Endometrial Gene Expression

An Emerging Paradigm for Reproductive Disorders

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Preface

Endometrial Gene Expression: An Emerging Paradigm for Reproductive Disorders is a book devoted to endometrial gene expressions. This book is the first to focus on endometrial gene expression and gynecological pathologies in terms of reproduction, endometriosis, and other gynecological diseases. A recent development in molecular genetic technology opens a new era of endometrial assessment for various gynecological conditions, including infertility.

Endometrium goes through structural changes cyclically under the hormonal influence to accommodate the implantation of an embryo. When implantation does not happen, menstruation occurs. Immune effectors in the endometrium dynamically change their populations during the menstrual cycle and set up immune responses with appropriate cytokine and chemokine milieus. The number of uterine natural killer (uNK) cells increases significantly during the luteal phase and reaches 70% of decidual lymphocytes in early pregnancy. Natural killer (NK) cell trafficking from the peripheral blood to the endometrium, as well as the proliferation of uterine NK cells, contributes to the rapid accumulation of NK cells. Cytokines and chemokines in the endometrium and decidua attract NK cell trafficking, and the evaluation of chemoattractant in the endometrium may predict endometrial immune responses and reproductive outcome. Due to recent advances in molecular genetic technology, changes in the endometrium can be detected, and molecules related to endometrial changes may serve as diagnostic and therapeutic markers for various gynecological conditions.

Endometrial immune responses are important for trophoblast invasion and early pregnancy. Indeed, dysregulated endometrial inflammatory immune responses are associated with reproductive failures, such as recurrent pregnancy losses, repeated implantation failures, endometriosis, and gynecological diseases with oncogenic potential. New molecular genetic techniques have been introduced for the gene expression studies, and the updated data have been accumulating. The time has come for a book dedicated to endometrial gene expression and which reviews the recent developments and findings in endometrial gene expression about reproduction and gynecological diseases.

Authoritative authors who are active in endometrial research and clinical medicine were invited to submit their research and findings. The book begins with the embryology of the uterus and explains the development and the structure of the uterus with the commentary of the development of urogenital systems. Genes directly regulate Mullerian duct development, and genes and pathways essential for urogenital tract formation and maintenance are reviewed. The molecular genetic mechanism underlying the physiology of menstruation and the development of endometrium will provide insight into the uterine condition and diseases as well as the adaptive value of menstruation in mammalian evolution.

Advances in molecular genetic technologies allow the high-throughput quantification of the gene expressions in the endometrium. Commonly used techniques for transcriptome profiling are systematically introduced for a better understanding of new technologies and an application to clinical medicine. Transcriptomics using high-throughput methods, such as microarray analysis, has been applied to the clinical medicine for the evaluation of endometrial receptivity. This transcriptomic approach has allowed identifying different mRNA expression patterns in the endometrium during the whole menstrual cycle, revealing a specific signature for each endometrial stage. Clinical application, predictability, and interpretation of the transcriptomic approach are meticulously reviewed. Epigenetic regulatory mechanisms such as DNA methylation; histone modification; noncoding RNA, for instance, miRNA; genomic imprinting; and X-chromosome inactivation contribute to embryo implantation, placental formation, organ formation, and fetal growth. Epigenetic dysregulation may result in aberrant endometrial receptivity and reproductive failures, and it can be detected by various sequencing techniques. The importance of precise timing of sampling and a well-defined category of the study population is emphasized.

The endometrium goes through changes when it is infected by pathogens. Therefore, gene expression studies to detect endometrial pathogens may predict endometrial immune responses. Pathogens induce a significant burden in the endometrium by changing immunophenotypes of endometrial immune effectors. HHV6 infection in the female genital tract has been implicated in female idiopathic infertility, causing a specific modification of gene expression in both endometrium and endometrial NK cells. In this book, HHV6 and endometrial changes, including gene expression and immune effectors, are comprehensively reviewed.

Endometriosis is characterized by the presence of endometrial tissues in extrauterine locations. Recent studies demonstrated the microRNAs, which are stable and specific modulators of gene expression, might serve as a biomarker for endometriosis. In the chapters reviewing endometrial gene expressions in women with endometriosis, endometrial mRNA expression, miRNAs, and related pathways in endometriosis are thoroughly reviewed by the world-renowned authorities in endometriosis research. Lastly, the role of immuno-metabolism is explored by investigating endometrial gene expression. The metabolic and nutritional impact on endometrial gene expression and reproductive disorders are thoroughly reviewed.

Investigation of the endometrium is often limited due to the difficulties in sampling and dynamic changes of the endometrium during the menstrual cycle. With

updated molecular genetic technology, various gene expressions can be assessed even with the small quantity of samples. In this book, we aimed to deliver the most updated, challenging, factual account of endometrial gene expressions. We hope this book will help to facilitate the clinical translation of updated scientific knowledge of endometrial gene expressions. Ultimately, we seek a readership enthusiastic about understanding the scientific foundation of advanced technologies and examining critically the implication of the current data in clinical medicine.

Evaluation of genome will preage a new era of drug recovery in which therapy will be tailored to aberration in DNA sequence.

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Chapter 1
Endometrial Development and Its Fine Structure

Takeshi Kurita and Jumpei Terakawa

Embryology of the Uterus

In this section, the development of the female reproductive tract will be discussed with an emphasis on the genes and pathways that control the process. In addition to normal development, pathologic human conditions as consequences of faulty development will be presented. The early developmental process of the female reproductive tract is essentially identical among mammalian species. In fact, molecular mechanisms of uterine embryogenesis described herein are mostly derived from studies utilizing genetically engineered mouse models. Nonetheless, the timing of events in embryogenesis is described by weeks after the first day of the last menstruation period (LMP) in human fetuses for clinical relevance.

Formation of the Müllerian Ducts

In mammals, most of the female reproductive tract, including the oviduct, uterine corpus (uterus), cervix, and vagina, arises from a pair of the Müllerian ducts (MDs) or paramesonephric ducts of mesodermal origin [1]. MD was named after Johannes Peter Müller, a German physiologist who described the structures in his book

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published in 1830 [2]. The initial segment of MDs arises as anterior-to-posterior invaginations of the thickened coelomic epithelium at the cranial end of the urogenital ridge and grows caudally through the urogenital ridge mesenchyme. The site of the initial invagination remains open throughout the development and becomes the abdominal ostium of the oviduct [3–5]. The tip of the MD comes into close contact with the Wolffian duct (WD) and elongates caudally using the WD as the guide [6–9]. Because the epithelial cells of MD and WD are in direct contact within a common basement membrane, it was speculated that WD and MD exchange epithelial cells during embryogenesis. However, a cell lineage tracing experiment with transgenic mouse lines disproved the hypothesis by demonstrating that the epithelium of MD arises solely from the coelomic epithelium [1, 7]. When WD epithelium was permanently labeled with enhanced green fluorescent protein (EGFP) by crossing dual-reporter tdTomato-EGFP (Cre reporter) mice and *Hoxb7-Cre* (WD epithelium-specific Cre) transgenic mice, EGFP-positive cells were never detected in the epithelium of MD-derived organs (uterus, cervix, and vagina) by a single-cell level screening [1].

Elongation and Fusion of MDs

Originally, the cranial portion of the MD lies laterally to the WD within the urogenital ridge mesenchyme (Fig. 1.1a). During the caudal growth, MDs ventrally cross over WDs to join and fuse with each other in the midline [3, 10] (Fig. 1.1b). Subsequently, the common wall (median septum) degenerates to form a single canal (uterovaginal canal). The caudal tips of MDs remain separated to keep physical contact with WDs on both sides even when the midportions of the MDs fuse together. The tips of MD finally become united immediately before the vertical fusion with the urogenital sinus [11] (Fig. 1.1c). In the human fetus, the formation, elongation, and fusion of MDs occur between 8 and 11 weeks after LMP, and the fused portion of MDs eventually develops into uterine corpus, cervix, and vagina [5].

At the site of the vertical fusion with MDs, urogenital sinus develops into the vaginal plate, a flat epithelial cord that connects the caudal end of MD epithelium to the urethral epithelium [5, 10]. The MDs further elongate caudally, led by the vaginal plate as it continues to migrate toward the posterior end of the body (Fig. 1.1d) [5, 8].

