implantation, showed that while LIF contributes to receptivity in women, it is not essential (Paiva, et al. 2009). Indeed treatment of infertile women with LIF failed to improve implantation rates (Brinsden, et al. 2009). We further established that another related cytokine, interleukin (IL)11, whose receptor (R), as for LIFR, is present on endometrial epithelium, and also plays a role in implantation, regulating the adhesiveness of primary endometrial epithelial cells, likely though upregulation of both flotillin-1 and annexin A2 (Yap, et al. 2011), which are themselves proposed to be essential for implantation. Furthermore, IL11, IL6 fibroblast growth factor (FGF)2, CXCL10, vascular endothelial growth factor (VEGF) and granulocyte-macrophage growth factor (GMCSF) are all regulated in endometrial epithelium by blastocyst-derived human chorionic growth factor (hCG) (Licht, et al. 2001, Paiva, et al. 2011), demonstrating the importance of the blastocyst signalling in establishment of pregnancy. IL11 is also one of a number of cytokines, including activin A, that drive decidualization via different pathways (Menkhorst, et al. 2010).

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The luminal epithelium is the first point of contact of the blastocyst with the endometrium. At the time of implantation this undergoes a 'plasma membrane transformation' (Murphy 2004) accompanied by loss of junctional integrity and adhesive molecule changes at the apical surface (Aplin and Ruane 2017). In women, PC6 acting via its proteolytic activity, post-translationally regulates anti-adhesion molecules (including dystroglycan, and integrins (Heng, et al. 2015, Paule, et al. 2012) and reorganizes the plasma membrane altering the apical architecture to provide a receptive surface (Heng, et al. 2011). Further, actin linkage to the apical plasma membrane is regulated by the ERM proteins, ezrin, moesin and radixin (Martin, et al. 2000): PC6 cleaves the ERM-binding phosphoprotein EBP50, which tethers ezrin to the membrane: knockdown of PC6, stabilizes membrane localization of ERMs, thus preventing the rearrangement of the actin microfilament web essential for receptivity (Heng, et al. 2011).

Changes in epithelial apical-basal polarity, first proposed by Denker (Denker 1993), are needed for progression of implantation following adhesion, enabling the trophectodermal cells to move between the epithelial cells and penetrate the stromal compartment. In the endometrial epithelial cell line ECC1 (the closest representative of luminal epithelium), combined estrogen/progesterone treatment to mimic the mid-secretory phase of the cycle downregulated polarity (measured by reduced transepithelial resistance). Importantly, defined polarity markers (Stardust, Crumbs and Scribble) were downregulated in endometrial biopsies during the progression from the proliferative to the secretory phase while knockdown of Scribble in the ECC1 cells, enhanced trophectodermal adhesion (Whitby, et al. 2018). Interestingly, this loss of polarity is further driven by hCG, a product of the pre-implantation blastocyst, via its receptor (the LHCGR) on the epithelial cells (Evans and Salamonsen 2013).

Uterine microenvironment of implantation

257 Uterine fluid provides the microenvironment for blastocyst hatching and final development, and for
258 the first stages of implantation (Salamonsen, et al. 2016) (Figure 2). It contains highly selected serum
259 proteins (albumin and immunoglobulins are particularly abundant) (Hannan, et al. 2009), along with
260 contributions from Fallopian tube fluid, leukocyte activation, semen and the blastocyst (in a
261 conception cycle). Salts, sugars, amino acids, lipids, hormones, carbohydrates, RNA forms and other
262 nutrients are also present. Importantly uterine fluid is enriched in soluble proteins secreted from the
263 endometrial luminal epithelium and glands and in secreted extracellular vesicles (EVs). Analysis of
264 uterine fluid may provide a useful window to detect whether or not receptivity has been achieved
265 (Salamonsen, et al. 2013) and to detect diseases of the reproductive tract (Lopata, et al. 2003).

