Placenta 168 (2025) 98–110

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

An overview of periconceptional pathology and pathophysiology: Where do we go next?

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ARTICLE INFO

Keywords:
Assisted reproductive technology
Corpus luteum
Decidua
Imprinting
Periconceptional
Uterine natural killer cells

ABSTRACT

It is becoming increasingly evident that studies of pregnancy and pregnancy complications must begin earlier in pregnancy than has typically been the case. Important events with effects through and beyond pregnancy take place in the periconceptional period, from shortly before to shortly after conception. During this time ovarian hormones act not only to facilitate successful implantation but also direct maternal physiological adaptations necessary for pregnancy. Decidua also undergoes changes necessary for successful implantation of the embryo. These involve not only maternal preparation of a receptive site for implantation but interactions, including immunological and others, of cells of the maternal decidua and embryo. All of this occurs in a setting in which the cells of the embryo are involved in complex epigenetic changes. Assisted reproductive technology (ART) has the potential to influence many of these since ART typically occurs at this time of these epigenetic change. Many of the variables involved in ART have the potential to influence epigenetic adaptation. In fall of 2023 the Global Pregnancy Collaboration brought together experts in these areas to consider the status of our understanding of the periconceptional period. This manuscript presents an update of this information including new approaches to study these very early events. The potential impact of ART on periconceptional events and the mechanisms involved are presented. Based upon this information recommendations are made for future research in these important areas.

1. Introduction

Pregnancy research is challenged by the complexity of the interaction of three environments, maternal, fetal, and external, and two genomes, mother and infant. An additional challenge is temporal. Pregnancy is dynamic and studies at only one or a few times in pregnancy limit our understanding of the complex longitudinal adaptations of pregnancy. Additionally, and the topic of this manuscript, another vastly understudied epoch essential to understanding pregnancy and in particular adverse pregnancy outcomes is the periconceptional period. The periconceptional period, the time span just prior to conception and encompassing the first few weeks of pregnancy, is becoming increasingly evident as a time of vital importance. Local preparation for successful implantation of the fertilized ovum, interactions, immunological and others, converging on implantation, and mechanisms of implantation must all be in sync for a successful pregnancy. Also, adaptations of maternal physiology to successfully support pregnancy must occur, beginning early in pregnancy. In this manuscript, we overview and update the state of available information on the periconceptional events influencing pregnancy outcome reviewed at a Workshop of the Global Pregnancy Collaboration in September 2023. We highlight opportunities for study and the importance of considering the periconceptional period in our effort to foster normal pregnancy outcomes.

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https://doi.org/10.1016/j.placenta.2025.06.010
Received 1 April 2025; Received in revised form 6 June 2025; Accepted 10 June 2025
Available online 11 June 2025

Abbreviations

CVS	chorionic villus sampling
ER	endoplasmic reticulum
EVT	extravillous trophoblast
FET	frozen embryo transfer
FGR	fetal growth restriction
ICSI	Intracytoplasmic plasmic sperm injection
IVF	*in vitro* fertilization
LIF	Leukemia Inhibitory Factor
NP	pregnancies with normal outcome
NP-CVS	CVS from normal pregnancies
PE	preeclampsia
uNK	uterine natural killer cells

2. Periconceptional adaptations

2.1. Decidualization

Dr. Kirk Conrad encouraged the consideration of not only the "seed", the fertilized ovum, but also the "soil", the decidua, modified endometrium of pregnancy, when considering the genesis of normal and abnormal pregnancies. Not only is this a rational "closing of the loop" but it provides a potential target for factors of established pre-pregnancy risks such as obesity and diabetes mellitus (Fig. 1).

Decidual modification begins in the secretory phase of the menstrual cycle (pre-decidualization) including changes in immune cells, glandular epithelium, and spiral arteries, which continue after implantation (decidualization). These changes are necessary for appropriate immunological interactions between the maternal uterus and fetoplacental semi-allograft, maturation of endometrial glands for histotrophic nutrition prior to the onset of intervillous blood flow, and ultimately, increased oxygen and nutrient delivery to the placenta and fetus in part facilitated by spiral artery remodeling.

Dr. Conrad and his colleagues performed innovative studies to investigate early pregnancy placental and decidual changes in the context of pregnancy outcomes. In collaboration with Allen Hogge MD, he obtained surplus decidual and trophoblastic tissue from women undergoing chorionic villus sampling (CVS), a procedure used for determining genetic normality or abnormality of the fetal genome. One hundred and thirty-two surplus CVS tissues were obtained, of which 4 were from women who in later pregnancy developed preeclampsia (PE) with severe features. In collaboration with Sandra Founds PhD and Arun Jeyabalan MD, global gene expression of CVS procured from these women with PE or pregnancies with normal outcome (NP) were compared. They identified 171 differentially expressed genes. Interestingly, they found no differences in HIF-alpha and oxidative stress related genes which they had predicted would be abnormal based upon findings from placentas delivered at the end of gestation. Intriguingly, several putative decidual genes were downregulated, suggesting decidual dysregulation as an antecedent in preeclampsia [1].

Next, with Maria Belen Rabaglino PhD, Dr. Conrad further explored the idea of decidual dysregulation as a potential cause of preeclampsia [2]. To this end, they utilized an innovative set of *in silico* experiments based on transcriptomic databases available in the public domain to establish a pathway analysis of normal decidualization both in the secretory phase and during early pregnancy, and then overlapped these pathways with those of the CVS tissues.

Specifically, they compared transcriptomic results of the mixed villous/extravillous trophoblast (EVT) and decidua in their CVS tissues with normal cycling endometrium (no EVT, no decidua), endometrium from ectopic, tubal pregnancies (no EVT) and early intrauterine pregnancy prior to elective termination (+EVT, +decidua). In this new bioinformatic analysis, 396 differentially expressed genes were identified between PE- and CVS from normal pregnancies (NP-CVS) [2]. One hundred and fifty-four of these 396 differentially expressed genes were associated with various stages of endometrial maturation. In turn, 112 of these from PE manifested changes opposite in direction to those with normal endometrial maturation, suggesting impaired predecidual

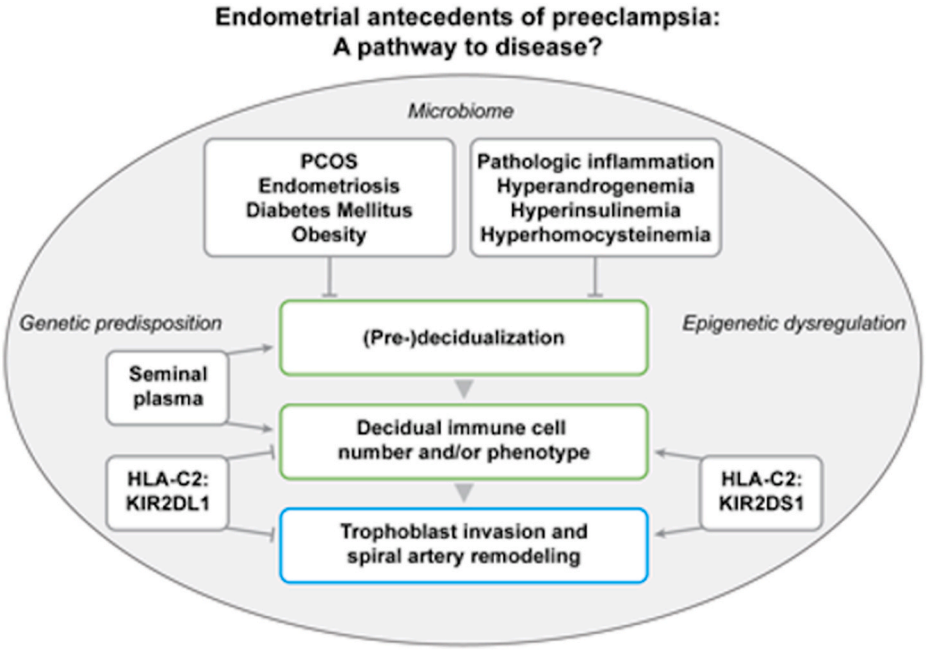


Fig. 1. Proposed Endometrial Antecedents of Preeclampsia. Polycystic ovarian syndrome, endometriosis, diabetes mellitus and obesity impair decidualization and increase PE risk. Normal decidualization is proposed to be regulated epigenetically by interactions of decidual natural killer cell KIR and trophoblast HLA-C ligands. The stimulation or inhibition of trophoblast by these antigen interactions influences PE risk. Seminal plasma may be protective through recruitment of T regulatory cells into the endometrium. An endometrial microbiome may contribute to the regulation of decidualization and its dysregulation to impaired decidualization (Conrad et al.[3] with permission).

maturation in PE. One hundred and sixteen of the 154 differentially expressed genes overlapped with differentially expressed genes in settings with no EVTs supporting primary defects in predecidualization. Relevant to early immunological aberrations of PE, 16 and 25 genes normally upregulated in decidual natural killer cells and decidual macrophages, respectively, were down regulated in the CVS samples from preeclamptic individuals [2,3].