Regression of MDs in the Male Fetus

During mammalian embryogenesis, there is a sexually indifferent stage in which the fetus has a potential to develop either male or female structures. The initial event of sexual differentiation in mammalian embryos is gonadogenesis, a complex process through which testes and ovaries arise from the genital ridge, a common somatic precursor of gonads [12]. In the human fetus, primordial germ cells reach the genital ridge by 7–8 weeks in both sexes [5]. In the male (XY) fetus, *SRY* (Sex Determining Region Y) expression in Sertoli cell precursors initiates testes determination by

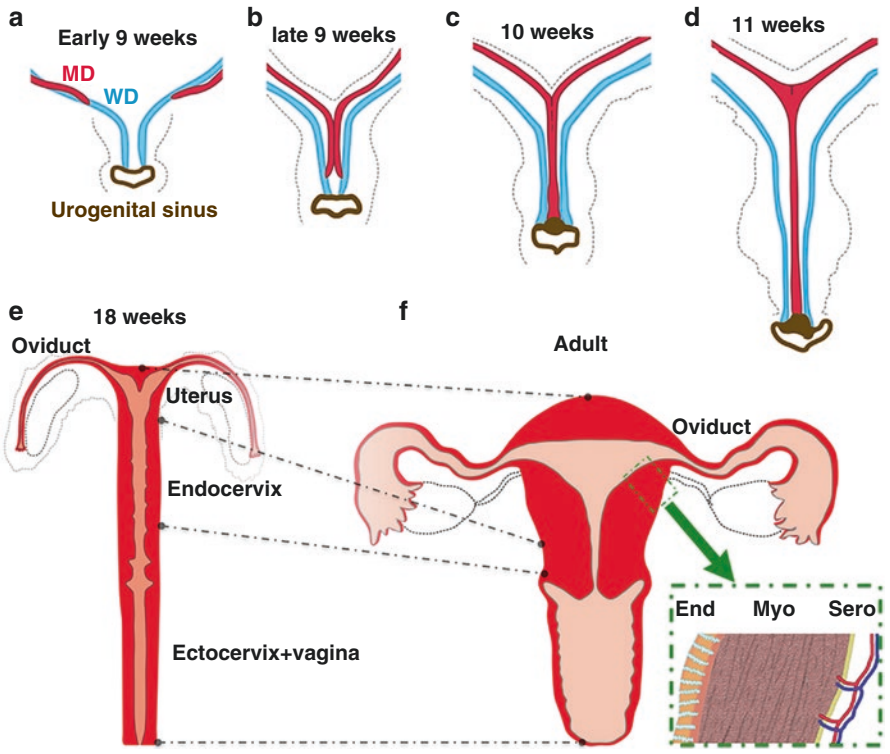

Fig. 1.1 Development of human female reproductive tract. (**a**–**d**: adapted from Koff 1933)

~9 weeks. SRY activates the gene regulatory hierarchy leading to testes differentiation in collaboration with another transcription factor SOX9 (SRY-box 9), which is also a target of SRY transcription factor [13, 14]. One of the target genes of SRY and SOX9 is anti-Müllerian hormone (AMH) or Müllerian inhibitory substance (MIS), a member of the transforming growth factor-β family [15]. In the male fetus (~10 weeks), immature Sertoli cells start secreting AMH, which triggers regression of MDs through activation of the receptors in the MD mesenchyme (for the detail, see section "Molecular Mechanism of MD Regression"). Accordingly, in the XY fetus, the caudal portion of MD disappears by 11 weeks. In the absence of SRY, the female pathway takes place, and the gonads develop as ovaries [16, 17]. As AMH secreting Sertoli cells do not develop, the XX fetus retains MDs. The further details of molecular and cellular mechanisms in gonadogenesis fall outside of the scope of this chapter.

Mayer–Rokitansky–Küster–Hauser Syndrome (OMIM: 277000)

Mayer–Rokitansky–Küster–Hauser syndrome (MRKHS), also referred to as Müllerian agenesis, is a rare gynecologic disorder characterized by the absence or underdevelopment of the uterus and vagina with normal ovarian functions and

external genitalia [18]. The etiology of MRKHS remains largely unknown in part due to its intrinsic heterogeneity in clinical presentations. When only reproductive organs are affected, the condition is classified as type I. However, it is very common to find MRKHS patients with several congenital anomalies (type II), including urinary tract, renal, craniofacial, and skeletal malformations. Hence, the acronym MURCS (*MÜ*llerian duct aplasia, *R*enal dysplasia, *C*ervical *S*omite anomalies) is also used. The association of defects in MD and other organs suggests that MRKHS results from the disruption of signaling pathways involved in the development of MD as well as other organs. A certain degree of familial association indicates the contribution of genetic factors to MRKHS (see reviews [19, 20]). Meanwhile, the incomplete penetrance of familial cases implies that the epigenetic and/or environmental factors also play a role in its etiology [21]. However, to the date, there has been no established link between an environmental factor and MRKHS.

Uterine Anomalies Caused by Fusion Failure

The lateral fusion of MDs occurs at different segments along the axis depending on the species. In human, the complete fusion in the uterine segment forms a simplex uterus, which consists of a single uterine corpus. Failure in the lateral fusion of MDs explains the Class II–VI Müllerian anomalies in the American Society for Reproductive Medicine (ASRM) classification [22]. Unilateral MD agenesis and subsequent lateral fusion failure explain the pathogenesis of unicornuate uterus (Class II). Uterus didelphys (Class III) and bicornuate (Class IV) reflect different degrees of incomplete lateral fusion at the uterine segment from two completely separated uteri (didelphys) to a caudally unified heart-shaped uterus (bicornuate). Septate (Class V) and arcuate (Class VI) also reflect different degrees of incomplete degradation of the median septum within a single uterine corpus formed by the fusion of two MDs.

On the other hand, MD fusion at vaginal or cervical segment generating two uteri is normal for some mammalian species [4, 23]. For example, in mice, the lateral fusion of MDs occurs at the caudal portion of the cervix, generating two uterine horns, two cervices, and one vagina.

Genes Essential for MD Development

MD development involves complex tissue interactions mediated by cross-talk between signaling pathways. Here, we list the genes/pathways that play essential roles in normal MD development, following the guidelines of the HUGO Gene Nomenclature Committee (HGNC) in the nomenclature of genes and proteins [24, 25] (Table 1.1). As the functions of these genes are identified through mouse genetic

Table 1.1 Mouse mutant models exhibit defects in MD formation

Gene symbol	Alternative names	Expression (mouse)	Mouse mutant model		Human case report of MD anomalies
			Mutation type	MD/uterine phenotypes	
Dach1 *Dach2*	DACH (*Dach1*)	MD Ep	Compound null [30]	MD agenesis	
Dlg1	*Dlgh1*, SAP97	WD Ep	Null [94]	MD fusion defect Caudal MD agenesis	
Emx2		WD Ep	Null [41]	MD agenesis (WD agenesis)	A heterozygous nonsense mutation (c.424G > T; p.E142X) in a woman with uterus didelphys [207]
Gata3	HDR, HDRS	WD Ep	WD Ep knockout [91]	MD agenesis (WD agenesis)	One case each for septate uterus and uterus didelphys with septate vagina in two affected individuals of a familial HDR syndrome (OMIM: 146255) with a heterozygous c.431delG mutation [93]
Hnf1b	FJHN, HNF2, LFB3, TCF2, HPC11, MODY5, VHNF1	WD Ep	Null [84]	MD agenesis (WD agenesis)	Vaginal aplasia with the rudimentary uterus [88] and bicornuate uterus [89, 90] in RCAD syndrome (OMIM: 137920) patients
Hoxa10	Hox1.8, HOX1, HOX1H, PL	Anterior MDMs (future anterior uterus)	Null [47]	Anterior homeotic transformation	A heterozygous missense mutation (c. G152A, p.G51D) in a woman with a bicornate uterus. Heterozygous frameshift stop mutation at p.R371 in a woman with the septate uterus [56]
Hoxa11	Hox1.9, HOX1, HOX1I, RUSAT1	Anterior MDMs (from the future caudal uterus to the cervix)	Null [48]	Anterior homeotic transformation	A heterozygous missense mutation (c.763G, p. E255K) in a woman with the septate uterus [57]
Hoxa13	Hox1.10, HOX1J	Posterior MDMs (from the future cervix to the vagina)	Null [45]	Caudal MD agenesis	Heterozygous mutations of *HOXA13* cause HFG syndrome (OMIM: 140000)

