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# Use of 'omics for endometrial timing: the cycle moves on

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**ABSTRACT:** For some years, the prospect of precise and personalized timing of the endometrial cycle for optimal embryo replacement has been held out as a potential solution to low implantation rates. It is envisaged that a receptive state can be defined and reached at a predictable time, and embryo replacement performed in synchrony. In the last century, morphological changes characteristic of the mid secretory phase were defined in precisely timed cycles in women of proven fertility, but when deviations from this standardized schedule occur, their significance for implantation has remained uncertain. 'Omics technologies have been widely advocated for staging the endometrial cycle and defining a set of biochemical requirements for implantation, but after two decades of research, improvements to pregnancy rates have not followed, and there is a striking lack of agreement regarding the molecular characterization of the receptive state. Some of the rationale underlying these problems is now emerging with the application of higher-level computational and biological methodology. Here, we consider the challenges of defining an endometrial phenotype that can support implantation and continuing pregnancy. Receptivity may be an emergent trait depending on contributions from multiple proteins that have low pathway connectivity. We recommend that authors choose language which rigorously avoids the implication that protocols for molecular staging of the mid secretory phase inherently identify a state of receptivity to the implanting blastocyst.

**Key words:** endometrium / embryo implantation / receptivity / window / transcriptomics / ERA / genomics / ART / embryo transfer / proteomics

## Evidence for a 'receptive window'

Studies in the mid-20th century (Psychoyos, 1973) unequivocally identified a maternally controlled period during which a blastocyst could implant in rat, mouse, rabbit and other species. In common laboratory rodents, the duration of the so-called 'window of receptivity' is about 24 h, after which the uterus becomes refractory. Some three decades later, after a few years of ART, observations suggested that embryos transferred during a period of 4 days from about 6 days after the LH peak could produce a pregnancy, while those transferred earlier or later could not (Bergh and Navot, 1992). As the human is a menstruating species, the state of refractoriness seen in mouse and rat at the end of the receptive phase is not needed (implanted embryos are lost), so the central hypothesis was born that receptivity is initiated in the endometrium in the mid secretory phase of the cycle, leading to questions as to how such a change may be identified and specified. The success of blastocyst transfer in human laboratory and farm animals suggests, though it does not prove, that receptivity is not dependent on the presence of an embryo.

When timed endometrial biopsies are obtained from women of proven fertility, clear morphological staging can be observed (Li *et al.*, 1988), building on the classical work of Noyes (Noyes *et al.*, 1975), but no clear connection has been identified between any combination of morphological features and pregnancy success either in ART or natural cycles (Creus *et al.*, 2002; Coutifaris *et al.*, 2004). Deviations from the 'normal' timing are common in women (Diaz-Gimeno *et al.*, 2017; Sebastian-Leon *et al.*, 2021; Lipecki *et al.*, 2022), and could arise from variation in the extent and quality of regrowth after menstruation, the kinetics of the response to progesterone or the extent of inflammatory cell infiltration, all potentially linked to genetic effects on cycle length (Laisk *et al.*, 2018), but there is not good agreement about their significance as regards receptivity. Thus even with standardized stimulation protocols, the endometrial response may be more variable in subfertile women than in those of proven fertility. Biologically it is reasonable to postulate that the luminal epithelium (LE) as the site of initial

attachment of the embryo may be crucial in control of early implantation (Aplin and Ruane, 2017), but morphological change is less pronounced in this cell type than in glandular cells (Dockery and Burke, 2008), and the LE is less abundant than other cell populations, so much more remains to be learned about it.