The concept of dysregulated decidualization was supported by studies of Garrido-Gomez et al. [4] These investigators examined mid-secretory endometrial biopsies from women with a history of preeclampsia with severe features and women with previously normal pregnancies. They isolated and cultured differentiated endometrial stromal cells from these samples and induced them to decidualize *in vitro*. Decidualization of endometrial stromal cells from women with prior preeclampsia was markedly reduced compared to those from women with normal pregnancy outcome. Significant overlap, including directionality, was also seen when comparing differentially expressed genes from PE-vs NP-CVS with differentially expressed genes between endometrial stromal cells isolated, cultured and decidualized *in vitro* from mid-secretory endometrial tissue of women with prior preeclampsia with severe features compared with similarly treated samples from women with previous normal pregnancy (*vide supra*). [5] Finally, Moran et al. presented intriguing data showing that, relative to women with prior normal pregnancy, serum obtained in the mid-secretory phase from women with a history of preeclampsia with severe features impaired decidualization of endometrial stromal cells in culture. These findings suggest that, in addition to intrinsic endometrial dysfunction, circulating factors in women with a history of preeclampsia may further impair endometrial maturation in the secretory phase [6].

Another approach to studying differences in decidualization is by obtaining endometrial samples at early pregnancy termination (9–14 weeks) with concomitant uterine artery Doppler examination. In such investigations, subjects with Doppler changes associated with an increased risk of preeclampsia in later pregnancy were more likely to exhibit changes consistent with impaired decidual natural killer cell function and decidualization [7]. Of course, pregnancy termination precluded definitive knowledge of pregnancy outcome in this study. Finally, in 6 studies, circulating IGFBP-1 was decreased in early gestation in women who later developed preeclampsia [3]. Insofar as circulating IGFBP-1 in early pregnancy is a biomarker of decidualization, these results are also consistent with the concept of decidual dysregulation in the genesis of PE.

The group also asked if the CVS genes that are differentially expressed in women who developed PE from those who experienced normal pregnancy, overlapped with genes differentially expressed between women with classic endometrial pathologies relative to their respective controls [5]. They analyzed transcriptomic databases available in the public domain of mid-secretory endometrial samples from women with recurrent implantation failure, recurrent miscarriages and the respective controls. They also examined the transcriptomics of endometrial stromal cells isolated from ovarian endometriomas and normal endometrial stroma that were decidualized *in vitro*. When comparing differentially expressed genes between PE- and NP-CVS with these endometrial pathologies and their respective controls, there was significant overlap of differentially expressed genes in the same, but not opposite direction suggesting common molecular pathways.

The interpretation of the data suggests that at least for some women who develop PE there may be a primary defect in predecidualization. However, the concept of endometrial origins of PE is in its infancy and requires further investigation. Finally, Dr. Conrad once again cautioned investigators about information from differentially expressed genes in tissues obtained at delivery, citing their findings and those of others that there is little or no molecular overlap with results obtained in early pregnancy of women who subsequently developed preeclampsia or secretory endometrium of women with a history of severe preeclampsia [3,5,8]. Thus, designing interventions to mitigate the molecular dysregulation of preeclampsia in the secretory endometrium or decidua of early pregnancy based on the molecular pathology of delivered tissues is likely to be misleading, and unlikely to yield preventative or early corrective measures.

2.2. Corpus Luteal function

The initial support and direction for pregnancy maintenance and adaptation beginning in the secretory phase and continuing after conception during the first trimester largely comes from the corpus luteum. Dr. Conrad and colleagues took advantage of variations in the methodologies of uterine preparation for embryo transfer after in vitro fertilization (IVF) to examine the potential role of factors secreted by the corpus luteum such as relaxin in maternal cardiovascular adaptations to pregnancy [9]. Relaxin is a peptide hormone that is a vasodilator and increases arterial compliance [10].

There are several approaches to prepare the endometrium for frozen embryo transfer. To a certain extent they provide a dose response of corpora luteal hormone production and maternal circulating concentration. In one approach using “programmed” cycles, endometrial development is achieved through exogenous administration of estrogen and progesterone, and in most cases folliculogenesis is inhibited. Consequently, a corpus luteum does not develop, and hence, circulating relaxin is undetectable throughout pregnancy. In a natural cycle, there is typically one corpus luteum resulting in normal plasma relaxin concentration. “Controlled ovarian stimulation” which uses exogenous gonadotrophin manipulation to stimulate the development of multiple follicles is used in fresh embryo transfer leads to multiple corpora lutea, and increased relaxin [11].

This “dose response” was used to assess the implications of corpora luteal function on pregnancy cardiovascular adaptation [12]. In the endometrial preparations yielding no corpora lutea and undetectable maternal circulating relaxin, there was generalized cardiovascular dysregulation (hypodynamic circulation). In the first trimester, there was attenuation of gestational increases in cardiac output and central aortic compliance, and decreases in systemic vascular resistance, as well as subdued pregnancy changes in left atrial dimensions and mitral E wave velocity. As was predicted, these deficits were largely restored after the first trimester associated with placental maturation and secretion of hormones, such as placental growth factor, a potent vasodilator. Interestingly, despite the increased relaxin concentrations after ovarian hyperstimulation associated with multiple corpora lutea, the maternal cardiovascular system was normal and not “hyperdynamic” [12].

In studies with Dr. Conrad’s colleague, Valerie Baker MD, they attempted to relate this dose response to the clinical outcome, preeclampsia. Importantly, they also needed to keep in mind the known impact of using embryos that were frozen compared to those freshly obtained. Several prior studies had found an increase of preeclampsia with frozen vs fresh embryos, and the freezing procedure was implicated in the increased risk for PE. However, the status of the corpus luteum (and endometrium) was not taken into consideration in these earlier reports [13]. Comparing “programmed cycles” in which folliculogenesis is inhibited, with natural (or mildly stimulated) cycles for frozen embryo transfer, the programmed cycle was found to be specifically associated with the majority of increased PE risk [14]. There was no increased PE risk after controlled ovarian stimulation and transfer of fresh autologous embryos. In their study and others in the literature, all or most of the cases of PE were late onset or term but there is currently not sufficient data to state that this is a true association [15].

Retrospective and prospective observational studies also indicate associations of IVF with increased risk for “idiopathic” placenta accreta (unassociated with prior cesarian section or concurrent placenta previa), postpartum hemorrhage consistent with morbidly adherence placenta, symmetrical fetal overgrowth and larger placentas, as well as post term birth, again most frequently observed after programmed cycles, in which there was no corpus luteum, the potential for suboptimal

endometrial preparation, and many reports of a thinner endometrium [15,16].