(continued)

Table 1.1 (continued)

Gene symbol	Alternative names	Expression (mouse)	Mouse mutant model		Human case report of MD anomalies
			Mutation type	MD/uterine phenotypes	
Hoxa13 *Hoxd13*	Hox4.8, HOX4I, BDE, BDSD, SPD, SPD1 (*Hoxd13)*	Posterior MD Ms (from the future cervix to the vagina)	Compound null [45]	MD agenesis (WD agenesis)	A double-heterozygous female for HFG allele (*HOXA13* polyalanine tract expansion mutation) and Synpolydactyly 1 (OMIM:186000) allele (*HOXD13* pR298W) had a narrow vaginal introitus
Lhx1	LIM1	MD Ep WD Ep	Null [28]	MD agenesis (WD agenesis)	A frameshift mutant allele (c.25dup; p. Arg9LysfsX25) in an MRKHS type II patient [29]
			MD Ep-specific knockout [26]	MD agenesis	
Pax2	Opdc, FSGS7, PAPRS	WD Ep	Null [33]	MD agenesis (WD agenesis)	A missense mutation (c.1284C > G, p.I428M) in a fetus with sever CAKUT with uterine agenesis [40]
Pbx1	CAKUHED	MD Ep/Ms Mesonephric Ms Coelomic Ep	Null [65]	MD agenesis	A 46 XY male patient carrying a missense mutant allele (p. R235Q) retained Müllerian structure, demonstrating uterus didelphys [208]
Wnt4	SERKAL	MD Ms Coelomic Ep	Null [70]	MD agenesis (WD agenesis)	Heterozygous missense mutations (p.L12P, p.R83C, p.E226G, p. A233T) in 4 MRKHS patients with hyperandrogenism [73, 74, 209, 210]
Wnt5a		MD Ms	Null [77]	Caudal MD agenesis	
Wnt7a		MD Ep	Null [157]	Anterior homeotic transformation, uterine hypoplasia	

(continued)

Table 1.1 (continued)

Gene symbol	Alternative names	Expression (mouse)	Mouse mutant model		Human case report of MD anomalies
			Mutation type	MD/uterine phenotypes	
Wnt9b	WNT14B, WNT15	WD Ep	Null [82]	MD agenesis	Heterozygous missense mutations (p.Q158E, p.R222H, p.R241H, p.R325H, p.C343X) in 5 MRKHS type I patients and p.R307W heterozygous mutation in a woman with the bicornuate uterus [83]
Rara *Rarb* *Rarg* *Rxra* *Rxrb* *Rxrg*	RARA: NR1B1, RAR RARB: HAP, MCOPS12, NR1B2, RRB2 RARG: NR1B3, RARC, RARD RXRA: NR2B1 RXRB: DAUDI6, H-2RIIBP, NR2B2, RCoR-1 RXRG: NR2B3, RXRC	MD Ep/Ms	Compound null [59, 60]	Partial or complete MD agenesis	

MD Müllerian duct, *WD* Wolffian duct, *Ep* epithelium, *Ms* mesenchyme, *MRKHS* Mayer–Rokitansky–Küster–Hauser syndrome, *CAKUT* congenital anomalies of the kidney and urinary tract, *HDR syndrome* hypoparathyroidism, deafness, and renal dysplasia syndrome, *RCAD syndrome* renal cysts and diabetes syndrome

studies, mouse gene symbols (italicized, with only the first letter in uppercase and the remaining letters in lowercase) are used primarily. Human gene symbols (all italicized uppercase) are also used when necessary.

Genes Directly Regulate MD Development

Lhx1 *Lhx1* (LIM Homeobox 1), which encodes a LIM-class homeodomain transcription factor, is essential for the formation of the WD and MD [26–28]. *Lhx1*-null mutant mice lack the derivatives of the WD as well as the MD [28]. Although

chimeric female mice of *Lhx1*-null and wild-type cells developed the MD normally, *Lhx1*-null cells contributed to the mesenchyme but not to the epithelium in the uterus and oviduct, indicating the cell-autonomous functions of *Lhx1* in MD epithelium [28]. This was further confirmed by MD epithelium-specific knockout mice of *Lhx1* by *Wnt7a-Cre*: Loss of *Lhx1* in epithelium inhibited the caudal elongation of MDs, which resulted in MRKHS-like phenotypes of female mice lacking the uterus, cervix, and upper vagina with a truncated oviduct [26]. In fact, deletion of 17q12, which contains *LHX1* locus, and *LHX1* mutations have been detected in a subpopulation of MRKHS patients [29]. Thus, a subtype of MRKHS might be due to the altered functions of LHX1.

Dach1/Dach2 Dachshund family transcription factors, *Dach1* and *Dach2*, play redundant roles in the development of multiple tissues/organs, including the MD [30]. Compound mutant mice of *Dach1* and *Dach2* demonstrated anaplasia in MD-derived organs in a gene-dosage-dependent manner. When both *Dach1* and *Dach2* were lost, MDs did not form even though WDs were intact [30]. Furthermore, *Lhx1* expression is reduced in the MD epithelium of *Dach1* and *Dach2* compound mutant mice, suggesting that *Dach1/2* are the upstream regulators of *Lhx1* in the MD [30].

Pax2/Pax8 Development of the urogenital system in mammals requires the coordinated differentiation of the ductal epithelium and the nephrogenic mesenchyme from the intermediate mesoderm [31]. *Pax2* and *Pax8*, encoding members of paired box transcription factors, play essential and redundant roles in this process [32]. Mouse embryos that lack both *Pax2* and *Pax8* are unable to form the pronephros or any later nephric structures. In WDs and MDs, both *Pax2* and *Pax8* are expressed in the epithelium, but *Pax2* plays a dominant role over *Pax8*. In *Pax2*-null mutant mice, WDs and MDs form partially but degenerate in both sexes [33]. In contrast, the embryonic development of WD and MD appears to be normal in *Pax8*-null mice [34]. Additionally, in kidney development, *Lhx1* expression in WDs requires *Pax2* [35], suggesting that *Pax2* may be upstream of *Lhx1* in the MDs. In fact, *Pax2* was expressed in the MD epithelium independent of *Lhx1* [26].

The critical roles of *PAX2/8* in kidney development have been demonstrated by the link between mutations and congenital anomalies of the kidney and urinary tract (CAKUT) [36–39]. MD agenesis was not reported in *PAX2/8* mutant patients with CAKUT. However, a study identified *PAX2* mutations in five fetuses with severe CAKUT, and one of these five fetuses demonstrated uterine agenesis [40], in agreement with mouse genetic studies.

Emx2 *Emx2* (Empty Spiracles Homeobox 2) encodes a homeobox-containing transcription factor that is expressed in the epithelium of MDs and WDs. In *Emx2*-null mutant mice, WDs were formed but degenerated, and MDs were not observed [41]. In kidney development, *Emx2* is in the downstream position of *Pax2/8* and *Lhx1*: *Emx2* expression was downregulated and completely lost in the pro-/mesonephros of *Pax2/8*-null and *Lhx1*-null embryos, respectively. Meanwhile, *Pax2* and *Lhx1* expression levels are unaffected in *Emx2*-null embryos [35]. Therefore, PAX2/

PAX8, LHX1, and EMX2 appear to comprise a transcription factor cascade that controls the formation of MDs.

