Success after failure: the role of endometrial stem cells in recurrent miscarriage

Emma S Lucas^{1,2}, Nigel P Dyer³, Katherine Fishwick¹, Sascha Ott^{2,3} and Jan J Brosens^{1,2}

¹Division of Biomedical Sciences, Warwick Medical School, Coventry, UK, ²Tommy's National Centre for Miscarriage Research, University Hospitals Coventry and Warwickshire NHS Trust, Coventry, UK and

³Warwick Systems Biology Centre, University of Warwick, Coventry, UK

Correspondence should be addressed to J Brosens; Email: J.J.Brosens@warwick.ac.uk

Abstract

Endometrial stem-like cells, including mesenchymal stem cells (MSCs) and epithelial progenitor cells, are essential for cyclic regeneration of the endometrium following menstrual shedding. Emerging evidence indicates that endometrial MSCs (eMSCs) constitute a dynamic population of cells that enables the endometrium to adapt in response to a failed pregnancy. Recurrent miscarriage is associated with relative depletion of endometrial eMSCs, which not only curtails the intrinsic ability of the endometrium to adapt to reproductive failure but also compromises endometrial decidualization, an obligatory transformation process for embryo implantation. These novel findings should pave the way for more effective screening of women at risk of pregnancy failure before conception.

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Introduction

Successful implantation of a human embryo is commonly attributed to binary variables; i.e. nidation of a 'normal', but not an 'abnormal', embryo in a 'receptive', but not a 'non-receptive', endometrium is required for a successful pregnancy. However, this implantation paradigm is based on animal studies, more specifically the mouse model (Wang & Dey 2006). Like many other rodents, mouse reproductive success is based on quantity and is characterized by rapid breeding cycles, multiple synchronous implantations, large litter size and, crucially, huge natural selection among offspring (Taylor 2016). Mouse offspring "quality" is arrived at mainly through sibling rivalry after birth. By contrast, human pregnancy requires prolonged investment in a single foetus at considerable cost to the mother (Haig 1993). Maternal cost, and the risk of neonatal death, increases sharply with each additional foetus (e.g. twins, triplets etc.) (Refuerzo 2012). From an evolutionary perspective, a reproductive strategy based on prolonged maternal investment in singleton pregnancies makes sense only if based on an intrinsically dynamic and adaptable implantation process (Macklon & Brosens 2014).

And it is. Cleavage-stage human embryos tolerate and actively generate aneuploid blastomeres through mitotic non-disjunction. As a consequence, most human embryos are mosaic (Vanneste *et al.* 2009, Taylor *et al.* 2014). With over 2500 distinct genetic errors documented to

date (Fragouli *et al.* 2013), each implanting blastocyst is arguably unique. Furthermore, transient aneuploidy during development may not be unequivocally as 'bad' as has been intuitively presumed because of the obvious link with cancer and congenital abnormalities. Emerging evidence suggests that aneuploidy drives rapid phenotypic adaptation (Kaya *et al.* 2015, Liu *et al.* 2015, Millet *et al.* 2015), confers resistance to cellular stress (Chen *et al.* 2012, Duncan *et al.* 2012, Kaya *et al.* 2015) and arguably imparts invasiveness on embryos necessary for implantation and deep placentation. Recent reports demonstrated unequivocally that embryos harbouring complex mosaic aneuploidies can give rise to healthy offspring, both in humans and, experimentally, in mice (Greco *et al.* 2015, Bolton *et al.* 2016).