Dr. Conrad theorized that the effects of dysregulated decidualization and systemic vascular effects are related either in origin or impact. Relaxin, for example, is not only a potent vasodilator and modifier of arterial compliance, but a known stimulus to decidualization [17]. Furthermore, in women receiving the preparative doses that resulted in no corpora lutea there was a subset of women who nonetheless ovulated. Preeclampsia occurred in 1.6 % of the women who ovulated and 15.3 % in those who did not [18]. The results of this provocative, but small study, support a role for the lack of corpus luteum hormones contributing to preeclampsia. However, in the same study the frequency of large infants was increased in the women who ovulated, 28.6 % vs. 10.7 %, thus disassociating the increased risk for PE and fetal overgrowth. (It should be noted, however, that in the numerous observational studies supporting an increased risk for the several pathologies in programmed cycles, including PE and fetal overgrowth as mentioned above, it was not specified whether there was significant overlap of these pathologies within subjects.)

Dr. Conrad presented a theoretical model to attempt to unify these results assuming common rather than distinct mechanisms (Fig. 2). For further details, the reader is referred to Reference. [16].

The currently available information suggests that in the majority of women expected to conceive using (modified) natural or mildly stimulated frozen embryo transfer (FET) cycles, these protocols may be preferable to mitigate the pathological maternal and neonatal pregnancy outcomes associated with "programmed" FET cycles. Moreover, clinical, ongoing pregnancy and live births rates may be higher, and early pregnancy loss lower in (modified) natural relative to programmed FET cycles [19], although not all agree [20].

2.3. The trophoblast-endometrial dialogue during implantation and early pregnancy development

Dr. Graham Burton addressed our increasing understanding of the steps by which the blastocyst attaches and implants into a receptive endometrium. He highlighted the extensive cross-talk between the endometrium and blastocyst by hormones, cytokines, miRNAs and extracellular vesicles [21]. Many factors have been implicated in regulating implantation but knock-out studies in the mouse have so far shown only Leukemia Inhibitory Factor (LIF) to be absolutely essential [22]. Mice lacking a functional LIF gene are fertile but their blastocysts fail to implant, although they are capable of doing so if transferred to primed wild-type recipient.

The interactions of the endometrium and blastocyst are similar to those between neutrophils and the endothelium during extravasation and involve three steps: apposition, attachment and invasion. Mucins present on the endometrial surface are key regulators. Usually, they prevent the attachment of pathogens and must be removed for implantation to occur. In some species there is a generalized shedding of all mucins but in the human there appears to be a localized loss at the contact point of the endometrium and blastocyst. This enables binding of L-selectin on the trophectoderm and glycosylated ligands on the luminal epithelium. Specific glycosylation of the endometrial proteins is increased by IL1β released from the trophectoderm, decidua, and maternal immune cells and by LIF secreted from endometrial glands in response to estrogen resulting in increased blastocyst binding [23]. Invasion follows attachment with the earliest finding of a partially embedded human conceptus 1 day after implantation. Importantly, the syncytiotrophoblast is close to, and breaks into, endometrial glands which provide essential histotrophic nutrition.

During the first trimester, when the spiral arteries are plugged by

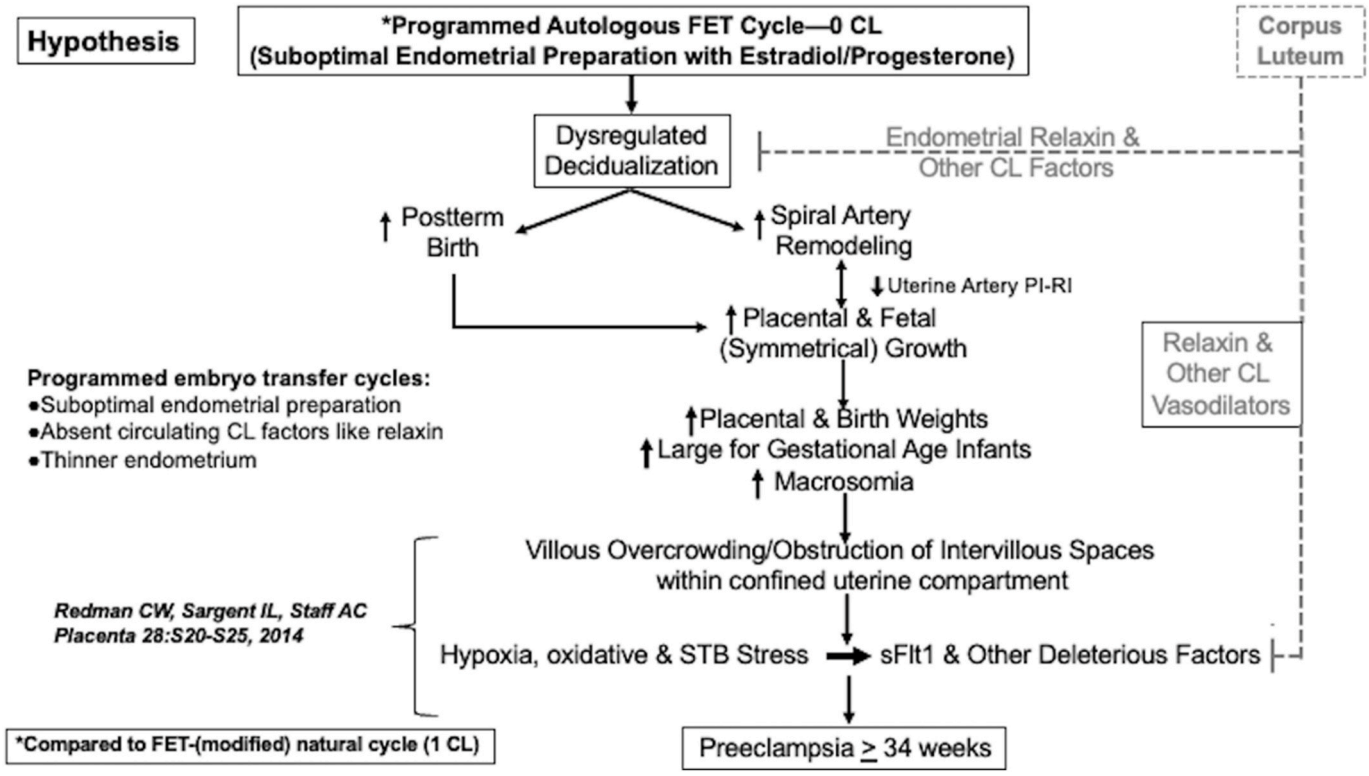


Fig. 2. Corpus Luteal and Endometrial Antecedents of Adverse Pregnancy Outcomes in Autologous Embryo Transfer
Compared to natural embryo transfer cycles with 1 corpus luteum, endometrial preparation with estradiol and progesterone may not be optimal in programmed cycles without a corpus luteum. Spontaneous ovulation, corpus luteum formation and release of factors like relaxin may contribute to reducing preeclampsia risk in programmed cycles. (light, dashed lines). FET, frozen embryo transfer; CL, corpus luteum; PI, pulsatility index; RI, resistance index; STB, syncytiotrophoblast; sFlt1, soluble fms-like tyrosine kinase 1 (Conrad et al.[15] with permission).

extravillous trophoblast as part of their remodeling, histotrophic nutrition is the major source of nourishment for the blastocyst. Histotroph provides plentiful lipids and glucose, and also importantly growth factors such as EGF, VEGF, and pathology [24]. These factors stimulate trophoblast proliferation and differentiation *in vitro* [25,26]. Studies in domestic species demonstrate that expression of these growth factors in the glands is upregulated in early pregnancy in response to trophoblastic hormones, particularly prolactin [27–29].This signaling loop creates a feed-forward drive that stimulates placental development. Dr. Burton speculated that an equivalent loop occurs in the human (Fig. 3), except that prolactin is secreted by decidual cells rather than the trophoblast [30,31]. This difference emphasizes the importance of correct decidualization during early pregnancy.