## Hypotheses underpinning 'omics studies of the endometrium

Several generations of 'omics technologies have been brought to bear on the problem of discriminating non-receptive from receptive endometrium, beginning with microarray studies at the beginning of the 21st century (Popovici *et al.*, 2000; Brar *et al.*, 2001). The primary hypothesis has been that progressive change in gene expression as the menstrual cycle progresses should enable a precisely timed, fresh biopsy transcriptome to reveal a reproducible molecular fingerprint of the receptive state (reviewed in Giudice *et al.* (2008)). Studies selected a limited number of informative genes from larger datasets (Diaz-Gimeno *et al.*, 2011; Ruiz-Alonso *et al.*, 2013). Later, RNA sequencing technologies were introduced, giving improved signal range and depth and also including microRNAs (Altmae *et al.*, 2017). Other studies continued to use RT-qPCR with smaller gene panels (Haouzi *et al.*, 2011; Enciso *et al.*, 2018, 2021; Haouzi *et al.*, 2021). Differential gene expression profiling led to panels of up to about 350 genes being identified as informative. A receptive state proteome might achieve the same end (Evans *et al.*, 2020); candidate protein markers have been localized to specific cell populations (Singh and Aplin, 2015) and their variation traced through the cycle, used to build mechanistic hypotheses (Quenby *et al.*, 2002; Aplin and Ruane, 2017; Heng *et al.*, 2021; Zhou *et al.*, 2021) or correlated with pregnancy failure (Germeyer *et al.*, 2014). Methodological advances will allow post-translational modifications to be mapped as well as more data-intensive tissue localization of proteins to be achieved. The uterine secretome (including protein and RNA in released vesicular material) has been accessed by analysis of flushings (Kasvandik *et al.*, 2020; Wang *et al.*, 2020; Giacomini *et al.*, 2021) and again success is reported in detecting progression through the secretory phase. There is a realistic possibility of sampling the fluid in a replacement cycle and correlating it with pregnancy establishment, but it will be challenging to define permissive protein/RNA concentration thresholds.

Studies have usually involved comparing 'normal' mid secretory phase tissue (usually at or near Day 7 after the LH peak, or after 6 days of progesterone exposure in artificial cycles) with tissues predicted not to be in condition to accept an implanting embryo. This 'non-receptive' state in practice has been drawn from several sources: anti-progestin-treated; proliferative phase; early secretory phase; or mid secretory tissue from women suffering recurrent implantation failure. Now, close to two decades after the first publication of a 'receptive' endometrial transcriptomic profile (Horcajadas *et al.*, 2004), there is no convincing evidence that pregnancy rates can be improved with the application of an endometrial gene expression test. Despite this, such tests are widely offered to patients; a commercially marketed endometrial receptivity array (ERA: www.igenomix.com/our-services/era/), which uses an RNAseq platform to measure expression of 238 genes, has been used by tens of thousands of women globally. Some other similar tests have been marketed (reviewed in Franasiak *et al.* (2021), Ben Rafael (2021), Scott (2021)).

When ERA-based staging at different days after the LH peak was compared with a numerically expressed histopathological evaluation in a group of 86 healthy oocyte donors below age 35 (Diaz-Gimeno *et al.*, 2013) the two assays were fully in agreement in samples in which mid secretory phase histology assumed the canonical pattern. However, in 13 biopsies, the ERA result was 'receptive' but at odds with the histopathology. Inspection of the tissue sections revealed features not associated with the normal mid secretory phase, such as prominent subnuclear glycogen masses in gland epithelium and absence of expected oedematous regions in the stroma. These results are not unexpected, since the ERA was designed to detect the gene profile of canonical LH + 7-day tissue architecture. Deviations such as 'advanced stromal maturation' or 'stromal-epithelial asynchrony' are not uncommon (Coutifaris *et al.*, 2004) but have not been addressed in terms of gene expression. Thus, in this design, reliable biomarkers of the mid secretory phase can be identified downstream of the global transcriptomic change that occurs after ovulation under the influence of progesterone (Sebastian-Leon *et al.*, 2021), but inevitably genes are missed that may be important when the trajectory of tissue maturation is non-standard.

We can see that a fundamental problem besetting the field is use of the term 'receptive'. Since it is not accepted practice to collect a mid secretory transcriptome and evaluate implantation success in the same cycle, most studies have sought to describe and evaluate progression through the secretory phase, testing a different hypothesis: that mid secretory tissue has a unique and reproducible gene expression profile. When deviations are detected, attempts are often made to subdivide the mid secretory into maturational substages, with terms such as 'late pre-receptive' and 'post-receptive'. These can arise from the choice of a particular clustering algorithm with or without supervision, coupled with a subjective choice of the number of clusters. In fact, transcriptomic datasets from the endometrial functionalis make a case for continuous development of the secretory endometrial phenotype, such that it is difficult to discern a specific time point at which the system might be switching to a receptive state (Enciso *et al.*, 2021). 'Post-receptivity', if it exists, could only be verified by transferring embryos after prolonged progestin exposure, which would be ethically unacceptable. Last but by no means least, patients are told their 'receptivity' is being assessed, which is misleading.