HOX Genes HOX genes, a subset of homeobox genes, are evolutionarily conserved and essential for the anterior-to-posterior pattering of developing embryos [42]. In vertebrates, HOX genes include 39 genes composed of 13 paralogue groups in 4 clusters (HOXA, HOXB, HOXC, and HOXD) each on a different chromosome with 3′-to-5′ alignment. The 3′-to-5′ order in each HOX gene cluster reflects an anterior-to-posterior order of expression. Accordingly, unique combinations of HOX proteins are expressed at different positions along the anterior-to-posterior axis of the developing embryo. In mouse MDs, Abdominal-B class HOX gene clusters, *Hoxa9-13* and *Hoxd9-13*, are expressed in a nested, partially overlapping pattern: The most anterior segment of MD expressing the most 3′ ortholog *Hoxa9*/*Hoxd9* becomes the oviduct. The uterine segments express *Hoxa10*/*Hoxd10* anteriorly and *Hoxa11*/*Hoxd11* posteriorly in a partially overlapping pattern. *Hoxa11*/*Hoxd11* and *Hoxd12* are expressed in the future cervix segments. The vaginal segment, the most posterior portion of MDs, expresses *Hoxa13*/*Hoxd13* [43–46]. The Abdominal-B class HOX genes specify the developmental fate of MD segments dose-dependently. For instance, the overlapping expression patterns of *Hoxa10/a11* and *Hoxd10/d11* are critical for the specification of uterine segments in the MDs. Inactivation of *Hoxa10* in mice transforms the anterior portion of the uterus into the oviduct-like structure [47]. The anterior conversion of the uterus to oviduct also occurs in *Hoxa11*-null mice to a lesser extent [48]. Furthermore, *Hoxa10*/*Hoxa11* transheterozygous mice demonstrate similar defects in the uterotubal junction [49]. The similar gene-dose effect was observed between *Hoxa13* and *Hoxd13* in the vaginal segment. While the loss of *Hoxd13* alone did not have an impact on MD development [50], heterozygous loss of *Hoxa13* in *Hoxd13*-null mice caused severe urogenital anomalies including agenesis of MD-derived organs [45]. Between *Hoxa13* and *Hoxd13*, *Hoxa13* has dominant effects on vaginal development: *Hoxa13*-null mutation results in agenesis [45] and lateral fusion failure in posterior MDs [51].

Human genetic studies also emphasize the significance of HOX genes in MD development. Human mutations in *HOXA13* cause hand–foot–genital syndrome (HFGS, MIM 140000), which shows an autosomal dominant pattern of inheritance [52]. Female HFGS patients present various degrees of incomplete MD fusion but are usually able to have children [53]. Most *HOXA13* mutations in HFGS patients result in the production of a nonfunctional HOXA13 protein. However, some mutations generate HOXA13 proteins that may function as a dominant negative, causing more severe phenotypes [54]. There has been a case report of double-heterozygous missense mutations for *HOXA13* and *HOXD13*: The double-heterozygous patient presented more severe digital abnormalities than heterozygous patients of *HOXA13* or *HOXD13* mutations, suggesting the gene-dose effects between *HOXA13* and *HOXD13* in human development [55]. However, the uterus of the *HOXA13* and *HOXD13* double-heterozygous patient appeared to be normal. To the date, hereditary syndromes caused by *HOXA10* or *HOXA11* mutations have

not been reported, likely because such mutations would result in sterility. Nevertheless, mutations in *HOXA10* and *HOXA11* have been identified in patients with sporadic cases of uterine malformations. There have been three non-MRKHS patients with bicornate or septate uterus carrying either a missense or a nonsense mutation of *HOXA10* [56]. Furthermore, a missense mutation of *HOXA11* that is predicted to reduce the DNA binding affinity has been identified in a woman with a septate uterus [57].

In both human and mouse, the expression patterns of HOX along the anterior–posterior axis are maintained in the adult MD-derived organs. In the uterus, HOX genes play critical roles during implantation [47, 48]. However, pregnancy-associated genes and pathways are outside of the scope of this chapter.

Retinoic Acid Receptors Retinoic acid (RA) is a vitamin A-derived small lipophilic molecule that acts as morphogens during embryogenesis. Maternal vitamin A deficiency causes agenesis of the posterior MD in female rat embryos [58], highlighting the significance of the RA signaling pathway in MD development. The morphogenetic effects of RA are mediated by two families of nuclear receptors: retinoic acid receptors (RARs) and retinoid X receptors (RXRs), each consisting of three types (α, β, and γ) encoded by *Rara*, *Rarb*, *Rarg*, *Rxra*, *Rxrb*, and *Rxrg*. RARs form heterodimers with RXRs, and the heterodimer activates expression of target genes via binding to RA response elements (RAREs) in the promoter. Due to the functional redundancies among RARs and RXRs, a null mutation in one receptor gene does not cause developmental defects in MD-derived organs. However, the compound mutations in *Rara* with *Rarb* or *Rarg* and *Rxra* with *Rara*, *Rarb*, or *Rarg* result in partial or complete agenesis of MDs in mice [59, 60]. The primary mechanism through which RA signaling controls anterior–posterior patterning of embryos is the regulation of HOX genes [61]. The responsiveness of a HOX gene to RA corresponds to its location within the HOX gene cluster: Low levels of RA induce the transcription of genes near the 3′ end of the cluster, while genes farther away from the 3′ end of the cluster require a longer period of exposure to a higher concentration of RA to be activated [62]. Thus, truncation of MDs in vitamin A-deficient rat embryos can be explained by the HOX gene deficiency in the posterior MDs due to reduced RA signaling activities.

Pbx1 PBX1 (PBX homeobox 1) is an atypical homeodomain transcription factor that regulates the development of multiple organ systems as a binding partner of multiple transcription factors. *Pbx1*-null mice exhibit widespread developmental defects and die at E15.5 [63]. PBX1 is expressed in both epithelium and mesenchyme of MDs [64], and *Pbx1*-null mice lack MDs [65, 66]. The exact mechanism of MD agenesis in *Pbx1*-null mice is unknown. Nevertheless, PBX1 is a co-transcription factor of HOX proteins. Since Abdominal-B class HOX genes play critical roles in the development of MD-derived organs, loss of MDs in *Pbx1*-null mice might be due to the impaired function of HOX genes.

Ctnnb1 The WNT signaling pathway plays critical roles in embryogenesis by regulating stem cell function, cell fate commitment, cell polarity, and cell prolifera-

tion [67, 68]. The activity of the WNT pathway is transduced by canonical and noncanonical pathways. β-Catenin (CTNNB1) plays the central role in the canonical WNT pathway: When WNT signaling is absent, ubiquitin-mediated proteasomal degradation by the destruction complex maintains low intracellular CTNNB1 levels. Binding of WNT ligands to the Frizzled membrane receptor and its co-receptor, low-density lipoprotein receptor-related proteins 5/6 (LRP5/6), stabilizes CTNNB1 by inactivating the destruction complex. The accumulated CTNNB1 translocates into the nucleus, forms a transcriptional complex with lymphoid enhancer factor (LEF)/T cell factor (TCF), and activates the transcription of target genes [69]. As described below, the canonical WNT pathway is activated by multiple WNT ligands, including WNT4, WNT7A, and WNT9B, in urogenital tract development. Thus, CTNNB1 is essential for the development of MDs.

Wnt4 *Wnt4*, encoding a member of the WNT family of secreted glycoproteins, is expressed in the coelomic epithelium and the mesenchyme at the site of MD formation [70]. *Wnt4*-null mice are defective in the initiation of MD formation, resulting in the absence of female reproductive tract including the uterus. While the invagination of the coelomic epithelium into urogenital ridge mesenchyme does not occur in *Wnt4*-null embryos, *Lhx1* turns on in the coelomic epithelium at the anterior end of the mesonephros, where the invagination should occur in normal embryos [28]. Therefore, differentiation of coelomic epithelium into MD precursor cells appears to occur independently of *Wnt4*. A neutralizing antibody against WNT4 inhibited the caudal elongation of MDs in the ex vivo urogenital ridge explant cultures, indicating that WNT4 is required not only for the initiation but also for the elongation of MDs [71]. *Wnt4* also plays an essential role in ovarian development: WNT4 represses male differentiation pathways in XX gonads by inhibiting the migration of endothelial and steroidogenic cells. Thus, null mutation of *Wnt4* results in masculinization of the gonad in XX mouse embryos [72]. In human, missense mutations in *WNT4* have been identified in four adolescent girls with MRKHS and hyperandrogenism [73, 74], confirming the observations in mouse studies.