266 Uterine gland secretions are unequivocally required for establishment of pregnancy as shown by
267 landmark studies in sheep (Gray, et al. 2001) and mice (Filant and Spencer 2013), in which the
268 development of uterine glands was totally inhibited (uterine gland knockout). The ewes showed
269 retarded conceptus development and no implantation while in the female mice there was no
270 decidualization and no implantation.

271 In women, the endometrium is rich in glands, with approximately 15 gland openings for every
272 millimetre of uterine surface in the secretory phase (Burton, et al. 2002), thus secretions into the
273 uterine cavity are abundant, particularly during the mid-secretory phase when the glands are fully
274 differentiated for secretion. Analysis of human uterine fluid has utilized either uterine aspirate or
275 lavage: these provide somewhat different results, probably since lavage washes the uterine surface,
276 removing loosely bound molecules or those trapped locally by the glycocalyx (Hannan, et al.
277 2012). Multiplex cytokine/chemokine analyses have measured many of these important mediators
278 in uterine fluid and shown that their levels differ between fertile and infertile women and in the
279 proliferative compared with the secretory phase (Boomsma, et al. 2009, Fitzgerald, et al. 2016,
280 Hannan, et al. 2011). In some cases, functional studies indicated their roles: for example, VEGF
281 promotes human endometrial epithelial cell adhesive capacity and mouse blastocyst outgrowth in

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282 vitro and along with placental growth factor (PLGF) enhances embryo development and
283 implantation in mice (Binder, et al. 2014, Binder, et al. 2016, Hannan, et al. 2011).

284 With the recent advent of sophisticated proteomics, uterine fluid has been examined in an unbiased
285 manner: many proteins already known as important for receptivity have been confirmed, and other
286 novel proteins also identified in secretory phase fluid (Casado-Vela, et al. 2009, Hannan, et al. 2010,
287 Scotchie, et al. 2009). Validation is typically absent while bioinformatics and functional assays could
288 indicate possible actions. Interestingly, proteomic analyses of proliferative phase proteins and
289 glycoproteins have indicated that in some idiopathic infertile women, the endometrium is developing
290 in an environment of increased inflammation, thus inadequately priming the endometrium for
291 development of receptivity (Fitzgerald, et al. 2018, Fitzgerald, et al. 2016) and personal
292 communication). Some of the proteins altered in the proliferative phase were further validated: for
293 example extracellular matrix protein 1 (ECM1) is secreted by both primary endometrial epithelial and
294 stromal cells and is not regulated by estrogen. It is a biotransporter, binding many partners among
295 which are a number of extracellular matrix proteins; hence ECM1 may influence endometrial
296 regeneration and development.

297 To determine the hormonal regulation of uterine fluid proteins, we analysed the soluble secreted
298 proteomes of ECC1 cells, appropriately treated to represent the proliferative (estrogen alone) and
299 secretory phases of the cycle (estrogen plus progesterone following estrogen priming), and also in
300 the presence of the embryo-derived hCG. There were substantial unique protein changes between
301 these. Of 1059 proteins identified, 123 were significantly altered by progesterone (mostly
302 downregulated) (Figure 3) and 43 proteins altered by hCG. The identified proteins were associated
303 with cellular adhesion, ECM organisation, developmental growth, growth factor regulation and cell
304 signalling. Many of the changes were validated in primary endometrial epithelial cells (Greening, et
305 al. 2016b). Several proteins were common to those in the secretory phase described by (Scotchie, et
306 al. 2009) and 15 had been identified in our previous analyses (Hannan, et al. 2010). Interestingly, the

enzymatic protein superoxide dismutase (SOD1), which is an important antioxidant defense, was elevated in response to hCG, and hence has a potentially important role in endometrial-embryo communication.