Progress in our understanding of the early interactions between the endometrium and blastocyst and their significance has been hindered by the fact that it is not possible to relate information from material from early pregnancy, usually obtained at pregnancy termination, with the progression of pregnancy to outcome. Recent innovative strategies have provided approaches to begin to overcome these shortcomings. Organoids of endometrial glands can be prepared from endometrial or scratch biopsies before implantation or noninvasively by collection of menstrual blood [32,33]. Such organoids display equivalent responses to early pregnancy hormones, including prolactin, as seen in domestic species. They should permit the full composition of the histotroph to be determined, and how it changes during early pregnancy. Equally, organoids of human trophoblast have also been derived [34,35], including from CVS samples for which pregnancy outcome is known [36]. This enables the effects of histotroph on trophoblast in healthy and complicated pregnancies to be tested systematically. Importantly, the biological activities of growth factors, such as VEGF, are inhibited by aberrant glycosylation secondary to endoplasmic reticulum (ER) stress [37], a feature of the pathophysiology of preeclampsia and fetal growth restriction [38]. Pilot data from Dr Cindrova-Davies in Dr Burton's group showed that endometrial scratch biopsies from women undergoing ART treatment displayed ER stress that correlated with early loss. Moreover, it was possible to therapeutically reverse this stress in the organoids. These data are preliminary but nonetheless indicate that preconceptional stress can be detected using organoid cultures, that this is associated with adverse outcomes, and may be reversible therapeutically.

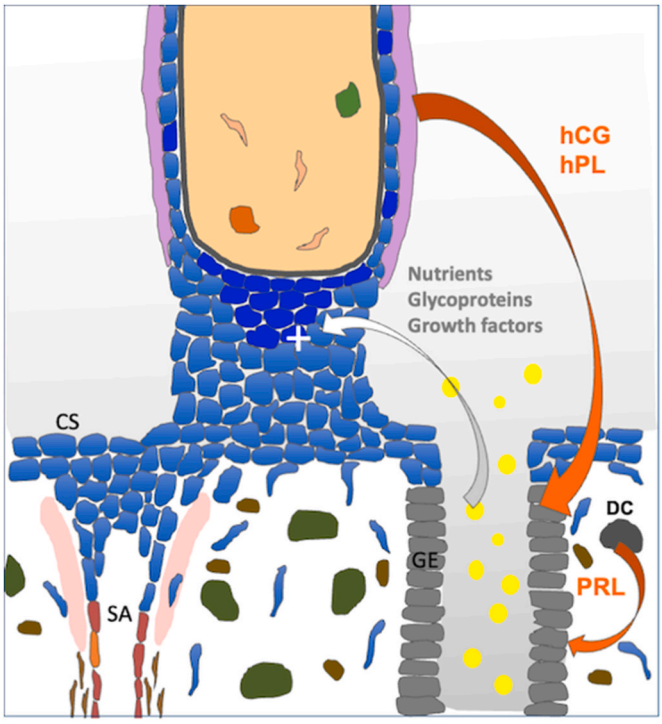


Fig. 3. Trophoblast-endometrial dialogue speculated to stimulate early placental development. Trophoblast hormones, including chorionic gonadotropin (hCG) and placental lactogen (hPL) along with prolactin (PRL) secreted by decidual cells (DC) stimulate gland epithelial cells (GE) to upregulate nutrients, including glucose, lipid droplets (yellow) and glycoproteins, and growth factors that feedback on the trophoblast and promote proliferation (+). CS, cytotrophoblastic shell; SA, spiral arteries. (Burton and Jauniaux[25] with permission).

In summary, our knowledge of the early interactions of blastocyst and endometrium indicate a two-way communication that leads to changes in receptivity and attachment and stimulates placental development. The role of many potential factors has not been delineated but appropriate glycosylation appears to be important [39]. Endometrial glands provide nutrients and stimulatory growth factors to facilitate implantation and promote trophoblast proliferation and differentiation. Early research with endometrial organoids derived from menstrual blood indicates that they are responsive to maternal and placental hormones and faithfully replicate *in vivo* endometrial gland pathology [33]. The combination of endometrial and trophoblast organoids has the potential to increase our understanding of the importance of gland function in normal and abnormal pregnancies, and to allow diagnosis of abnormal implantation and direct potential therapies.

2.4. Immunology of placentation

The genetic and consequent immunological differences between mother and her infant, which converge at the intimate contact of the placenta and uterus have been a topic of intense study. Dr. Ashley Moffett presented her findings on the immunology of placentation and also described techniques that will facilitate future mechanistic studies. Interactions between uterine immune cells and the implanting placenta are likely to be relevant to many disorders of placentation, from recurrent miscarriage to unexplained stillbirth, preeclampsia and fetal growth restriction (FGR) [40].

Although the outcome of this interaction is frequently compared to an allograft, there are fundamental differences. It is better to view the dialogue between maternal and fetal cells in early decidua as a physiological "balancing act", where a delicate boundary is established between two individuals. Placental cell invasion must be neither too deep nor too shallow, a relationship orchestrated by decidua, as seen when the placenta invades too deeply when decidua is absent. Such a relationship is indicated by the fact that placentally-mediated preeclampsia seems to be determined by both maternal and paternal genes.

Distinctive immune cells present in the decidua, once known as endometrial granulocytes, but now defined as uterine natural killer cells (uNK), are involved in this process. Uterine natural killer cells make up 70 % of leukocytes in the decidua, are hormonally regulated, and are temporally and spatially associated with implantation. Despite their name they are very poor at killing [41]. They are a rich source of cytokines (CSF-1 GM-CSF) and express receptors that recognize HLA-E, -C and -G, ligands on the invading trophoblast cells. Two of the interacting pairs, paternal HLA-C on trophoblast and maternal KIR on uNK, are especially relevant because they are both highly polymorphic raising the question: are there maternal KIR/fetal HLA-C combinations associated with pregnancy disorders? HLA-C alleles can all be divided into two groups, C1+HLA-C and C2+HLA-C. C1+HLA-C allotypes bind weakly to the inhibitory KIR2DL3/2DL2 [42]. However, the C2+HLA-C allotypes are strongly inhibited by the KIR2DL1 and this can be counteracted by an activating receptor KIR2DS1 (Fig. 4).

In a UK population, about 30 % of men with European ancestry have a C2+ HLA-C allele [43]. Women whose babies have a paternally derived C2+ HLA-C allele are at increased risk for preeclampsia unless the mother also has KIR2DS1 (present in ~40 % of women) [43]. Interestingly, in other populations activating and inhibitory KIRs may