Wnt5a *Wnt5a* activates the noncanonical WNT/planar cell polarity (PCP) or WNT/Ca^{2+} pathways. In the WNT/PCP pathway, WNT ligands are recognized by the receptor complex consisting of Frizzled and retinoic acid-related orphan receptor (ROR)/receptor tyrosine kinase (RYK) receptor [75], and the signal is transduced independent of CTNNB1. *Wnt5a* is expressed in the mesenchyme of developing female reproductive tract [76]. The null mutant mice for *Wnt5a* lacked the caudal portion of MD-derived organs due to impaired caudal elongation of the MDs [77]. However, *Wnt5a* is dispensable for the lateral fusion of two MDs, and the anterior portion of the fused cervix was present in some *Wnt5a*-null embryos targeted deletion of *Wnt5a* and *Wnt4* in MD mesenchyme by *Amhr2-Cre* resulted in the complete loss of vagina [78], confirming the essential role of mesenchymal WNTs in the caudal elongation of MDs.

Mutations in *WNT5A* and its receptor *ROR2* cause the Robinow syndrome (OMIM: 180700), a rare genetic condition characterized by short stature, limb shortening, genital hypoplasia, and craniofacial abnormalities [79]. While renal defects are reported in a subpopulation of patients, both male and female patients are fertile, and MD agenesis has not been reported in patients affected with the Robinow syndrome. To date, there is no report that links human *WNT5A* mutation and MD agenesis.

Genes Required for Development of the Urinary System

The developmental origin and process of the reproductive system are closely related to those of the urinary system [3]. Three developmentally overlapping excretory systems, the pronephros, mesonephros, and metanephros, form during embryogenesis of amniotes, including mammals [80, 81]. The genes listed below are essential for the formation of the urinary system including WD (mesonephric duct). Since WD development is prerequisite, these genes are also required for the normal development of MD.

Wnt9b *Wnt9b* is essential for the development of WDs and metanephric tubules: nephric tubule formation through mesenchymal-to-epithelial transition does not occur in *Wnt9b*-null embryos [82]. While *Wnt9b* is not expressed in MDs, the initial segment of MDs fails to elongate in *Wnt9b*-null embryos due to the WD defects. When the WDs were rescued in *Wnt9b*-null embryos by targeted expression of *Wnt1* in the WD epithelium, *Wnt9b*-null MDs elongated normally [82]. This observation confirms the essential role of WDs in the caudal elongation but not in the initial formation of the MDs.

In a small subset of MRKHS patients, several *WNT9B* mutations that are potentially pathogenic by in silico analyses (PolyPhe-2, SIFT, and MutationTaster) have been reported [83]. However, all patients are classified in type 1 MRKHS. Given the MD agenesis in *Wnt9b*-null mouse embryos was secondary to the defects in WD development, the absence of renal malformation in these patients questions the link between the *WNT9B* mutations and MRKHS.

Hnf1b HNF1B (HNF1 Homeobox B), a POU homeodomain transcription factor, is required for multiple steps of early kidney development, acting directly upstream of *Wnt9b*. In *Hnf1b*-null embryos, the WDs show reduced expression levels of *Lhx1* and *Pax2* and prematurely degenerate. Although MDs do not develop in the absence of *Hnf1b*, supplementation of WNT9B rescued MD in the urogenital tract explants from *Hnf1b*-null embryos [84], suggesting that MD defects in *Hnf1b*-null embryos are secondary to WD defects [85]. In agreement with mouse studies, *HNF1B* mutations in human are associated with hereditary urogenital dysplasia, a familiar condition with uterine and renal malformations [86]. Human *HNF1B* mutations are also associated with Renal Cysts and Diabetes Syndrome (RCAD) (OMIM: 137920), an autosomal dominant disorder comprising abnormal renal development and diabetes

[87]. There have been reports of vaginal aplasia, rudimentary uterus [88], and bicornuate uterus [89, 90] in individuals affected with RCAD.

Gata3 *Gata3* (GATA Binding Protein 3) encodes a member of the GATA transcription factor family. GATA3 acts as a crucial mediator of canonical WNT signaling pathway in the formation of the urinary system. Thus, WD epithelium-specific inactivation of *Gata3* by *Hoxb7-Cre* resulted in a spectrum of urogenital malformations, reminiscent of the CAKUT observed in human [91]. Targeted deletion of *Gata3* in WD epithelium resulted in complete loss of the uterus in more than 85% of female mice, suggesting that the uterine defects are secondary to the defects in the WD development.

In human, haploinsufficiency of *GATA3* causes hypoparathyroidism, deafness, and renal dysplasia (HDR) syndrome (OMIM: 146255), a rare disease with wide phenotypic variability. A review of 115 cases found hypoparathyroidism and deafness in 95% and renal abnormalities in 60% of affected individuals [92], but MD defects were not reported. There has been a Spanish familial case (mother and daughter) of HDR syndrome with female genital tract malformation: The mother had a nonfunctional right kidney and septate uterus, whereas the daughter had right renal agenesis and uterus didelphys with septate vagina [93].

Dlg1 *Dlg1* (discs large MAGUK scaffold protein 1) encodes a member of the membrane-associated guanylate kinase (MAGUK) scaffolding proteins, expressed throughout the body in epithelial cells. The null mutation of *Dlg1* in mice causes agenesis of lower MDs with the absence of lateral fusion. The fusion defect in MDs appears to be secondary to the defects in the urinary system. In *Dlg1*-null mice, the lower end of the ureter did not descend to the bladder wall and remained connected to WD at E14.5 [94]. Furthermore, the MD was connected to the common nephric duct in some *Dlg1*-null embryos, suggesting that descending and lateral fusion of MDs are physically obstructed by malformations of the nephric system.

Molecular Mechanism of MD Regression

As described in section "Regression of MDs in the Male Fetus", MDs are degenerated in male fetuses by the action of AMH. Binding of AMH to its type II receptor AMHR2 induces the formation of a heterodimer complex with a type I receptor, either BMPR1A (bone morphogenetic protein receptor, type 1A) [95] or ACVR1 (activin A receptor type 1) [96]. AMH action on MDs is transduced by the SMAD pathway, which is triggered by phosphorylation of receptor-regulated SMADs (R-SMADs) leading to the formation and nuclear translocation of transcriptionally active trimers consisting of two phospho-R-SMADs and one SMAD4 [97, 98]. Three R-SMADs, SMAD1, 5, and 9, play redundant functions in AMH signal transduction. Phosphorylation of SMAD1/5/9 by AMHR2/BMPR1A or AMHR2/ACVR1 complexes induces the formation and nuclear translocation of the SMAD transcription factor complex and activates expression of target genes. WNT7A

secreted by the MD epithelium induces expression of AMHR2 in the MD mesenchyme [99]. Thus, male *Wnt7a*-null mice retain MDs [100]. Similarly, the deletion of *Ctnnb1* in MD mesenchyme by *Amhr2-Cre* inhibits regression of MDs in male mice [101], suggesting that WNT7A induces AMHR2 expression through the canonical WNT pathway. Paradoxically, constitutive activation of CTNNB1 by deletion of exon 3 also blocks MD regression [102].

Wif1 (WNT Inhibitory Factor 1) and *Sp7* (Sp7 transcription factor) are the downstream effectors of AMH in the regression of MDs. Both genes are induced in the MD mesenchyme by AMH. *Wif1* knockdown in male urogenital tract inhibits the regression of MDs [103], and the null mutation of *Sp7* delays the regression of MDs in male embryos [104]. AMH also induces *Wnt4* expression in the MD mesenchyme. However, the function of WNT4 in MD regression remains unclear because the deletion of *Wnt4* floxed allele by *Amhr2-Cre* did not inhibit the regression of MD in male mice [99].

A defect in the pathways mentioned above can result in Persistent Müllerian duct syndrome (PMDS, OMIM:261550), which presents the development of the female reproductive tracts in males with normal male reproductive organs. Typically, PMDS patients also present bilateral cryptorchidism and inguinal hernias [105]. PMDS is caused by deleterious mutations in *AMH* (PMDS Type I) [106] or *AMHR2* (PMDS Type II) [107]. On the other hand, there is no evidence that some Müllerian agenesis in women is caused by the uncontrolled activity of the AMH pathway.