Extracellular vesicles: part of the cellular secretome

The term 'secretome of a cell' has recently been redefined to include the totality of organic and inorganic elements secreted from cells either as soluble forms or within EVs produced via endosomal-exocytosis (Wikipedia). EVs are nano-sized particles, released from all cells. They provide communication with other cells, even at some distance, by delivery of their complex cargo via cell-specific docking sites. This alters the phenotype of the recipient cells, contributing to both physiological and pathological processes. Importantly, the molecular 'cargo' (that includes RNA, miRNA, DNA, lipids and proteins) is protected from extracellular degradation. EVs can be divided by size and specific marker proteins into apoptotic bodies, microvesicles and exosomes and separated by differential ultracentrifugation. Apoptotic bodies are removed at 10,000g and the others pellet at 100,000g: it is the latter fraction that is generally used in functional studies. Further separation can be achieved by density gradient centrifugation (Nguyen, et al. 2016) to provide the highly purified fractions essential for proteomic analyses.

We were the first to identify EVs in human uterine fluid and that these contained a unique cohort of proteins and miRNA (Greening, et al. 2016a, Ng, et al. 2013) respectively, differing from those of the parent endometrial epithelial cells. The contents of endometrial epithelial exosomes, prepared from the secretions of ECC1 cells, are programmed by ovarian steroid hormones (as are the soluble secreted products), with the protein profiles differing from those of the soluble secretome and the cellular proteome of the same cells. Analyses of the exosomal proteomes defined a total of 1043 exosomal proteins, of which 254 were regulated by estrogen (E) alone and 126 by combined estrogen plus progesterone (EP). More exosomal proteins were downregulated, than upregulated by EP vs E,

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and these were in the categories of basement membrane, cell adhesion, and extracellular matrix/cytoskeletal proteins (Greening, et al. 2016a). Importantly these endometrial epithelial exosomes have a unique proteome compared with exosomal proteins from other tissues and cancers (www.exocarta.com) with ~10% of the proteins not detected in exosomes from other sources. Functionally, endometrial exosomes are taken up by trophoblast (HTR8) cells, by the trophectodermal stem cell line TSC (Evans J, Greening DW, Salamonsen LA, unpublished observations), by mouse blastocysts (Catt S, Nguyen H, Salamonsen LA, unpublished observations) and also by endometrial epithelial cells themselves (Salamonsen LA, Greening DW, Evans J, Gurung S, Roslee AM unpublished). In the HTR8 cells, exosomal uptake enhanced trophoblast adhesive capacity (as measured by xCelligence) via the Fak-kinase pathway (Greening, et al. 2016a), while in mouse blastocysts, exosomal uptake enhanced outgrowth. Most physiologically relevant is that in a human implantation model that quantitates trophectodermal spheroid attachment to an endometrial epithelial cell monolayer (Evans J, Greening DW, Walker KJ, Bilandzic M, Kinnear S, Hutchinson J, Salamonsen LA, submitted), inclusion of endometrial exosomes enhanced both spheroid adhesion and attachment (Evans J, Salamonsen LA, unpublished observations).

Clinical applications

Our emphasis has been on studying human endometrium with a view to clinically relevant outcomes; while many studies have 'placed another piece in the jigsaw' of knowledge that will subsequently lead to translation, some have/will have direct clinical implications.

Change in IVF practice to 'Freeze-all'

Regrettably, infertility clinics have had an embryocentric view since IVF was introduced, with the main aim being to produce a good blastocyst for transfer. However the endometrium, which is equally as important, has remained the 'black box of early pregnancy loss" and indeed implantation (Macklon, et al. 2002). In 2014, we presented a strong case for frozen embryo transfer, based on both

355 scientific and clinical evidence (Evans, et al. 2014) which underpinned what has been a slow change
356 from previous international practice. In Australia, such 'freeze-all cycles, increased from 5% of fresh
357 cycles in 2011 to almost one in five cycles in 2015, with numbers still rising but statistics not yet
358 available (National Perinatal Epidemiology and Statistics Unit, University of NSW's Centre for Big
359 Data Research in Health and School of Women's and Children's Health maintained on behalf of the
360 Fertility Society of Australia).