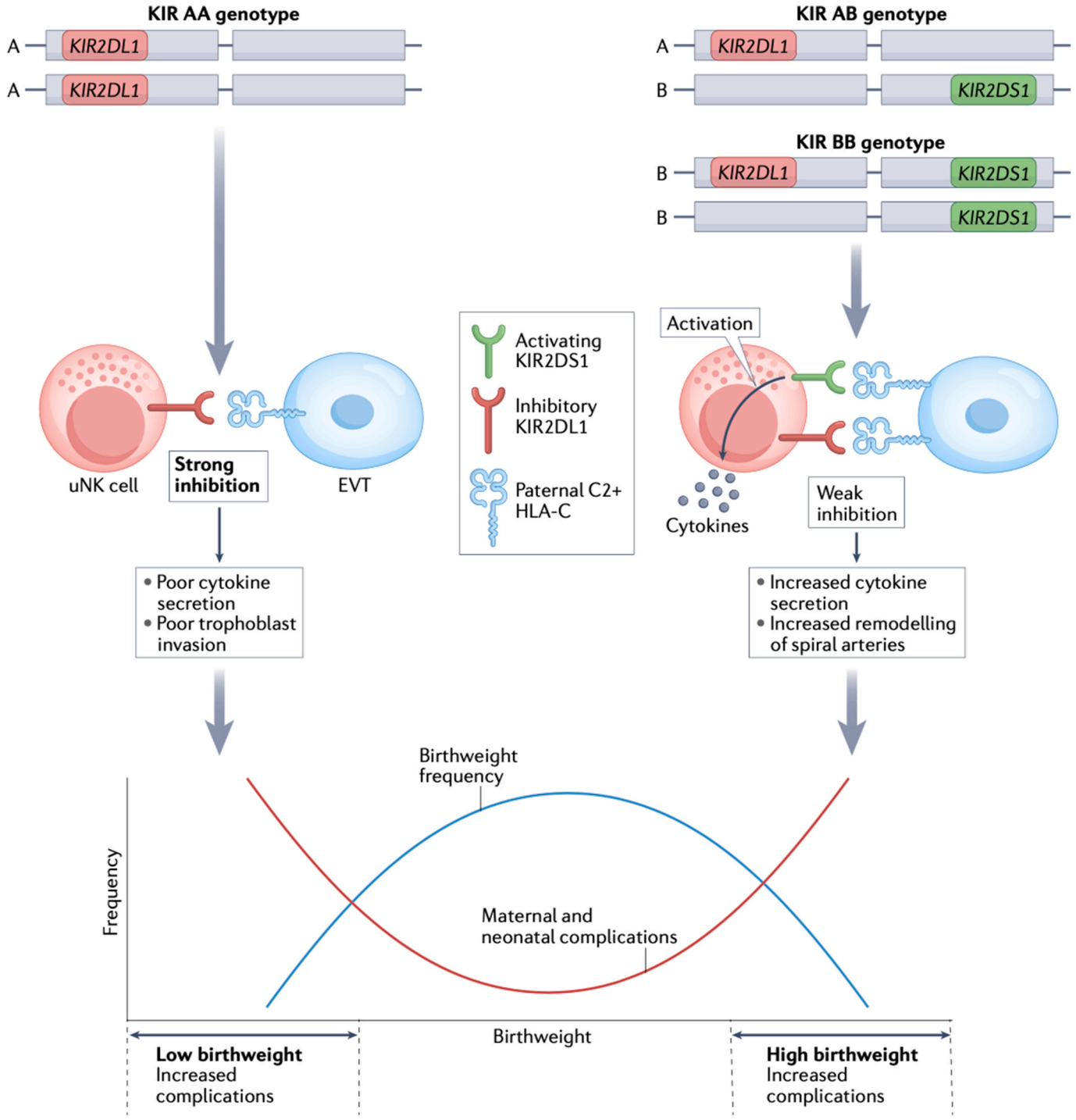


Fig. 4. The effect of different combinations of maternal KIRs and paternal HLA variants on pregnancy outcome.
Maternal killer cell immunoglobulin-like receptor (KIR) AA haplotypes, which encode two copies of KIR2DL1 (a potent inhibitory receptor for C2+ HLA- C epitopes), result in strong inhibition of uterine natural killer (uNK) cells when C2+ HLA- C is inherited paternally and expressed on extravillous trophoblast (EVT). This is associated with low birthweight, increased risk of pre-eclampsia and recurrent miscarriage, probably secondary to reduced remodeling of the maternal vasculature and poor placentation. The presence of the activating KIR KIR2DS1, encoded in women with KIR AB or BB haplotypes, in combination with paternal C2+ HLA- C results in less uNK cell inhibition and increased secretion of cytokines such as CCL4, XCL1 and GM- CSF. This is associated with a greater frequency of large for gestational age infants. Increased birthweight is associated with maternal and neonatal complications, including dysfunctional labor, shoulder dystocia, maternal trauma and post-partum hemorrhage. Human birthweight is thus an example of balancing selection partially dependent on KIR and HLA gene families (Moffett et al.[34] with permission).

vary but similar relationships to pregnancy outcome are manifest with combinations of HLA-C and activating or inhibiting KIR.

This raises the question, what is the role of uNK cells? One emerging important role is to contribute to the decidual anti-inflammatory and T cell immunosuppressive environment. However, after binding to trophoblast how do uNK cells affect its invasive behavior? It is now possible to test this because trophoblast cells can also be cultured as organoids [34,35]. These organoids differentiate into villous trophoblast

secreting hormones such as hCG and can be stimulated to differentiate to invasive extravillous trophoblast. When compared with samples of full thickness uterus, single cell transcriptomic analysis reveals, within the organoids, all forms of extravillous trophoblast lineages other than endovascular trophoblast and placental bed giant cells.

These cells can now be used experimentally to serve as targets for uNK cells. Unfortunately, it is not yet possible to culture organoids together with uNK cells. A reductionist approach is to expose trophoblast organoids to cytokines, whose secretion is increased after stimulation of uNK through KIR2DS1. The results are an enhancement of extravillous trophoblast differentiation toward more invasive forms and increased expression of genes with functions relevant to invasion and associated with preeclampsia and FGR [44].

Dr. Moffett then addressed some of the "dogmas" of placentation. Although it is frequently stated that inflammation is a major component of implantation, there are many mechanisms in place to avoid this. In addition, there is still no convincing evidence that T cells have a major role in preeclampsia.

Information on the complex interactions between the placenta and uterus is beginning to be unraveled. The use of trophoblast organoids to explore this will inform potential therapeutic interventions.

2.5. Infertility and assisted reproduction: influences on pregnancy outcomes

The first infant conceived ex utero was born in 1978. The introduction of this first step in ART was followed by extensive approaches providing methods that have resulted in 10 million pregnancies and accounts for up to 6 % of pregnancies in countries in which ART is practiced [45]. As Dr. Conrad pointed out, this is accomplished with various hormonal manipulations to recover more oocytes, and with fresh and frozen embryos. In addition, other variables involved in culturing and maintaining early embryos are utilized including culture media, oxygen content and numerous others. One of the most invasive of these is Intracytoplasmic sperm injection (ICSI), which involves injecting an entire sperm into an ovum for fertilization. This was originally developed for settings in which there were very few sperm available but is now the most common form of *in vitro* fertilization, accounting for 65 % of ART cycles worldwide [45]. A second very common variable is the use of frozen embryos, which now accounts for 50 % of ART infants in the US [45]. Also, with advanced maternal age, there is often a need for donor oocytes, which accounts for 7.3 % of cycles in the US [45]. There is remarkably little data on the impact of these variables.

Fig. 5 shows the rise in the use of IVF in the USA during 1997–2020. Over this time period there has been a 4.5-fold increase in the number of IVF cycles, a 4.4-fold increase in the number of deliveries, and a 3.25-fold increase in the number of liveborn infants. Currently, 38 % of all IVF cycles are freeze-all, with no intent to transfer, and 62 % are fresh IVF cycles [46].

It is becoming evident that the various approaches to ART are not without risk. Dr. Barbara Luke reviewed the risks associated with ART. One problem in assessing the risk of ART is that subfertility which leads to the use of ART is itself associated with increased adverse pregnancy outcomes. In studies in which ART results have as their controls non-ART offspring of subfetile pregnancies there is this combined effect, but the influence of ART remains. Another consideration is the implantation of more than one embryo. Although it is well recognized that implantation of multiple embryos is associated with increased maternal and perinatal morbidity from multiple pregnancies, only 40 % of ART cycles are with just one embryo [47].