Anatomy and Physiology of the Uterus

Basic Structure of the Uterus

During female reproductive tract development, MDs undergo a dynamic transformation from a homogeneous cellular structure (Fig. 1.1e) into anatomically and functionally distinctive organs, including oviduct, uterus, cervix, and vagina (Fig. 1.1f). In humans, the uterus develops from the upper portion of fused MDs. Although the gross morphology of the adult uterus is quite diverse among species, the developmental process and basic structure are common among the uteri of all mammals. Mature uteri consist of three layers: the endometrium (uterine mucosa), the myometrium (smooth muscle wall), and a serosa (peritoneum) (Fig. 1.1f). The endometrium, the innermost layer of the uterus, is the mucosal lining of the uterine cavity (Fig. 1.2). The myometrium is the main bulk of the uterus, which is composed of layers of smooth muscles. The serosa consists of superficial mesothelium and a thin layer of loose connective tissue beneath and encapsulates the uterine corpus.

Morphogenesis of the Uterus

Prenatal Development In the human fetus, the future uterine corpus and cervix become anatomically recognizable in the upper portion of the fused MDs by 16 weeks after LMP (Fig. 1.1e). While the boundary of future endocervix and uterus

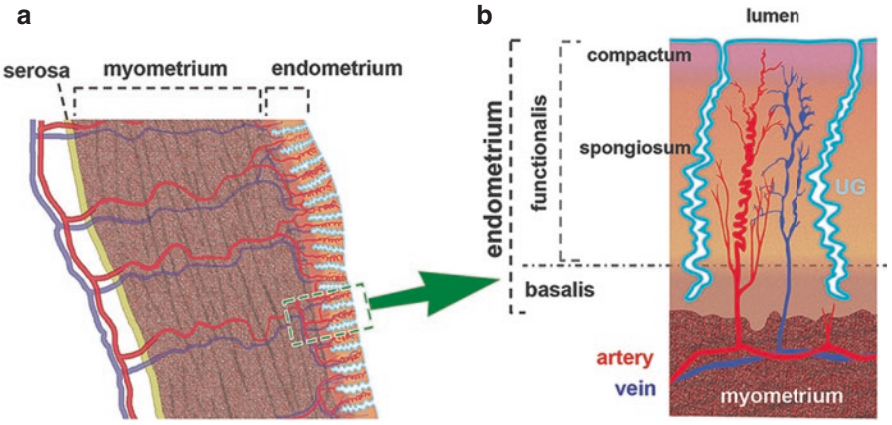

Fig. 1.2 (**a**, **b**) Structure of human endometrium

is unclear at this stage, the expression of TP63 transcription factor in the ectocervical and vaginal epithelia marks the future boundary of the endocervix and ectocervix [108–111]. The formation of the outer smooth muscle layer is first observed in the lower segments of MD mesenchyme by 9 weeks and progresses cranially, as assessed by the expression of α-smooth muscle actin (ATCA2) [5]. By 20 weeks, the differentiation of urogenital ridge mesenchyme into the myometrium and endometrial stroma becomes apparent in the uterine segments. Differentiation of epithelial cells in the fused segments of MD appears to progress from caudal to cranial. The formation of the cervical gland is initiated as an invagination of the luminal epithelium into the stroma by 20 weeks, and uterine glands appear around 28 weeks. While the uterine glands gradually increase the size and depth in the female fetus, the structure remains rudimental at birth [112].

Postnatal Development Uterine volume significantly decreases in the first few months of postnatal development, likely due to the withdrawal of maternal 17β-estradiol (E2) [113]. In the first 7 years, the overall uterine volume remains relatively constant as measured by MRI and pelvic ultrasonography [114], even though the size of the uterine corpus and the complexity and depth of the uterine glands gradually increase [112]. Dramatic increases in the uterine volume and endometrial thickness occur when ovaries become active in puberty [113–117].

Structure of the Endometrium (Fig. 1.2b)

The endometrium consists of columnar luminal epithelium, supported by cellular stroma containing tubular glands, and lines the uterine cavity providing the environment for fetal development. The endometrium is divided into stratum basalis (basal layer) and stratum functionalis (functional layer). The stratum functionalis is the thick superficial/inner layer, which can be further subdivided into two zones, the

stratum compactum and the stratum spongiosum: The stratum compactum is nearest to the uterine cavity with dense stroma directly supporting luminal epithelial cells; the stratum spongiosum is the middle layer between the stratum compactum and the stratum basalis, consisting of dilated and coiled portions of the uterine glands. The stratum basalis is adjacent to the myometrium, containing the permanent stroma and deep ends of glands, which are not shed at menstruation.

Menstrual Cycle and Endometrial Structure

Menstruation, the shedding of the stratum functionalis with associated bleeding, is triggered by the fall of progesterone (P4) due to regression of the corpus luteum at the end of an unsuccessful reproductive cycle [118]. The structures of endometrium dramatically change during the menstrual cycles as the functionalis grows anew from the stratum basalis. The first day of menstrual flow marks the first day of a new menstrual cycle. Menstruation lasts for 3–7 days on average. At the end of menstruation, follicle-stimulating hormone (FSH), secreted by the anterior pituitary gland, initiates the maturation process of ovarian follicles and stimulates E2 production in ovaries. As stimulated by rising levels of E2, the endometrial mucosa regenerates from the deep glands and stromal cells within the stratum basalis. As the functionalis layer gradually increases the thickness during the proliferative phase, the endometrial glands increase in length size, and spiral arteries elongate into the stroma toward the surface. Once the levels of E2 peak, the pituitary gland begins to secrete luteinizing hormone (LH), while the secretion of FSH wanes. LH surge triggers ovulation. Then, differentiation of the burst follicle into corpus luteum leads to the secretion E2 and P4, which initiates the secretory phase or luteal phase. In this phase, the microscopic features of the endometrium change almost daily, allowing the pathologists to date the menstrual cycle [119–121]. During the secretory phase, the endometrial lining reaches its maximal thickness through cellular proliferation and the accumulation of stromal extracellular matrix. The uterine glands become even more intricately coiled, and glandular epithelial cells accumulate glycogen-rich vacuoles at their base. The spiral arteries also increase the coiling and rapidly lengthen. In response to elevated P4, decidual changes are observed in the stratum compactum [122]. Few days after ovulation, edema appears in the surface layer of the stroma, but the edema is gradually replaced by contiguous large stromal cells with abundant cytoplasm and large pale nuclei [123]. By the end of the secretory phase, the superficial endometrial stroma appears nearly solidified, and the decidua-like stromal cells in the stratum compactum express the decidual markers, such as prolactin (PRL) [124] and insulin-like growth factor binding protein-1 (IGFBP1) [125]. If conception and implantation do not occur, P4 levels decline as the corpus luteum regresses, leading to menstruation.

Menstruation occurs only in a small number of mammalian species including the higher primates, some fruit bats, the elephant shrew, and the spiny mouse [126–129]. In non-menstruating mammals, the fall in P4 regresses endometrial tissues through apoptosis without shedding of functionalis [130, 131].

Endometrial Stem Cells

Many mammalian adult tissues contain tissue stem cells, which proliferate to compensate for tissue loss [132]. Adult tissue stem cells generally self-preserve and persist in a quiescent and undifferentiated state for an extended period, as enforced by the microenvironment of the stem cell niche [133]. The highest-turnover tissues, such as the intestinal epithelium, epidermis, and hematopoietic system, organize their stem cell compartments into a hierarchical structure, in which a slow-cycling stem cell residing at the top of the hierarchy gives rise to transient amplifying cells that bear the proliferative burden. Hayflick and Moorhead discovered that a normal human fetal cell population could divide only a finite number of times (average 40–60) in culture (Hayflick limit) [134]. The Hayflick limit is explained by telomere dysfunction. The telomere is a region of repetitive nucleotide sequences at each end of linear DNA strand, which forms large loop structures called telomere loops that protect chromosome ends from fusion and being recognized as DNA damage [135]. Telomere dysfunction can be repaired only by telomerase, a ribonucleoprotein complex that extends telomeres [136]. Since telomerase activity is generally low in normal somatic cells, telomeres are progressively shortened in each cell cycle due to the end replication problem [137]. Consequently, when telomeres become too short of forming the telomere loop, the exposed chromosome ends trigger DNA damage response, leading to cellular senescence, a state of irreversible cell cycle arrest [138]. The hierarchical stem cell system helps to maintain the regenerative capacity of tissues throughout the lifetime of the organism by limiting the number of cell division that the stem cells undergo.