361 The scientific evidence supporting this change came largely from a detailed immunohistochemical
362 study of endometrial biopsies taken at ovum pickup, with final analyses comparing women who
363 did/did not become pregnant following embryo transfer in that IVF cycle. Critically, most tissues
364 were highly disturbed by the hormonal treatments compared with samples from unstimulated women.
365 Only endometrium with a score close to normal controls supported pregnancy (Evans, et al. 2012).
366 Furthermore, a separate investigation showed that hCG stimulation of ovulation, makes the
367 endometrium refractory to subsequent embryonic hCG signalling, which promotes receptivity (Evans
368 and Salamonsen 2013).

369 Lifestyle factors influencing women's fertility

370 Many lifestyle factors contribute to infertility in both men and women; these include age, nutrition,
371 weight, exercise and environmental exposures. Reduction in cigarette smoking, illicit drug use,
372 alcohol and caffeine consumption are all of proven benefit and some of the underpinning molecular
373 mechanisms are known (Sharma, et al. 2013). Obesity is a known risk factor for ovulation defects,
374 but growing evidence implicates obesity in mediating endometrial dysfunction (reviewed in
375 (Antoniotti, et al. 2018). By explanation, a family of post-translational modifications of fat and
376 sugar-related molecules, known as advanced glycation end products (AGEs), which are elevated
377 systemically in women with high BMI, are at high concentrations in the uterine cavity of these
378 women. Importantly AGEs adversely impact both endometrial function and embryo implantation

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379 competence (Antoniotti, et al. 2018), Since AGE levels can be lowered by diet (de Courten, et al.
380 2016) or by pharmaceutical means (Coughlan, et al. 2007), this offers hope to those infertile women
381 with obesity and/or related metabolic disorders.

382

383 Blood test for endometrial receptivity

384 A major need in IVF clinics is for a test to indicate whether fresh embryo transfer is likely to be
385 successful. Following our extensive analysis of uterine fluid, a cohort of cytokines measured in
386 uterine fluid taken on the day of ovum pickup, has proven to be a highly effective test for uterine
387 receptivity in the cycle of sampling: it clearly differentiates between women who will have a
388 successful transfer in that cycle and those who will not (Edgell, et al. 2018). A modification of this
389 test is equally effective if applied to serum samples similarly taken at the time of ovum pickup
390 (Edgell and Salamonsen, unpublished data).

391

392 We anticipate that manipulation of the uterine microenvironment using one or more of the factors
393 dysregulated in infertile women may be effective in treating endometrial infertility without need for
394 IVF.

395

396 Beyond the endometrium: scar-free wound repair

397 Endometrial repair following menstruation is unique among adult tissues in that it occurs very
398 rapidly and without scarring. Endometrial destruction occurs at focal points and re-epithelialization
399 follows immediately to cover the endometrial surface: thus repair occurs in the presence of
400 menstrual effluent. From the proteomic data discussed above, several menstrual fluid proteins were
401 functionally active in endometrial repair assays (Evans, et al. 2018). Testing was also successfully
402 applied to wound repair models, including in vivo wound repair in pigs. Unlike other known wound

repair proteins, which promote cellular proliferation, and have a potential hazard of stimulating cancers, the menstrual fluid proteins enhanced cellular migration and initial re-epithelialization of wounds. It is predicted that these factors will extend current wound repair paradigms, maybe reducing the risk of scarring and providing effective treatment of chronic non-healing wounds.