Several mechanisms ensure survival of mosaic embryos, including self-correction through apoptosis and possibly sequestration of aneuploid cells into the trophoblast lineage (Bolton *et al.* 2016) (Fig. 1). However, invasiveness combined with the exceptional diversity of human embryos necessitates the need for additional, external (i.e. uterine) selection to limit the risk of maternal investment in a failing pregnancy (Gellersen & Brosens 2014, Macklon & Brosens 2014). The first evidence that the endometrium is an intrinsic biosensor of embryo quality actually originated from studies in cattle. Microarray studies showed that the pregnant bovine

Figure 1 Embryo selection at implantation and recurrent miscarriage. Pre-implantation human embryos are remarkably diverse. Most human embryos are mosaic. Normal blastomeres are indicated in green whereas a different colour indicates a distinct aneuploidy. While embryonic mosaicism bestows adaptability onto the species through genetic diversity, it also increases the risk of prolonged maternal investment in a failing pregnancy. Several embryo-intrinsic and -extrinsic mechanisms operate in early pregnancy to limit this risk, including embryonic self-correction (1), biosensing of embryo quality by decidualizing (purple) cells (2), corpus luteum rescue by placental fitness hormones such as hCG (3), and oxidative stress associated with the onset of placental perfusion at the end of the 1st trimester of pregnancy, effectively stress-testing the placental-decidual interface (4). Lack of embryo selection at implantation inevitably increases the risk of clinical miscarriage. Conversely, as a pregnancy transits to the 2nd trimester, selection pressure decreases and the risk of further miscarriage drops markedly.

endometrium mounts a transcriptional response that is distinct for embryos generated by artificial insemination, IVF, or somatic cell nuclear transfer (Mansouri-Attia *et al.* 2009). Decidualizing human endometrial stromal cells have since emerged as exquisite sensors that respond to as yet unidentified embryonic serine proteases in a manner that either supports further development (positive selection) or ensures rapid disposal through menstruation-like shedding (negative selection) (Brosens *et al.* 2014). Quality control may not necessarily cease once the conceptus is embedded in the endometrium, but likely continues throughout the first trimester of pregnancy. For example, the gradual shift from ovarian to placental progesterone production arguably means that the endometrium will *de facto* select against embryos that are perceived to lack fitness because of insufficient human chorionic gonadotrophin production. Similarly, the onset of placental perfusion around week 10 of pregnancy causes dramatic changes in local oxygen tension and triggers bursts of free radicals (Burton *et al.* 1999) (Fig. 1), effectively stress-testing the foeto-maternal interface. Thus, suboptimal selection at implantation inevitably increases the risk of clinical miscarriage; and conversely, once all selection pressures have been endured successfully by the end of the 1st trimester, the likelihood of further catastrophic failure drops markedly (Fig. 1). The corollary of inefficient embryo selection at implantation is rapid conception, defined by short time-to-pregnancy interval. It has been estimated that 40% of recurrent miscarriage patients are superfertile, defined by the fact that each pregnancy is achieved

within three cycles (Salker *et al.* 2010, Teklenburg *et al.* 2010, Orlando & Coulam 2014).

Endometrial stem cells are perhaps the least appreciated and least understood drivers of reproductive plasticity. Considering the unrivalled regenerative capacity of the endometrium, it is remarkable, if not baffling, that the first experimental study demonstrating the presence of resident endometrial mesenchymal stem cells (eMSCs) was reported only 12 years ago (Chan *et al.* 2004). eMSCs are abundant, multipotent, immuno-privileged and highly regenerative in various pre-clinical models of disease (reviewed in Mutlu *et al.* 2015).

In this commentary, we explore the role of endometrial MSCs in effecting one of the most salient aspects of human reproduction, i.e. the ability to achieve a live birth after multiple pregnancy failures.

Persistent reproductive failure

One of the most disappointing aspects of modern reproductive medicine is the pervasive puerile view of implantation and early pregnancy. Patients suffering from reproductive failure, i.e. repeated IVF implantation failures or recurrent miscarriage, continue to be subjected to a battery of screening tests for subclinical 'disorders', which are presumed to converge somehow onto a 'fragile' implanting embryo, causing reproductive failure. Numerous anatomical, endocrine, immunological, thrombophilic and genetic perturbations have been invoked to explain persistent reproductive failure (Rai & Regan 2006, Agenor & Bhattacharya 2015).