The stratum functionalis is a highly regenerative tissue that undergoes monthly cycles of growth, differentiation, and shedding. It has been estimated that the average women in developed countries undergo menstruation over 400 times in life [139]. Therefore, the presence of endometrial stem cells in the stratum basalis has been postulated [139–142]. However, the identity and characteristics of endometrial stem cells remain elusive. A recent genome-sequencing analysis of individual uterine glands (UGs) within normal endometrium has revealed that UGs of the same individual carry distinct somatic mutations with an allele frequency as high as 0.5, suggesting that UG in the adult uterus arises through the expansion of a small number of epithelial stem cell [143]. Meanwhile, substantial levels of telomerase activity have been detected in the epithelial and stromal cells of regenerating endometrium [144–146]. Therefore, the regenerative capacity of the endometrium may be maintained independently of the hierarchical stem cell system.

Uterine Glands

Structure and Function of Uterine Glands

UGs or endometrial glands of the adult human uterus are simple tubular (no brunching) and lined by secretory simple columnar epithelial cells. Development of human UGs begins in the third trimester as the invagination of the luminal epithelium into

underlying stroma. However, most growth and differentiation occur during the menstrual cycle as discussed above.

Mouse UG formation begins around postnatal day 5 (PD5) as an epithelial invagination consisting of a small number of epithelial cells differentiated from the luminal epithelium [147] and nearly completes before puberty [148]. Unlike UGs in human endometrium, UGs in adult mice are branched (compound glands) [147, 149]. In mice, epithelial cells in each UG are not monoclonal: The expression of an X-linked GFP transgene was heterogeneous among a cluster of UGs [150], which shares the main duct that opens to the lumen [149].

Mouse studies demonstrated that the main function of UG is to secrete substances that are essential for the establishment of pregnancy and subsequent development of the fetus. In mice, secretion of leukemia inhibitory factor (LIF), an interleukin-6 (IL6) class cytokine, by UGs is essential for pregnancy [151]. The *Lif* null female mice are defective in blastocyst implantation and decidualization [152]; however, a one-time administration of exogenous LIF can restore fertility [153]. Although the requirement of UG and LIF in human pregnancy remains to be determined, secretion of IL6 class cytokines by UGs is generally considered to be essential for the establishment of pregnancy in women [154].

Genes and Pathways Essential for UG Formation and Maintenance in Mice

Foxa2 FOXA2 (Forkhead Box A2) belongs to the forkhead transcription factor family and plays essential roles in the development of multiple systems. In the uterus, FOXA2 is expressed exclusively in glandular epithelial cells. Conditional inactivation of *Foxa2* in the uterus after birth by using Pgr^{Cre} results in loss of UGs and subsequent infertility with reduced expression of LIF [155].

Genes Involved in the Canonical WNT-CTNNB1 Pathway The loss of genes encoding the WNT ligands, *Wnt4* [156] and *Wnt7a* [100, 157, 158], and the downstream transcription regulators of the canonical WNT pathway, *Ctnnb1* [155, 159] and *Lef1* [160], in the uterine cells resulted in the absence of UGs, indicating importance of the canonical WNT pathway in UG formation during uterine development. A recent mouse study indicated that cell-autonomous activation of the WNT-CTNNB1 pathway in uterine epithelial cells is sufficient to initiate the UG formation: Stabilization of CTNNB1 protein in the fully differentiated luminal epithelium of mouse endometrium triggered the formation of UGs [161]. Furthermore, the continuous presence of the canonical WNT pathway activity is essential for the maintenance of UGs: Deletion of *Ctnnb1* in the epithelium of mature uteri resulted in the loss of FOXA2 expression in the UGs [161]. A mouse study suggested that activity of the canonical WNT pathway may be involved in the pathogenesis of adenomyosis, a benign gynecologic condition defined by the presence of endometrial tissues including UGs within the myometrium. In mice, constitutive activation of CTNNB1 in the endometrial epithelium and stroma by Pgr^{Cre} drove the myometrial

invasion of endometrial tissues [162]. However, the involvement of the canonical WNT pathway in the pathogenesis of human adenomyosis remains to be demonstrated.

Porcn Critical role of WNT pathways in the maintenance of UG was also demonstrated by conditional deletion of *Porcn* (porcupine O-acyltransferase), a gene encodes a membrane-bound O-acyltransferase that is essential for acylation of all WNT ligands. Since acylation is essential for secretion and receptor binding of WNT ligands, deletion of *Porcn* by Pgr^{Cre} inactivates WNT pathways in both epithelial and stromal cells of the uterus. As deletion of *Porcn* by Pgr^{Cre} occurs postnatally, UG initially developed normally, but the postnatal loss of *Porcn* resulted in progressive regression of endometrial stroma and UGs in the adult uterus [163].

Lgr4 LGR4 (leucine-rich repeat-containing G-protein-coupled receptor 4) is a G-protein-coupled receptor that binds R-spondins and potentiates the WNT signaling pathway [164]. Thus, loss of LGR4 attenuates WNT pathway activity. Deletion of *Lgr4* in the embryonic uterine epithelium by *Krt5-Cre* significantly reduced the number of UGs, resulted in subfertility of female mice [165].

Sox17 *Sox17* (SRY-box 17) encodes for a transcription factor that is essential for UG formation. In the mouse uterus, SOX17 is expressed in luminal and glandular epithelium as well as endothelial cells [161, 166, 167]. Guimaraes-Young et al. proposed that mesenchymal *Sox17* is essential for the UG formation because the deletion of *Sox17* in the uterine epithelium and stroma by Pgr^{Cre} – but not the epithelial-specific deletion by *Sprr2f-Cre* – ablated UGs [167]. The result was however misinterpreted as the relative timing of Cre expression in UG formation was not considered. The endogenous PGR turns on in the uterine epithelium around PD3 [168], whereas PGR expression in uterine stroma does not occur in the neonatal uterus, as it is estrogen-dependent [169]. In addition, PGR is not expressed in endothelial cells. Therefore, Pgr^{Cre} deletes *Sox17* only in the epithelium of the neonatal uterus before UGs formation, whereas the deletion of *Sox17* in the uterine epithelium by *Sprr2f-Cre* occurs after UG formation.

In endoderm formation, SOX17 is an essential downstream transcription factor of the canonical WNT pathway [170]. During endoderm specification in *Xenopus*, SOX17 regulates FOXA2 expression as a co-transcription factor of CTNNB1 [171]. Hence, SOX17 may regulate UG formation as a part of the transcription factor network under the control of the canonical WNT pathway.

Dlx5/Dlx6 Distal-Less Homeobox 5 (*Dlx5)* and *Dlx6* are clustered homeobox genes required for craniofacial, axial, and appendicular skeletal development [172]. The activity of *Dlx5* allele was detected in the median part of MDs, from which uterus develops. In the mature uterus, both *Dlx5* and *Dlx6* were detected in the luminal and glandular epithelium [173]. The *Dlx5/Dlx6* bigenic cluster is essential for UG formation, given conditional deletion of the cluster by Pgr^{Cre} resulted in a drastic reduction of UGs. It has been shown that *Dlx5* is a target gene of the canonical

WNT pathway in semicircular canals [174]. Furthermore, in human endometrial cancer, exon 3 mutations in *CTNNB1*, which stabilize the CTNNB1 protein, are associated with overexpression of *DLX5* and *DLX6* [161]. These observations suggest that DLX5/DLX6 are downstream transcription factors of the canonical WNT pathway in the formation of UGs.

Wnt5a Although *Wnt5a* does not activate the canonical WNT pathway, a mouse study suggests that *Wnt5a* may be involved in the UG development: When uterine pieces from *Wnt5a*-null mice were rescued by kidney grafting, only 17% of uterine explants developed UGs [77].