## What a mid secretory biopsy contains

Biopsies recover a mixture of luminal and glandular epithelium, stromal, vascular, blood and inflammatory cells from the functionalis. Depth, location in the uterine corpus and size all vary. The epithelial response to progesterone is more rapid than in the stroma, so that from an implantation standpoint, a normal LE will be primed for embryo attachment at this time (Aplin and Ruane, 2017). Evidence from gene expression studies suggests that the stroma has begun the programme of gene expression that leads to decidualization (Lucas *et al.*, 2020), including initiation of a stress response and extracellular matrix

remodelling with the appearance of areas of oedema, but these resident cells are not overtly decidualized and have not yet diverged to produce active and acutely senescent subpopulations. Divergence has been postulated to help create the dynamic tissue environment required for implantation; natural killer (NK)-cell infiltration has been initiated by the mid secretory, but this process is dependent on recruitment signals emitted by decidualizing stroma and it is still at an early stage. The NK cells eventually act to clear the senescent decidual subpopulation (Brighton *et al.*, 2017). It has been suggested that the differentiated decidua acts as a biosensor of embryonic viability, with the capacity to support continuing development, or switch to an embryotoxic phenotype (Macklon and Brosens, 2014). However, biosensory capacity arises only after 6–8 days of progesterone exposure (at least *in vitro*) (Teklenburg *et al.*, 2010) and is effected partly by embryonic signalling to the uterine NK-cell population (Kong *et al.*, 2021), so it is not predicted to be an attribute of the mid secretory phase.

## Observations on transcriptomes and proteomes

Naively one might suppose that making many independent measurements of a wide variety of mRNA or protein species might enhance the precision of an estimate of the stage of maturation of the endometrium as it responds acutely to rising progesterone, in comparison with measuring one or a few known biomarkers. However, this is by no means the likely endpoint.

Studies have revealed variation between different tissue samples that are nominally of the same cycle phase, with measurements blurred by high levels of noise. Indeed, the lack of agreement in different transcriptomic studies as to what genes may be informative for mid secretory staging is very striking: 8 transcriptomic profiles published between 2011 and 2018 identify a total of ~300 genes but only about 10% of them appear in more than one panel (Diaz-Gimeno *et al.*, 2021). The same was already evident in the era of microarray transcriptomics (Giudice *et al.*, 2008).

There are several reasons why in a group of tissue biopsies, multiple genes may appear to be differentially expressed with no relevance to receptivity. Expression of some genes may happen not to be tightly controlled in this tissue; variation of complex genetic origin between healthy individual women is suggested by genome wide association studies (GWAS) studies of menstrual cycle length (Laisk *et al.*, 2018); gene expression is affected by factors not related to stage of the cycle—for example, a sub-clinical infection or sterile inflammation. Variable representation of different cell subpopulations is a likely source of apparent variation in gene expression. In some studies, attempts have been made to normalize data, for example based on an estimate of the proportion of epithelial cells, and indeed, these studies confirm that this is one source of transcriptomic variability (Evans *et al.*, 2018; Suhorutshenko *et al.*, 2018). At the same time, genuine variation can be obscured by changes in a given gene that occur in opposing directions in different cell types. Importantly, single-nucleotide polymorphisms in promoter regions can affect gene expression several-fold, as can more distant sequence variants leading to variability, generalized genetically as expression quantitative traits loci (eQTL). Some of these issues were identified in a publication in 2014 that called for improved consistency and rigour in study design and execution (Altmae *et al.*, 2014).

## Meta-approaches to the use of endometrial gene expression for secretory phase staging

Recently, two groups have approached the analysis of gene profiles extracted from tissue biopsies in new ways, using experimental computational methodology to address the complexity that arises from many measurements of mRNA abundance across the genome in sample sets obtained through the implantation phase from diverse populations.

Diaz-Gimeno *et al.* (2021) applied machine learning approaches to test how adding genes progressively to a panel improves the match between molecular dating and days of progesterone exposure in artificial cycle. They compared four datasets from public archives (with low levels of overlap between gene panels, as mentioned above) and also carried out a new clinical study to test their own panel of 310 genes, repeat-reordering the genes through multiple iterations to improve the fit and using a cut-off of 90% on a fidelity scale calibrated against timing from initiation of progesterone. Despite the gene panels being very different to one another, all performed better than random, and the data shows nicely how different gene sets on a scale up to a few hundred can achieve similar results. Starting from their own panel, 73 genes was the minimum number required to achieve 90% prediction confidence, though >80% could be achieved using only 10 genes. Adding genes in random order to the confidence plot eventually allows equal confidence levels to be achieved, as all informative genes are included. Tissues exhibited the expected variation in speed and quality of progesterone response; the authors attempted to cluster their data into four maturational substages; one cluster of tissues with slightly longer progesterone exposure trended towards a later secretory phenotype and showed segregation from the other samples in a principal components analysis.