Yet every diagnostic test currently in clinical practice lacks specificity, meaning that many women with normal pregnancies also test positive. Nevertheless, it remains standard practice to label a 'positive' test as 'causal', ignoring the lack of clinical evidence, biological plausibility or the absence of interventions that are even remotely effective. In the absence of a 'positive' test, many clinicians resort to exalting the virtues of vitamins, micronutrients and other soft interventions. Others advocate an interventional approach, using a range of combinatory empirical treatments (e.g. heparin, aspirin, steroids, human chorionic gonadotrophin, intravenous immunoglobulin, hydroxychloroquine, intralipids, TNF α inhibitors etc.) as well as pre-implantation genetic screening of IVF embryos. None of these interventions have been conclusively shown to improve reproductive success; and some may well be harmful.

Yet, despite this lamentable state of affairs, most women suffering either repeated implantation failure or recurrent miscarriage do achieve a successful pregnancy (Brigham *et al.* 1999, Ogasawara *et al.* 2000, Practice Committee of the American Society for Reproductive Medicine 2012, Saravelos & Regan 2014, Vlaanderen 2014), irrespective of treatment. For example, several randomized control trials on recurrent miscarriage, defined here as three consecutive pregnancy losses, reported life births rate of 65% or more in the placebo group (Coomarasamy *et al.* 2015, Pasquier *et al.* 2015). In recurrent miscarriage, the incidence of euploid foetal loss increases with each additional miscarriage, whereas the likelihood of a future successful pregnancy gradually decreases (Ogasawara *et al.* 2000). These observations indicate that RM is a graded disorder with the level of severity defined by the number of previous pregnancy losses. Nevertheless, even after five consecutive miscarriages, the likelihood of a life birth in the subsequent pregnancy remains in excess of 50% (Brigham *et al.* 1999, Rai & Regan 2006).

A parsimonious explanation for these clinical observations is that embryo–endometrial interactions are intrinsically dynamic and capable of adapting from pregnancy to pregnancy to ensure reproductive success. However, the more severe the defect, the higher the likelihood of repeated pregnancy failures in consecutive conception cycles.

The decidualizing endometrium in recurrent miscarriage

Much of the work on the endometrium in the context of recurrent miscarriage has focused on decidualization, an obligatory transformative process for pregnancy in all mammalian species where implantation involves breaching of the luminal endometrial epithelium by the conceptus (Ramsey *et al.* 1976). The decidual process is foremost characterized by transformation of endometrial fibroblasts into specialized epithelioid

cells. In parallel, the endometrium undergoes extensive remodelling, effected by the influx of specialized immune cells, predominantly uterine natural killer cells and macrophages (reviewed by Gellersen and Brosens 2014). Associated vascular changes then prepare the human endometrium for endovascular trophoblast invasion and the formation of a functional haemochorial placenta (Brosens *et al.* 2002). Decidual cells encapsulate and safeguard the conceptus against various stressors. For example, stress-induced signalling through c-Jun N-terminal kinase and p38 mitogen-activated protein kinase pathways is selectively inactivated upon decidualization of human endometrial stromal cells (Yoshino *et al.* 2003, Leitaio *et al.* 2010, 2011). In parallel with a marked induction of various free radical scavengers, silencing of stress-signalling pathways renders decidual cells extraordinarily resistant to oxidative cell death (Kajihara *et al.* 2006). Moreover, circadian oscillations within the endometrium are firmly disabled upon decidualization (Muter *et al.* 2015), which further isolates the implanting blastocyst from the maternal environment. Decidual cells are also gatekeepers and chief modulators of local immune cells at the embryo–endometrial interface. In pregnancy, decidual cells also actively prevent influx of antigen-specific cytotoxic T lymphocytes by silencing of genes encoding key chemokines (Nancy *et al.* 2012).