With these considerations, there is a 1.5-to-2-fold increased risk for preterm birth, preeclampsia, low birth weight, small for gestational age, stillbirth and perinatal mortality, and placental pathology with ART, particularly with multiple embryos transferred resulting in singleton births (vanishing twins) [48,49]. Many reports of other possible long term adverse effects, neurological, birth defects, malignancies, respiratory disorders, and others are inconsistent and can be related to subfertility or pregnancies with multiples. However, an increased risk of

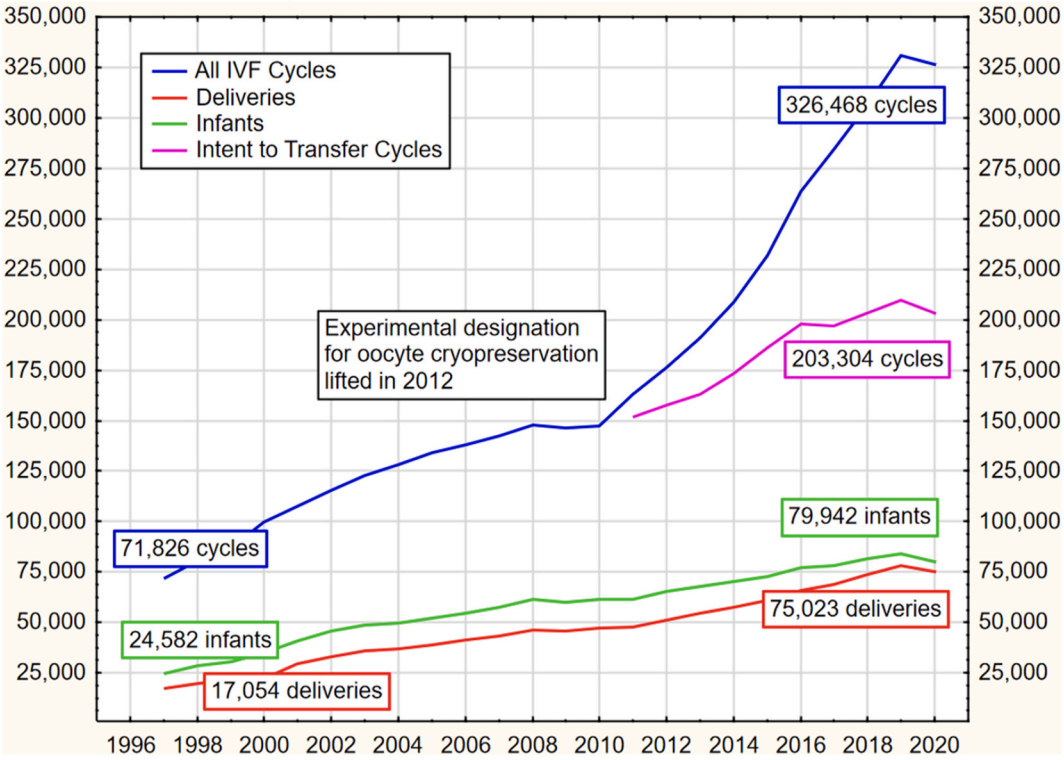


Fig. 5. Increased and changing pattern of Assissted Reproductive Technology. The use of ART (number of cycles) has increaased more than 4 times since 2007 accellerating with approval of clinical usage of frozen embryos. The reduction in the use of multiple embryos is indicated by the reduction of births per delivery from an average 1.4 in 1997 to 1.05 in 2020.

cardiovascular disease in later life is a feature of ART that does not seem to be explained by these variables [50]. In addition, there is an increase of epigenetic alterations in ART pregnancies that is not accounted for by accompanying subfertility. This is likely the explanation for an almost 4-fold increase in abnormalities involving imprinted genes with ART [51].

The mother even without multiple embryo transfer is at increased risk for bleeding, pregnancy hypertension, placenta previa and abruption [52]. Although the contribution of accompanying subfertility cannot be ruled out, the fact that the increase in several of these adverse outcomes is greater with frozen embryos indicates the likelihood that ART plays a role [52]. Not surprisingly, based on the role of immunological factors in the genesis of preeclampsia there is an increase of this disorder with the use of donor oocytes.

The very nonphysiological strategy of injecting an entire sperm into an ovum with ICSI raises the possibility of special risks with this approach. However, with the increasing use of ICSI it is difficult to sort out whether this maneuver accounts for the infant and maternal risks described. There is no evidence of increased risk of the hazards of preterm birth, SGA, stillbirth, bleeding, or preeclampsia from ICSI compared with usual fertilization, but the risks of major birth defects are increased [53]. There are, nonetheless, adverse outcomes in later life associated with ICSI. Boys conceived by ICSI have lower semen quality and testosterone [54,55]. Girls conceived with ICSI have greater risk of obesity including the obesity most associated with cardiovascular disease, central obesity [55]. Interestingly, this is not as severe in boys.

The use of frozen embryos is increasing. There is no evident increase in preterm birth, low birth weight, SGA, stillbirth and perinatal mortality with frozen embryos. Dr. Conrad pointed out the increased frequency of preeclampsia in mothers in whom frozen embryos were use for ART. Dr. Luke pointed out another maternal issue with the use of frozen embryos. Bleeding, as reflected in the number of women with blood transfusions, was doubled in women who received frozen compared to those receiving fresh embryos [52]. The risk of freezing also extends to the fetus conceived with this approach. When compared with siblings that are born from nonfrozen embryos the sibling conceived from a frozen embryo are larger by at least 70 % [56].

Assisted reproductive approaches and the difference outcomes with different approaches could provide insights into periconceptional physiology and also guide modifications to increase the safety of the procedure. Unfortunately, thus far there has been little information gained from studies of these issues with ART which provides new insights. This is an area that deserves further study. Continued tracking of child health may also be critical in evaluating latent risks, such as cancer [57,58], and the intergenerational effects of ART treatment on the fertility of IVF-conceived individuals.

2.6. Potential mechanisms by which ARTs lead to adverse pregnancy outcomes

As described above, the alterations in pregnancy and long-term outcomes, and the up to 10-fold increase in imprinting syndromes with ARTs suggests that these treatments may alter outomes by epigenetic modifications [59–72]. Dr. Mellissa Mann presented work from her laboratory on the effects of ART treatments on maternal and paternal DNA methylation at imprinted genes. Under normal development, there are three genome-scale phases of DNA methylation programming (Fig. 6). During gametogenesis, parental alleles undergo universal DNA demethylation (Phase 1, Erasure), after which DNA becomes subsequently "tagged" by sex-specific DNA methylation (Phase 2, Acquisition), albeit, before birth in sperm, and after puberty and prior to ovulation with oocytes (Fig. 6A) [73,74]. Phase 3 (Maintenance) occurs after fertilization, where parental-specific alleles at imprinted genes maintain their methylation status, while most other sequences are demethylated. ART procedures overlap the important phases of Acquisition and Maintenance (Fig. 6A). Dr. Mann and her group tested the hypothesis, "Embryos are predisposed to imprinting defects because ARTs disrupt crucial genomic imprinting regulatory events in gametes and embryos". To test this hypothesis, studies used a mouse model that bypasses confounding infertility, and focused on genes that when aberrantly regulated result in imprinting syndromes.

The Mann lab began by testing whether a common ART strategy, hormone-induced superovulation (also called ovarian stimulation), resulted in loss of DNA methylation at imprinted alleles in blastocyst stage embryos. With three such maternal-methylated genes, they found a dose-related reduction in DNA methylation [75]. Interestingly a "control gene" with paternal inactivation, also manifested a dose-dependent reduction in methylation with superovulation. They posited that DNA methylation was reduced because of an ART effect on the Acquisition phase for maternal alleles, and on the Maintenance phase for the paternal allele. However, when the impact of superovulation was examined in oocytes, there was no difference in methylation of maternal alleles, i.e. the Acquisition phase occurred normally [76]. These results indicate an effect of superovulation on a maternal factor necessary for the Maintenance phase.