Contraceptive targets

One major ambition has remained unfulfilled: to provide a novel non-hormonal contraceptive for women that need only be utilized in any cycle in which coitus occurs. In the early 1990s we proposed that maintenance of the endometrium in a non-receptive state throughout the cycle (by inhibiting the development of receptivity), would provide very effective contraception. Funding for such development was available in the 1990s -2000s: we proved that blocking the actions of PC6 and its substrates, or IL11 would be effective. PC6 is a particularly exciting target since it is also essential for HIV infectivity through the vagina. Subsequently, numerous other potential targets have been identified. In women, it is difficult to directly target the endometrium or uterine cavity. The teams of Nie and Dimitriadis, working with inhibitors of PC6 and IL11 respectively, investigated modes for local administration in mice, particularly vaginal gels which could provide simultaneous protection from pregnancy and infection (Ho, et al. 2014, Menkhorst, et al. 2011). The inhibitors used were effective and clearly reached the mouse endometrium via a “first pass effect’ from the vaginal circulation. Whether this would also be the case in women is not known: progestins which are of very low molecular weight, can be delivered via the vagina. We now anticipate that delivery of inhibitors via exosomes/nanoparticles with target specificity to endometrial epithelium (a current major interest of ours) may provide a solution to local delivery. A major advantage of targeting the endometrium, is that it is shed in each menstrual cycle and thus actions of inhibitors in one cycle would not be maintained and hence not be systemically harmful.

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The major obstacles remaining to achieve such contraception, are twofold. Firstly systems for direct delivery to the uterine cavity need to be developed, and secondly, the political demands that contraception for women be targeted only at pre-fertilization events, needs to be overcome. This is clearly regrettable in a world where non-steroidal contraceptive methods for women remain a global need and in which a majority of women are not confined by the values of a few (Crosignani and Glasier 2012). Indeed, promotion of family planning so that women can avoid unwanted pregnancy, is central to achieving the Millennium Development Goal on improving maternal health, reducing child mortality and eliminating extreme poverty (Cleland, et al. 2006).

Conclusions.

It has been a privilege and a great pleasure to contribute to our knowledge of endometrial function and women's health. However, this would not have been possible without the many others working in the field, a number of whom have become collaborators and friends. Together we have built a strong body of knowledge from which clinical solutions to a number of disorders affecting women will evolve and which will resolve often 'silent suffering'. My generation's field of endeavour is now passing to the hands of the wonderful younger scientists we have trained: I have every confidence that they will deliver the important outcomes needed.

Conflicts of interest

No conflicts of interest to report.

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Figure legends

Figure 1. The menstrual cascade is a highly controlled process of inflammation and tissue degradation. In brief, during the late secretory phase of the cycle and in the absence of a pregnancy, the falling levels of progesterone and estrogen stimulate production of chemokines and cytokines by endometrial decidualized stromal cells and epithelial cells. These result in entry of large numbers of leukocytes into the tissue, which become activated locally and stimulate production of degradative enzymes, particularly matrix metalloproteinases in their latent forms which also become activated. These then degrade the extracellular matrix of the tissue, resulting in shedding and concomitant bleeding. The photomicrographs show: (A) staining of menstrual tissue with CD45, indicating that 40-50% of the cells in the tissue are of leukocyte origin: (B) inactive mast cells in the tissue, become activated releasing their granular contents (shown here staining for mast cell tryptase); (C) in situ zymography of day 2 menstrual tissue, indicating active MMP2 and MMP9, at very focal points in the tissue, thus explaining the piecemeal tissue shedding.

Figure 2. The early stages of human implantation. The unhatched blastocyst enters the uterine cavity, where it sheds the zona pellucida, and undergoes further development as it becomes apposed to the uterine surface. At this time decidualization is initiated close to the blood vessels from which macrophages and uterine natural killer cells are attracted into and through the endometrium along a chemokine gradient. The microenvironment within the uterine cavity (including soluble factors and extracellular vesicles secreted from both the epithelium and trophectoderm) promote phenotypic

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475 changes in both apposing cell types, necessary for implantation. Changes in adhesive properties
476 enable blastocyst attachment to the endometrial epithelial surface, which is undergoing a partial
477 epithelial to mesenchymal transformation – the reduced polarity enables trophectodermal cells to
478 penetrate the epithelial surface, under which they form a syncytium; some cells escape to invade the
479 blood vessels which they transform. M; macrophage, NK; uterine natural killer cells, bv; blood
480 vessel.
481
482 Figure 3. The total secretome of the ECC1 cell line (representative of endometrial epithelial cells),
483 comprises both a soluble proteome and a proteome contained in secreted exosomes. These
484 proteomes were analysed following incubation of the ECC1 cells under conditions representing the
485 proliferative (estrogen) and the secretory (estrogen plus progesterone) phases of the menstrual cycle.
486 The Venn diagrams clearly establish that while there are proteins in common between the two
487 proteomes, the majority of proteins are specific to either the soluble or exosomal compartments.
488 There were also many protein differences between the two hormonal treatments (modified from
489 Greening et al, 2016b).