Importantly, decidualization is not an all-or-nothing phenomenon. Instead, differentiating human endometrial stromal cells transit through distinct functional phenotypes upon transformation into decidual cells (Salker *et al.* 2012, Lucas *et al.* 2016). This transitional pathway, which can be recapitulated in culture, is characterized initially by an acute pro-inflammatory response, lasting several days. Release by differentiating endometrial stromal cells of 'alarmins' like interleukin-33, a potent activator of the innate immune system, has emerged as an important driver of this transient inflammatory process (Salker *et al.* 2012). These inflammatory mediators in turn up-regulate the expression of key implantation genes, including leukaemia inhibitory factor, interleukin 1- β , heparin binding EGF, bone morphogenetic protein 2, wingless-related MMTV integration site 4, and homeobox protein 10. Acquisition of a mature decidual phenotype curtails the inflammatory response through induction of anti-inflammatory soluble decoy receptors (Salker *et al.* 2012) and pronounced up-regulation of 11 β -hydroxysteroid dehydrogenase type 1, leading to increased local cortisol production (Kuroda *et al.* 2013a,b). Decidual factors such as LEFTY2 contribute to the active closure of the window of receptivity (Tabibzadeh *et al.* 2000, Tang *et al.* 2005). As aforementioned, mature decidual cells are exquisitely responsive to embryo signals, especially trypsin-like proteases, and engage in active rejection by triggering menstruation-like shedding mediated by a decidual stress response (Brosens *et al.* 2014).

Taken together, these observations illustrate how the decidualization pathway contributes to the functional transition of the endometrium from a nonreceptive (early-secretory phase) to a receptive (mid-secretory phase) and then a selective (late-secretory phase) state.

The hallmark of the endometrium in recurrent miscarriage is a disordered and prolonged pro-inflammatory decidual response. This excessive inflammatory response in turn prolongs the 'window of receptivity', promotes out-of-phase implantation, and disables embryo selection (Salker *et al.* 2010, 2011, 2012, Lucas *et al.* 2016). Consequently, poor-quality embryos are not disposed of in a timely manner and high-quality embryos implant in an unsupportive environment. Both scenarios lead to clinical miscarriage.

'Memory' of endometrial stromal cells

A truly prodigious finding, reported first 10 years ago, is that human endometrial stromal cells from individual patients closely phenocopy the decidual response *in vivo* upon differentiation in culture (Klemmt *et al.* 2006). Aberrant decidualization in culture has not only been reported for recurrent miscarriage patients (Francis *et al.* 2006, Salker *et al.* 2010) but also for endometriosis (Klemmt *et al.* 2006, Aghajanova *et al.* 2011, Ahn *et al.* 2016) and polycystic ovary syndrome (Piltonen *et al.* 2013, Piltonen *et al.* 2015). However, while progesterone resistance, defined by the refractoriness of cultured human endometrial stromal cells to decidualogenic cues (Barragan *et al.* 2016), characterizes endometriosis patients; an excessive and prolonged inflammatory decidual response is typically associated with recurrent miscarriage (Salker *et al.* 2012).

We hypothesized that an epigenetic mechanism may underlie this pathological 'memory' of endometrial stromal cells associated with recurrent miscarriage. Consequently, we sequenced immunoprecipitated methylated DNA (MeDIP-seq) to compare the global cytosine methylation profiles in primary cultures established from mid-luteal biopsies from recurrent miscarriage patients and control subjects (Lucas *et al.* 2016). Disappointingly, the methylation signature at CG dinucleotides, the most common context of DNA methylation, was largely similar between the clinical groups, although there were notable differences in genes associated with decidualization and implantation processes, including the progesterone receptor co-activator high-mobility group box 2 (*HMGB2*) (Boonyaratanakornkit *et al.* 1998).

Some MeDIP-seq analysis pipelines utilize protocols based on the assumption that methylation is confined to CpG dinucleotides. When we re-analysed the sequencing data using an unrestricted approach, a striking signature became apparent in endometrial stromal cells isolated from recurrent miscarriage samples, characterized

by hypomethylation of 2741 loci. These differentially methylated regions (DMRs) overwhelmingly mapped to CA-rich regions that were largely devoid of CpG dinucleotides. Many DMRs not only clustered within 15 Mb of the telomeres but also contained several DNA motifs that were selectively hypomethylated in recurrent miscarriage patients (Lucas *et al.* 2016).