Lipecki *et al.* (2022) chose a small panel of six gene products, four of which are expressed predominantly in epithelium, from a larger repertoire of published cycle-variable mRNAs, and examined expression in natural cycles relative to the LH peak. Five of these exhibit a 'conventional' pattern of rising progesterone-induced expression, while the sixth declines after ovulation. They derived a gene expression-based progression parameter and compared it with the actual timing of the biopsy, demonstrating anew the variability (usually in the range ±2 days) of the speed of the progesterone response in endometrium from different women, and allowing iterative reprofiling to arrive at a 'corrected' time with advancement through the mid secretory phase as a continuous variable. Significant differences, or shifts in the corrected timing plot, were not detected between control subjects and women suffering recurrent miscarriage or repeated IVF failure. Nor did a history of increasing miscarriages alter the result. As with the whole tissue 'omics studies, while variations between women emerge, the data do not give unambiguous insight into the reasons for defective endometrial function.

Together these studies confirm that progression through the secretory phase can be characterized using a small number of genes and

show that the reason that previous studies have limited agreement regarding gene sets is that numerous different choices can give information of comparable accuracy. Some genes will be weakly controlled or vary in patterns not related to the hormonal cycle, so adding more genes may impair accuracy by increasing biological variability. Conversely, where blocks of genes are coregulated, a greatly reduced number of measurements can be informative (known as 'compressive biology' (Cleary *et al.*, 2017)). Bearing in mind these new approaches, the design of further clinical studies needs careful consideration. The authors have made a strong case not to apply the ERA across the board to ART patients (Ben Rafael, 2021; Riestenberg *et al.*, 2021; Scott, 2021). We also counsel caution because it has been demonstrated repeatedly that tissue not conforming to the canonical pattern of mid secretory gene expression can be receptive (Diaz-Gimeno *et al.*, 2017; Neves *et al.*, 2019; Enciso *et al.*, 2021; Riestenberg *et al.*, 2021).

## scRNAseq

Deriving single-cell transcriptomes allows escape from data bias arising from different proportions of cell types. It is proving useful in characterizing the diverse and fluctuating cell populations present in this dynamic tissue, tracking temporal and ontological relationships between cell types and identifying signalling pathways that are differentially important in subsets. It should not be viewed as a way of validating the full tissue transcriptome; when the same laboratory-derived transcriptomes from mouse uterine tissue and single cells, the emergent data were largely non-overlapping (He *et al.*, 2019; Yang *et al.*, 2021). For example, of 16 'hub' genes with the greatest changes in whole uterus, only *5* are represented at all in the single-cell differential expression profile. Four of these are in T or NK T cells, one in stromal cells; in three of these five genes the direction of change is opposite to what is seen in the whole tissue.

The human single-cell profile confirms the presence of populations well known from earlier morphology studies: ciliated as well as secretory epithelial cells in both the glandular and luminal epithelial populations, stromal cells, vascular endothelial cells and inflammatory cell populations. There is some suggestion that the secretory epithelial cell may undergo a discrete phenotypic transition that coincides with onset of the receptive period (Wang *et al.*, 2020). RNA velocity (La Manno *et al.*, 2018), which tracks processing of pre-mRNA to mature mRNA, thus identifying a direction of transcriptomic change, can be added to assist the interpretation of relationships between cell populations. It should be noted however that a new source of technical error arises: tissue-resident cells alter their gene expression quite rapidly when removed from the normal *in vivo* environment. In general, some hours will often have elapsed between biopsy collection and lysis of the final purified cell populations, allowing for phenotypic drift and even anoikis. To combat this, scRNAseq can be coupled with immunohistochemisty or spatial transcriptomics in tissue, as for example in the identification of Notch and Wnt activity in ciliated and secretory epithelial populations respectively (Garcia-Alonso *et al.*, 2021a). However, scRNAseq reveals new challenges that will have to be met in defining the 'normal' mid secretory fingerprint. Strikingly different cell clustering patterns are evident in nominally similarly staged biopsies (though so far without detailed clinical information) (Garcia-Alonso *et al.*, 2021b). We do not know what the significance for receptivity may be of variation in the ratios of ciliated, non-ciliated and secretory cells in the endometrial epithelium. Similarly, complexity is increasingly being documented in stroma, where stem cells and senescent cell populations cohabit with fluctuating immune and vascular populations.