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Box 1: Professor Lois A. Salamonsen

Lois has spent her professional life following her passion: understanding the cellular and molecular events that underpin the extraordinary cyclical remodelling of the human endometrium.

She obtained her BSc(hons) in biochemistry at Otago University in New Zealand, where she met her lifelong partner Bob Salamonsen. He and the two sons that followed their marriage have provided continuous support and encouragement to her career. The first ten years focussed on his career development in medicine, which led them to Melbourne, Bergen and Manchester (UK) – in all of these she worked as a research assistant in various laboratories which provided her with a wealth of laboratory skills. On return to Melbourne where they made their home, and with the boys now at school, she obtained a PhD from Monash University and subsequently developed her own laboratory at Prince Henry's Institute.

Lois's career was funded through the Fellowship scheme of NHMRC of Australia where she finally became Senior Principal Research Fellow (NHMRC, 2006-2016). When the Hudson Institute for Medical Research was created through merger of two Institutes, she was appointed Head of the Centre for Reproductive Health. She is also adjunct Professor in the Department of Obstetrics and Gynaecology at Monash University. While now partly retired she still heads the Endometrial Remodelling laboratory at the Hudson Institute.

Her honours include election as a Fellow of: The Australian Academy of Sciences (FAA); the Royal Australasian College of Obstetrics and Gynaecology; the Society for the Study of Reproduction

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(USA); and the Society for Reproductive Biology (SRB, Australasia). She is recipient of the Beacon award from Frontiers in Reproduction, and the Founder's lecture of the SRB.

Her team, through >260 publications, are recognized for their contributions to our understanding of endometrial remodelling, the mechanisms underlying menstruation and abnormal uterine bleeding, uterine receptivity, embryo implantation, along with new approaches to female contraception as detailed in the accompanying article. Their focus is on the human, with strong emphasis on translational research.

Current research focuses on the microenvironment of implantation. Identification of the proteins and exosomes in uterine fluid, their regulation and functions both on the endometrium and on the developing embryo and trophectoderm, is providing insights into the complexity of implantation and how it is disturbed in infertile women. The demonstration of strongly detrimental effects of the ovulation induction regimes used in IVF clinics on endometrial receptivity and the potential for implantation, combined with new tests to predict receptivity, should lead to changes in clinical practice and improved IVF outcomes.

Lois has a passion for training young scientists, with a number of her trainees subsequently developing highly productive careers. Some now hold professorial appointments worldwide. Equally, she guides others to appropriate and fulfilling careers outside of research: the adage being that a PhD provides important skills beyond just the research, that can be applied broadly, and that a scientifically informed population is important to society as a whole. Lois is particularly known for her mentorship of young women, who face difficult decisions in combining motherhood and childcare with a high-pressure career in scientific research. She comes to this from her own life experience, that even starting a PhD at 40 is not prohibitive of a productive life in science.

This life has been a privilege and a joy – who could ask for more.

Some important articles

Dimitriadis, E, CA White, RL Jones, and LA Salamonsen 2005 Cytokines, chemokines and growth factors in endometrium related to implantation. *Hum Reprod Update* **11** 613-630.

Evans, J, NJ Hannan, TA Edgell, BJ Vollenhoven, PJ Lutjen, T Osianlis, LA Salamonsen, and LJ Rombauts 2014 Fresh versus frozen embryo transfer: backing clinical decisions with scientific and clinical evidence. *Hum Reprod Update* **20** 808-821.