Methylation at CpH (H=A, C, T) is an epigenetic hallmark of stem cells, embryos and gametes (Ramsahoye *et al.* 2000, Shirane *et al.* 2013). It is lost in most somatic tissues but can be re-established upon pluripotency reprogramming of somatic cells (Ziller *et al.* 2011). Hence, we reasoned that the global CpH hypomethylation signature in endometrial stromal cells from recurrent miscarriage patients could be accounted for by lack of eMSCs or stemness.

eMSCs in recurrent miscarriage

The defining feature of the human sexual cycle, shared with few other mammals, is menstruation. This remarkable phenomenon is triggered by falling progesterone levels in species that exhibit cyclic decidualization of the endometrium independently of an implanting embryo. MSCs regulate the main phases of wound healing via modulation of inflammatory response, promotion of angiogenesis and stimulation of cell movement (reviewed by Wong *et al.* 2015, Wang *et al.* 2016); thus, a role for these cells in endometrial regeneration following menstruation, miscarriage or parturition is obvious. Endometrial MSCs share the classic properties of bone marrow MSCs, including clonogenicity, multipotency, the ability to reconstitute endometrial stroma *in vivo* and expression of surface markers that distinguish them from leucocytes, haematopoietic and endothelial cells (reviewed by Gargett *et al.* 2016).

As described for other organs, eMSCs predominantly reside in the perivascular niche of both the basal and functional layers (Masuda *et al.* 2012, Ulrich *et al.* 2014). Screening the endometrium with a panel of perivascular markers identified SUSD2 (W5C5) as a powerful marker for selection of clonogenic endometrial cells (Masuda *et al.* 2012). The SUSD2/W5C5-positive cell population isolated by magnetic activated cell sorting constitutes approximately 6–7% of endometrial stromal cells. The SUSD2/W5C5-positive cell fraction contains on average 2–4% clonogenic cells (Masuda *et al.* 2012, Murakami *et al.* 2013). However, clonogenic eMSCs can also be isolated from the SUSD2/W5C5-negative cell fraction, although the relative abundance is much lower (0.7%) (Murakami *et al.* 2013). A recent gene expression profiling study provided compelling evidence that clonogenic eMSCs residing in the perivascular niche are the lineage precursors of the more committed non-perivascular eMSCs (Barragan *et al.* 2016).

To explain the CpH hypomethylation signature in endometrial stromal cells of recurrent miscarriage patients, we systematically measured the total number of freshly isolated SUSD2/W5C5-positive cells, the abundance of clonogenic SUSD2/W5C5-positive eMSCs and the abundance of clonogenic SUSD2/W5C5-negative eMSCs in mid-luteal biopsies obtained from 31 recurrent miscarriage patients and 28 control subjects. The total number of SUSD2/W5C5-positive cells did not differ between the study and control groups. However, recurrent miscarriage was associated with a 41% reduction in the abundance of clonogenic SUSD2/W5C5-positive eMSCs, respectively. Strikingly, no clonogenic SUSD2/W5C5-negative eMSCs were recovered from 13 out of 31 (42%) recurrent miscarriage samples compared with 3 out of 28 (11%) control samples (Lucas *et al.* 2016).

Both the level of methylation and the abundance of clonogenic cells correlated inversely with the severity of the miscarriage phenotype, defined by the number of previous pregnancy losses. Furthermore, eMSC deficiency has profound ramifications downstream of the differentiation pathway by accumulating senescent cells in the endometrial stromal compartment. Cellular senescence is associated with distinct pro-inflammatory cytokines and chemokines, matrix metalloproteinases and growth factors, termed senescence-associated secretory phenotype (SASP) (Acosta *et al.* 2013). Importantly, induction of senescence in primary endometrial stromal cells triggered a blunted but prolonged inflammatory secretory response upon decidualization, akin to the secretory response of primary cultures from recurrent

miscarriage patients and likely reflecting the contribution of SASP (Lucas *et al.* 2016).