## Endometrial receptivity as an emergent trait

In genetics, a complex trait arises when many different genes each make a small contribution to an emergent phenotype (Goldstein, 2009). For example, one can predict the height a child will reach with reasonable accuracy based on the heights of their parents, but this cannot be done so readily from gene expression studies. Could this be the case with the receptive endometrium? There is evidence that eQTLs affect endometrial gene expression in a cycle-dependent way (Fung *et al.*, 2017), and the development of methodology to estimate a polygenic risk score for a set trait (Lu *et al.*, 2021; Polygenic Risk Score Task Force of the International Common Disease Alliance, 2021) offers promise of progress in identifying the genetic basis of reproductive phenotypes. Omnigenic theory (arising from GWAS studies of disease) (Boyle *et al.*, 2017) suggests that associations can be spread widely through the genome, including genes that lie outside core disease-related (or as here, phenotype-related) signalling pathways. This might go some way to explaining why so few consistent connecting mechanistic threads have been gleaned from mid secretory-enriched gene panels. It does not exclude the possibility that transcriptomic biomarkers may be useful (Stevens *et al.*, 2021), but rather presents a challenge in terms of characterizing a receptive state that depends on the embryo-endometrial discourse, even when timely and precise gene expression profiles are achieved.

## Future/conclusions

Epithelial (luminal and glandular), stromal, immune and vascular aspects of receptivity play out along a temporal axis in early pregnancy, and engagement between the conceptus and these different maternal cellular compartments occurs in specific time windows, varying in length. This in effect creates a series of checkpoints, as implantation is followed by the establishment of trophoblast lineages, decidualization, influx of inflammatory cells, invasion of extravillous trophoblast and the onset of local and uterine (organ-level) vascular remodelling (Aplin *et al.*, 2020). Passing the endocrine checkpoint at 8 weeks requires preceding events to have culminated in development of syncytiotrophoblast sufficient to supply steroid hormones, as systemic requirements overtake the capacity of the corpus luteum.

Much work has gone into the creation of transcript and protein expression libraries representative of mid secretory endometrium in which sampling variation, 'true' biological variation and pathology come together to create diverse data characteristics in which functionally important products may be discovered, but others may be obscured or overlooked. Though the possibility has been mooted of altering ('personalizing') the timing of embryo replacement when a skewed profile suggests delayed tissue maturation, the consequences

for pregnancy outcome (if any) remain to be established. Criteria of increased stringency should be applied to the definition of a functional mid secretory phase; for example, the ability to accept a high-quality donor embryo rather than the assumption of normal fertility sometime (perhaps years) after a successful pregnancy was achieved.

Given new data emerging from single-cell analysis, it may be that a holistic cell-centred approach to preparing the endometrium for subsequent replacement cycles could be more effective, given the potential for improved post-menstrual regeneration, including the recruitment of stem cell populations into the new tissue (Tewary *et al.*, 2020; Salamonsen *et al.*, 2021). The use of blastoids (Kagawa *et al.*, 2022; Yanagida *et al.*, 2021; Yu *et al.*, 2021) or embryos (Rawlings *et al.*, 2021) to 'probe' (implant into) 3D models of endometrium *in vitro* has the potential to add mechanistic information at a cellular and molecular level to deepen our understanding of implantation in ways that will never be possible with 'omics. Even so, the demands of modelling the expanding implantation site through to later times at which pregnancy loss is still common are steep and ethically challenging.

Even with high-order analytical tools and the guarantee of a viable transferred embryo, there must be doubt as to whether a single mid secretory biopsy taken in a previous non-conception cycle can attain the predictive capacity not only to improve the likelihood of implantation but also of a viable ongoing pregnancy. This supports the use of single embryo transfer to maximize chances of pregnancy establishment. Finally, to avoid further confusion we propose that the terms 'receptivity' and 'window of implantation' should be confined to studies that have yielded observations involving well-characterized embryos implanting *in vivo*.

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J.D.A. conceived the piece based on discussion with A.S. J.D.A. made the initial draft, A.S. reviewed and added to it. Both authors approved the final version.

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The authors have no conflict of interest to declare.

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