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Evans, J, LA Salamonsen, A Winship, E Menkhorst, G Nie, CE Gargett, and E Dimitriadis 2016 Fertile ground: human endometrial programming and lessons in health and disease. *Nat Rev Endocrinol* **12** 654-667.

Greening, DW, HP Nguyen, K Elgass, RJ Simpson, and LA Salamonsen 2016 Human endometrial exosomes contain hormone-specific cargo modulating trophoblast adhesive capacity: Insights into endometrial-embryo interactions. *Biol Reprod* **94** 38.

Hannan, NJ, P Paiva, KL Meehan, LJ Rombauts, DK Gardner, and LA Salamonsen 2011 Analysis of fertility-related soluble mediators in human uterine fluid identifies VEGF as a key regulator of embryo implantation. *Endocrinology* **152** 4948-4956.

Jones, RL, NJ Hannan, TJ Kaitu'u, J Zhang, and LA Salamonsen 2004 Identification of chemokines important for leukocyte recruitment to the human endometrium at the times of embryo implantation and menstruation. *J Clin Endocrinol Metab* **89** 6155-6167.

Ng, YH, S Rome, A Jalabert, A Forterre, H Singh, CL Hincks, and LA Salamonsen 2013 Endometrial exosomes/microvesicles in the uterine microenvironment: a new paradigm for embryo-endometrial cross talk at implantation. *PLoS One* **8** e58502.

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Rawdanowicz, TJ, AL Hampton, H Nagase, DE Woolley, and LA Salamonsen 1994 Matrix metalloproteinase production by cultured human endometrial stromal cells: identification of interstitial collagenase, gelatinase-A, gelatinase-B, and stromelysin-1 and their differential regulation by interleukin-1 alpha and tumor necrosis factor-alpha. *J Clin Endocrinol Metab* **79** 530-536.

Salamonsen, LA, J Evans, HP Nguyen, and TA Edgell 2016 The microenvironment of human implantation: Determinant of reproductive success. *Am J Reprod Immunol* **75** 218-225.

Salamonsen, LA, and G Nie 2002 Proteases at the endometrial-trophoblast interface: their role in implantation. *Rev Endocr Metab Disord* **3** 133-143.