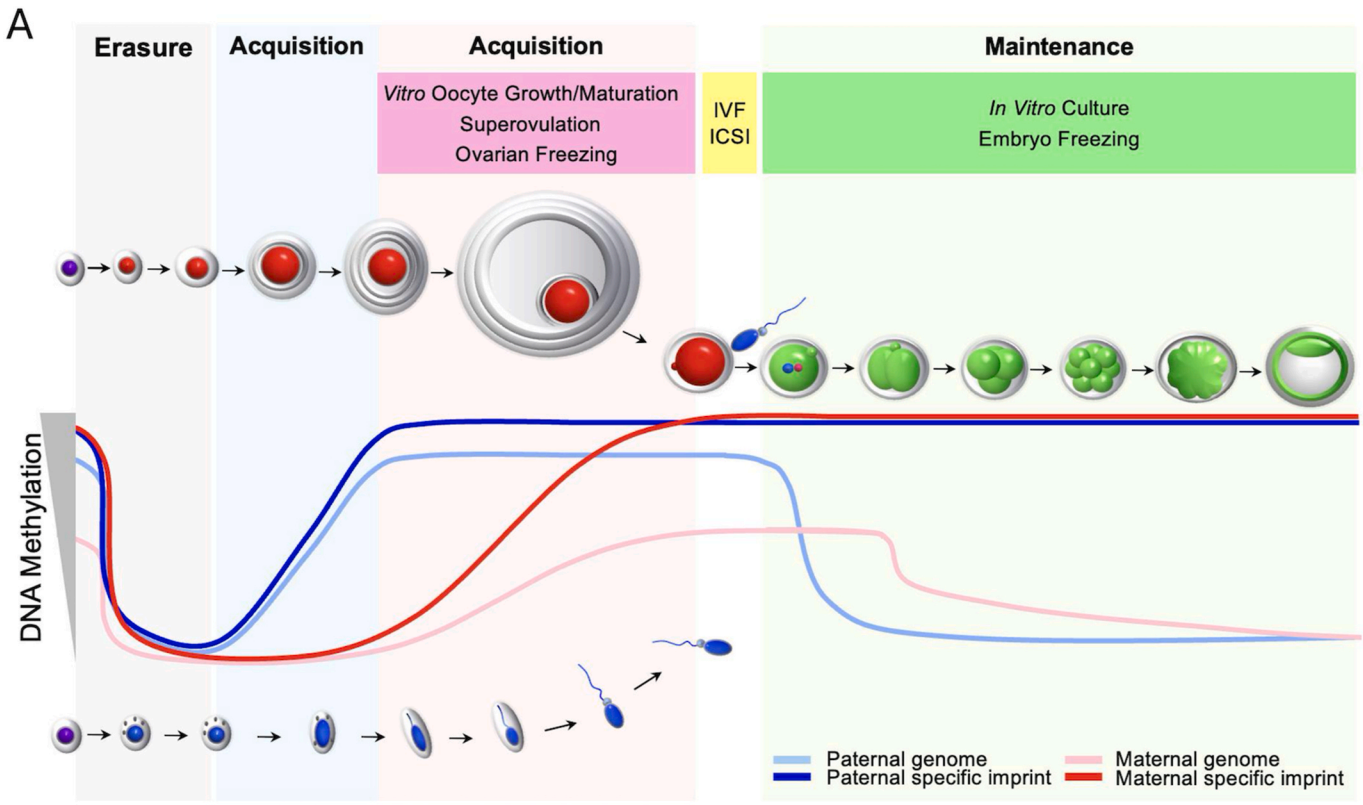
The Mann lab also examined the effects of *in vitro* embryo culture, where embryos were cultured from the 2-cell to blastocyst stages (Fig. 6B). Unexpectantly, a subset of embryos displayed rapid rates of development to the 8-cell stage, with a subsequent reduction of imprinted methylation at the blastocyst stage [77]. By comparison, in vivo-derived and slow developing embryos maintained imprinted methylation. The lab also examined the impact of superovulation and embryo culture, together and separately, on three genes involved in imprinting disorders [78,79]. They found that there was an effect of both treatments, and that when combined, produced increased numbers of embryos with aberrant DNA methylation.

Studies performed later in gestation provide interesting insights. Imprinted genes examined at Day 9.5 of gestation in embryos produced via superovulation, embryo culture and embryo transfer demonstrated decreased DNA methylation primarily in the placenta compared to the embryo proper [80]. Further examination of increasing numbers of ART treatments at later stage of gestation found larger placentas with an increased junctional zone, and reduced methylation at imprinted genes, compared to embryo transfer alone [81].

Studies to fine tune the timing of ART effects were done with superovulation and embryo culture, varying the time of *in vitro* culture: 1-cell to blastocyst, 1-cell to morula, 4-cell to morula, or morula to blastocyst stages, compared with *in vivo* controls (Fig. 6B) [82]. All groups experienced placental overgrowth and reduced DNA methylation, except the morula to blastocyst group, narrowing the effect of ARTs between the 4-cell to morula stages. These data, together with rapid development to the 8-cell stage affecting imprinted methylation, support a window of susceptibility between the 4- and 8-cell stages, during the Maintenance phase of imprinted methylation.

An important question from these studies is what mechanisms are changed in ART-produced embryos to cause rapid developmental rates and aberrant imprinted methylation. The focus going forward is that ARTs disrupt maternal-effect factors in oocytes that are required in embryos during the 4- to 8-cell stages to maintain imprinted methylation. While Dr. Mann's group previously examined the impact of different culture media, the findings that each culture medium led to aberrant DNA methylation, although some media had less of an effect, indicates a complexity that will require molecular strategies of investigation on single embryos. However, they also, as demonstrated by Dr. Mann's studies, provide the variability to begin to understand the importance of the periconception environment to embryo development.

While generally considered safe, studies on the use of different ART treatments in mice and humans found that a combination of ARTs increased the chance of imprinted methylation and expression errors when compared to single treatments [75,78,83–86], indicating that ARTs are a contributor to aberrant imprinted methylation. Importantly, a window of susceptibility to ARTs has been identified between the 4- to