Perspective

The advances in endometrial stem cell biology have generated innovative tools to accurately quantify and characterize eMSC populations associated with reproductive failure. Importantly, these advances are also rewriting our understanding of the biology of endometrial stromal cells. Rather than being a homogenous population, endometrial stromal cells, *in vivo* and *in vitro*, consist of a community of cells, ranging from quiescent and active MSCs, transit-amplifying cells, mature fibroblasts and senescent cells. The observation that relative MSC deficiency and heightened cellular senescence is associated with recurrent miscarriage exemplifies that the decidual response, or more precisely the transitional decidual pathway, is determined by the balance of subpopulations that make up the community of stromal cells (Fig. 2). By default, the constituents of this community, and thus the nature of the decidual response, changes with increasing distance away of the perivascular niche. A recent study illustrated this spatial organization in the endometrium by demonstrating that perivascular SUSD2/W5C5-positive cells mount a distinct decidual response that could account for preferential homing of invading trophoblast to the spiral arteries (Murakami *et al.* 2014).

What causes eMSC deficiency in the uterus is a pertinent but as yet unanswered question. The properties and potential of adult stem cells are determined by a

Figure 2 Endometrial stromal compartment: a 'community' affair. The stromal compartment of the endometrium is a mixed community of cells of quiescent and active MSCs, transit-amplifying cells, mature fibroblasts and senescent cells. The composition of this community changes with increased distance from the perivascular niche. The effect of eMSC deficiency is amplified downstream of the perivascular niche. Failure of homeostatic balancing of the constituents of the stromal community accounts for aberrant decidualization associated with recurrent miscarriage.

combination of intrinsic characteristics and a tissue-specific microenvironment. High Notch activity is a common niche feature and essential for cell-fate specification and maintenance of stem cells in a poised quiescent state (Bjornson *et al.* 2012, Cheung & Rando 2013). For example, silencing of Notch signalling in skeletal cells leads to stem cell depletion and gives rise to muscles that lack the ability to regenerate in response to injury (Bjornson *et al.* 2012). By analogy, it seems plausible that pathological cues arising from dysmetabolic conditions associated with adverse pregnancy outcome, such as obesity, interfere with Notch signalling in the endometrium, thus gradually depleting the tissue of quiescent stem cells and rendering it vulnerable to damage in pregnancy. In support of this conjecture, a previous study reported that body mass index negatively correlates with cloning efficiency of endometrial SUSD2/W5C5-positive and -negative eMSCs (Murakami *et al.* 2013). Age, on the other hand, has no or little impact on the abundance of eMSCs (Murakami *et al.* 2013, Ulrich *et al.* 2014).

These observations lead to other pertinent questions. How does the endometrium maintain its MSC populations over 400 or so cycles of tissue breakdown and regeneration? Is expansion of the eMSC population required to accommodate pregnancy? A widely held assumption is that eMSCs residing in the basal layer, which is not shed during menstruation, maintain the regenerative capacity of the endometrium from menarche until the menopause and beyond. However, this seems unlikely as age does not seem to impact on the abundance of eMSCs. An alternative scenario is that influx of immune cells, and specifically uterine natural killer cells, during the luteal phase plays a key role in homeostatic balancing of the stromal subpopulations through selective clearance of senescent cells (Hoenicke & Zender 2012). This attractive but as yet unproven scenario would link aberrant immune cell function to subsequent pregnancy failure without having to invoke a host-versus-graft response to the invading placental semi-allograft. Finally, it is not beyond the realms of possibility that decidual or trophoblast cues lead to expansion of eMSC population, in parallel with the expansion of the vascular bed. If this is the case, it would explain how the endometrium could self-correct over time and enable recurrent miscarriage patients to achieve a successful pregnancy after consecutive previous failures.

In summary, the discovery that eMSC deficiency is linked to recurrent miscarriage has provided new insights into the mechanisms of aberrant decidualization, lack of embryo selection and pregnancy failure. At the same time, new questions have arisen regarding the mechanisms that control the maintenance of eMSCs from cycle to cycle and from pregnancy to pregnancy. Screening for eMSC deficiency may identify women at risk of recurrent reproductive failure and, conversely,

harnessing the mechanisms that control endometrial stem cell populations may lead to effective interventions that reduce the physical and emotional trauma caused by recurrent miscarriage.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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