Figure 1

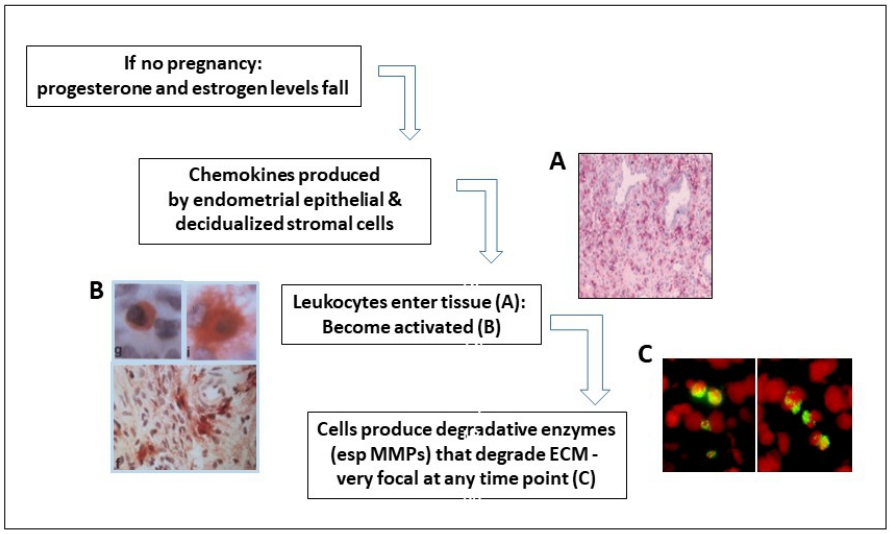

Figure 1. The menstrual cascade is a highly controlled process of inflammation and tissue degradation. In brief, during the late secretory phase of the cycle and in the absence of a pregnancy, the falling levels of progesterone and estrogen stimulate production of chemokines and cytokines by endometrial decidualized stromal cells and epithelial cells. These result in entry of large numbers of leukocytes into the tissue, which become activated locally and stimulate production of degradative enzymes, particularly matrix metalloproteinases in their latent forms which also become activated. These then degrade the extracellular matrix of the tissue, resulting in shedding and concomitant bleeding. The photomicrographs show: (A) staining of menstrual tissue with CD45, indicating that 40-50% of the cells in the tissue are of leukocyte origin: (B) inactive mast cells in the tissue, become activated releasing their granular contents (shown here staining for mast cell tryptase); (C) in situ zymography of day 2 menstrual tissue, indicating active MMP2 and MMP9, at very focal points in the tissue, thus explaining the piecemeal tissue shedding.

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Figure 2

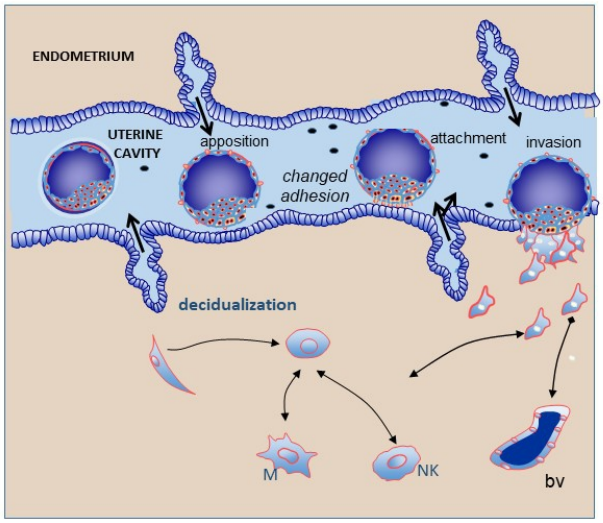

Figure 2. The early stages of human implantation. The unhatched blastocyst enters the uterine cavity, where it sheds the zona pellucida, and undergoes further development as it becomes apposed to the uterine surface. At this time decidualization is initiated close to the blood vessels from which macrophages and uterine natural killer cells are attracted into and through the endometrium along a chemokine gradient. The microenvironment within the uterine cavity (including soluble factors and extracellular vesicles secreted from both the epithelium and trophectoderm) promote phenotypic changes in both apposing cell types, necessary for implantation. Changes in adhesive properties enable blastocyst attachment to the endometrial epithelial surface, which is undergoing a partial epithelial to mesenchymal transformation – the reduced polarity enables trophectodermal cells to penetrate the epithelial surface, under which they form a syncytium; some cells escape to invade the blood vessels which they transform. M; macrophage, NK; uterine natural killer cells, bv; blood vessel.

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Figure 3

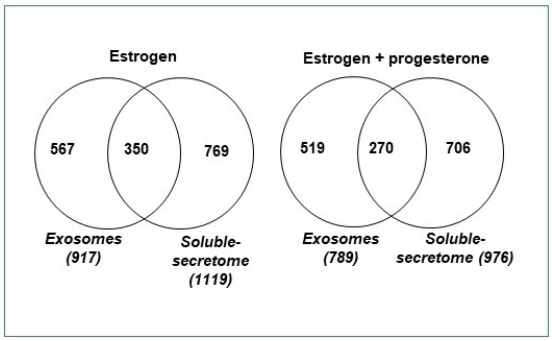

Figure 3. The total secretome of the ECC1 cell line (representative of endometrial epithelial cells), comprises both a soluble proteome and a proteome contained in secreted exosomes. These proteomes were analysed following incubation of the ECC1 cells under conditions representing the proliferative (estrogen) and the secretory (estrogen plus progesterone) phases of the menstrual cycle. The Venn diagrams clearly establish that while there are proteins in common between the two proteomes, the majority of proteins are specific to either the soluble or exosomal compartments. There were also many protein differences between the two hormonal treatments (modified from Greening et al, 2016b).

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