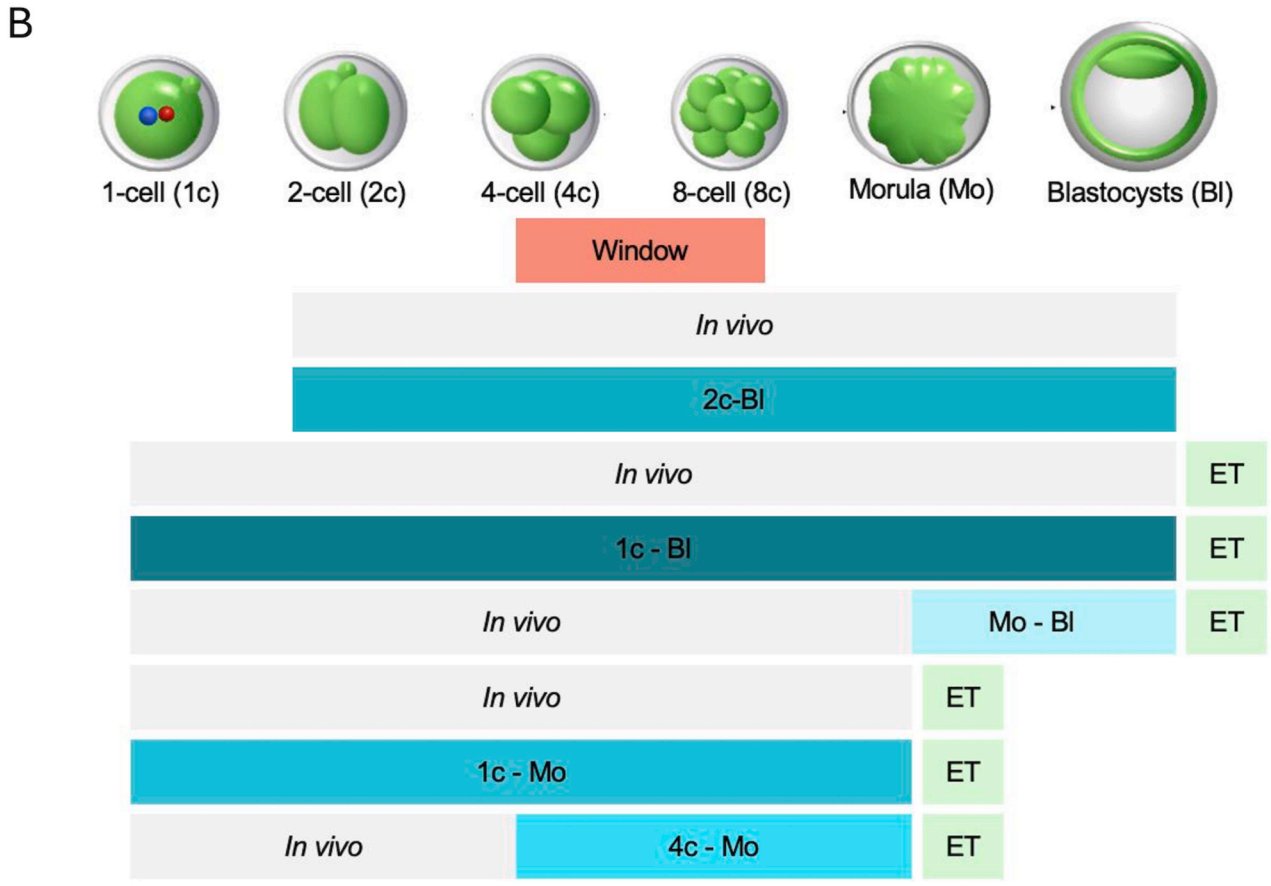


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Fig. 6. DNA methylation programming in relation to with assisted reproductive technology usage. (A) There are three phases of genome-scale DNA methylation programming. *Erasure phase* (grey shading): paternal (blue lines) and maternal (red and pink lines). DNA methylation is removed in primordial germ cells. *Acquisition phase* (blue and pink shading): sex-specific *de novo* DNA methylation is established in gametes, earlier in males compared to females. *Maintenance phase* (green shading): after fertilization, paternal and maternal genomes (light blue and pink lines) are demethylated, while imprinted genes maintain paternal (dark blue line) and maternal (red lines) specific methylation. Assisted reproductive techniques lead to aberrant DNA methylation during the Maintenance phase. B) Embryo culture across preimplantation exposure times reveals a window of susceptibility between the 4- to 8-cell stages. Culture of mouse embryos from the 2-cell to blastocyst stages reduces imprinted methylation in blastocysts, especially for embryos with rapid development to the 8-cell stage. When culture extends across the 4-cell to morula stages, DNA methylation was perturbed in late-stage placentas. However, when culture began at the morula stage, DNA methylation was maintained similar to *in vivo* controls. (Modified from Denomme and Mann[64] with permission.).

8-cell stages of mouse development [79,82]. Going forward, it is crucial to ascertain the molecular mechanisms regulating imprinted methylation during these early developmental stages, and how and when ARTs alter these mechanisms, leading to imprinted as well as other DNA methylation errors. Gaining this knowledge will advance ART treatments that will reduce the frequency of embryos with imprinting perturbations, and ultimately, imprinting disorders and other adverse pregnancy outcomes.

3. Summary and recommendations

3.1. Summary

The data presented support the importance of the periconceptional period to the establishment of normal pregnancy and pregnancy outcome. Decidua promotes normal implantation and provides initial nutrition, growth factors and oxygen to the implanted embryo. Early events in placentation are modified by immune interactions between embryo and decidua. Emerging information implicates variability of HLA-C genes expressed by the fetal trophoblast and the maternal KIR genetic system of uNK cells to modulate trophoblast activation.

Detailed understanding of the importance of periconceptional events is limited by the lack of information on the implications of these events at the termination of pregnancy. Studies utilizing non-used tissue from the time of CVS in early gestation form the basis of current information. However, CVS is being replaced by non-invasive strategies. Several other strategies have been utilized to attempt to gather information usually only available with CVS. Of these, the most exciting is work with organoids, which can be obtained from endometrial biopsy or menstrual fluid. The interactions mirror those of intact tissue and do not adversely affect the pregnancy. Hence, endometrial function can now be assessed, and possibly if necessary manipulated therapeutically, preconceptionally.

It is also evident that ovarian hormones, including relaxin are crucial to the maintenance of early pregnancy and reprogramming non-pregnant to pregnant physiology. Interestingly much of the information about the impact of these hormones comes from ART where different manipulations of the hormonal milieu can result in pregnancies in which ovarian hormones are absent or present in excess.

ART, as presently practiced, is performed at a time in early pregnancy when many crucial events in genetic maturation are occurring including the erasure and acquisition of epigenetic modifications (Fig. 6). It is not surprising that there are implications of ART for progression of normal pregnancy and infant outcomes particularly events related to genetic imprinting. These can be mimicked in animal experiments, which demonstrate the roles of different components of ART on misregulation of genomic imprinting. However, the enormous number of variables involved in the current practice of ART and minimal formal studies of the impact of these variable approaches challenges identifications of relevant components.

Several recommendations are directed by current findings and evident limitations of current research strategies.

3.2. Policy suggestions

1. Greater importance should be directed to studies and clinical management of the periconceptional period than at present. Preventing complications of pregnancy may be a more successful strategy than designing therapeutic interventions to treat them once they are established.
2. One of the challenges of periconceptional research is that it crosses several disciplines. Collaboration among physicians and scientists across the disciplines of reproductive endocrinology, immunology, infertility, maternal fetal medicine, placentology, physiology and bioengineering should be encouraged to facilitate research progress.
3. Chorionic villus sampling provides the only window into the early pregnancy maternal-fetal interface with known pregnancy outcomes. However, it has been largely replaced by noninvasive strategies to assess fetal genetic abnormalities. Nonetheless, it is likely that some CVS samples are currently biobanked. Efforts should be made to catalogue these materials and to make them available to investigators.

3.3. Research suggestions

1. There is a need to define in greater detail a healthy preconceptional endometrium in functional terms, such as the capacity for decidualization, responsiveness of histotroph secretion to trophoblast hormones and activity of immunological cell populations. It is also important to understand the normal variability between cycles, and the effects of factors such as high BMI, maternal age and ethnicity that are related to increased risk of adverse pregnancy outcomes.
2. There is little formal testing of the myriad of variables included in ART. Identifying different short- and long-term maternal and fetal outcomes with different approaches could provide insights into periconceptional physiology and also guide modifications of ART to increase the safety of the procedure.
3. ART provides the opportunity to test for correlation between endometrial function assessed using organoids derived immediately prior to, or during, a treatment cycle and the clinical outcome. Large studies are needed to control for confounders.
4. Ovarian products are crucial to the initiation of normal pregnancies. It would be useful to identify the full suite of factors secreted into the maternal circulation during the luteal phase and early pregnancy.
5. There is probably enough evidence for the importance of relaxin to justify a trial to replace relaxin in ART pregnancies without a corpus luteum to determine restoration of maternal pregnancy cardiovascular physiology.
6. Going forward, it is crucial to ascertain the molecular mechanisms regulating imprinted methylation during these early developmental stages, and how and when ARTs alter these mechanisms, leading to imprinted as well as other DNA methylation errors. Gaining this knowledge will advance ART treatments that will reduce the frequency of embryos with imprinting perturbations, and ultimately, imprinting disorders and other adverse pregnancy outcomes.
7. Development of a biomarker panel of decidualization (e.g., IGFBP-1, glycodelin, decorin, others) for application to maternal blood or uterine secretions would have diagnostic potential.

8. Organoids and other innovative strategies to model the maternal-fetal interface and study early pregnancy events with minimally invasive strategies should be exploited and encouraged. The use of both endometrial and trophoblast organoids to study events early in pregnancy now allow experiments to be performed in human placentas *in vitro*. These will allow a deeper understanding of how the pathophysiology underlying clinical scenarios at full-term develop during gestation, and identification of opportunities for interventions.

CRediT authorship contribution statement

James M. Roberts: Writing – review & editing, Writing – original draft, Validation, Supervision, Conceptualization. **Graham J. Burton:** Writing – original draft, Validation, Investigation. **Kirk P. Conrad:** Writing – review & editing, Validation, Investigation. **Barbara Luke:** Writing – review & editing, Validation, Investigation. **Mellissa RW. Mann:** Writing – review & editing, Validation, Investigation, Conceptualization. **Ashley Moffett:** Writing – review & editing, Validation, Investigation. **McKenzie K. Jancsura:** Writing – review & editing, Conceptualization.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sector.

Declaration of competing interest

None.

Acknowledgements

We thank the participants and attendees of the Global Pregnancy Workshop that was held September 18 and 19, 2023. Their discussion was integral to the development of the recommendations.

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