Pik3ca The phosphatidylinositol 3-kinase (PI3K)>AKT pathway is a key regulator of fundamental cellular processes including intracellular trafficking, metabolism, cell growth, proliferation, motility, and survival. Class I PI3Ks catalyze phosphatidylinositol-4,5-biphosphate to phosphatidylinositol (3,4,5)-trisphosphate (PIP3), and the elevated PIP3 level triggers the cascade of PI3K > AKT pathway: The binding of PIP3 anchors AKT to the plasma membrane, where AKT is activated by phosphoinositide-dependent kinase 1 (PDK1). In turn, AKT activates multiple downstream targets, including mammalian target of rapamycin (mTOR), to promote cell survival and growth. Thus, aberrant activation of the PI3K > AKT pathway is commonly observed in many human cancers. Class I PI3Ks are heterodimers consisting of a p110 catalytic subunit and a p85 regulatory subunit. In human endometrium, p110α encoded by *PIK3CA* and p85α encoded by *PIK3R1* are the dominant isoforms of PI3K subunits, and *PIK3CA* is one of the most frequently mutated genes in human endometrial cancers [175]. In mice, the formation of UGs can be induced by an activating mutation of *Ctnnb1*. However, the growth of UGs requires an additional mutation that activates PI3K [161]. In fact, conditional deletion of *Pik3ca* in uterine epithelium and stroma by Pgr^{Cre} results in a significant reduction of UGs [176], indicating the critical role of PI3K > AKT pathway activity in innate UG formation.

Nf2 *Nf2* (Neurofibromin 2) encodes FERM domain-containing cytoskeletal protein Merlin/NF2, which organizes the cell cortex by assembling membrane complexes and linking them to the cortical actin cytoskeleton [177]. Accordingly, the loss of NF2 affects epithelial morphogenesis in many tissues [178]. Inactivation of *Nf2* in the MD epithelium (*Wnt7a-Cre*) or neonatal uterus (Pgr^{Cre}) ablates UGs without affecting the canonical WNT pathway activity and induction of FOXA2 expression in the luminal epithelial cells [179], confirming the essential role of NF2 in epithelial morphogenesis.

Developmental Genes in the Adult Endometrium

Many developmental genes described above persist in the adult endometrium. For instance, HOX genes and WNT pathway comprise a gene regulatory loop (e.g., WNT5A-WNT7-HOXA10/HOXA11) in the mouse adult uterus [8, 44, 77,

155–157, 180]. The expression patterns of developmental genes appear to reflect the dynamic nature of the endometrium, which undergoes cycles of regeneration and regression in response to changes in systemic hormone levels. For example, Abdominal-B class HOX genes are regulated by P4 in the uterine stroma of adult mice [44]. Multiple WNT ligands are also regulated by E2 and P4 in the uteri of cycling female mice [76, 181, 182]. Thus, hormone actions in adult endometrium are likely mediated by developmental signaling pathways.

E2 and P4 effects on endometrium are primarily mediated by estrogen receptor α (ESR1) and progesterone receptor (PGR), respectively. Mouse studies have demonstrated that E2 and P4 regulate epithelial functions in the uterus via receptors in stromal cells [131, 169, 183–188]. Meanwhile, it is unclear if the functions of human endometrial epithelial cells are regulated by E2 and P4 via ESR1 and PGR in stromal cells.

In Utero Exposure to Diethylstilbestrol (T-Shaped Uterus)

A T-shaped uterus refers to a type of uterine malformation wherein the radiographic appearance of uterine cavity resembles the letter T. A T-shaped uterus is one of the most common urogenital abnormalities associated with in utero exposure to diethylstilbestrol (DES) [189, 190], an orally active nonsteroidal estrogen [191, 192]. Several studies revealed the poor reproductive performance of women with T-shaped uteri when untreated [193, 194]. In 1947, The United States Food and Drug Administration (FDA) approved DES for preventing miscarriages, based on an uncontrolled study with a small number of participants. In 1953, a large-scale double-blind clinical trial involving 1646 pregnant participants concluded that DES did not reduce the incidence of abortion but may actually "favor" premature labor [195]. Nonetheless, DES remained a routine treatment for pregnant women until 1971, when a retrospective case–control study identified a statistically significant association between in utero DES exposure and risk of vaginal adenocarcinoma [196]. This finding was supported by another case–control study published in the same year [197]. Consequently, the US FDA banned its use during pregnancy. However, it is estimated that more than two million mothers, daughters, and sons were already exposed to DES in the United States alone [198].

The most common condition found in women who were exposed to DES in utero is vaginal adenosis, a putative precursor of vaginal adenocarcinoma, characterized by the development of columnar epithelia in the vagina [199, 200]. Several mouse studies have revealed the molecular mechanisms underlying the pathogenesis of vaginal adenosis [108, 109, 201–203]. On the other hand, the molecular etiology of the most common uterine defect, a T-shaped uterus, remains unclear, primarily due to the absence of appropriate animal models, as the anatomy of human and rodent uteri is distinctively different. Nonetheless, it has been shown in mice that DES action via ESR1 inhibits the expression of genes critical for uterine development, including *Wnt7a*, *Hoxa10*, and *Hoxa11* [44, 204, 205]. In utero DES exposure may have caused the T-shaped uterus through deregulation of the WNT-HOX gene regulatory loop.

Challenges in Human Uterine Biology Research

Mechanistic insight into uterine development has been developed through mouse genetic studies. Since the early organogenesis of human and mouse uteri is fundamentally identical, the molecular mechanisms are also expected to be common between these two species. Indeed, human mutations replicate the urogenital phenotypes of corresponding mutant mice. However, the reproductive cycles are fundamentally different: 4-week menstrual cycle in women versus 4-day estrus cycle in female mice. The physiological difference is reflected to the innate growth characteristics of epithelial cells: in vivo proliferation of human uterine epithelial cells requires exposure to E2 for a longer duration compared to the mouse uterine epithelial cells [206]. The regeneration of the stratum functionalis from the stratum basalis also raises a critical question: Does the UG growth during the menstrual cycle share the molecular mechanisms with the developmental formation of UGs? A xenograft study of human UGs suggested that the luminal epithelium in human endometrium regenerates from UGs: Human endometrial xenografts consisting of isolated UGs and singly dissociated endometrial stromal cells developed normal human endometrial tissues containing both luminal and glandular epithelia [206]. The absence of animal models suitable for mechanistic studies of menstrual cycle limits research on the molecular mechanism of endometrial shedding and regeneration, including the presence and nature of endometrial stem cells. Nevertheless, the current knowledge gap in the physiology of human uterus will be eventually filled by advances in human genetics and molecular analysis technologies. The molecular mechanism underlying the physiology of menstruation will provide insight into the uterine condition and disease as well as the adaptive value of menstruation in mammalian evolution.

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References

1. Kurita T. Developmental origin of vaginal epithelium. Differentiation. 2010;80(2–3):99–105.
2. Müller J. Bildungsgeschichte der Genitalien aus anatomischen Untersuchungen an Embryonen des Menschen und der Thiere; nebst einem Anhang über die chirurgische Behandlung der Hypospadia. Düsseldorf: Arnz; 1830.
3. O'Rahilly R. The embryology and anatomy of the uterus. In: Wynn RM, editor. The uterus. International Academy of Pathology, Monograph. 14. Baltimore: The Williams & Wilkins Co.; 1973. p. 17–39.
4. Kobayashi A, Behringer RR. Developmental genetics of the female reproductive tract in mammals. Nat Rev Genet. 2003;4(12):969–80.
5. Robboy SJ, Kurita T, Baskin L, Cunha GR. New insights into human female reproductive tract development. Differentiation. 2017;97:9–22.
6. Grünwald P. The relation of the growing tip of the Müllerian duct to the Wolffian duct and its importance for the genesis of malformations. Anat Rec. 1941;81:1–19.

7. Orvis GD, Behringer RR. Cellular mechanisms of Müllerian duct formation in the mouse. Dev Biol. 2007;306(2):493–504.
8. Kurita T. Normal and abnormal epithelial differentiation in the female reproductive tract. Differentiation. 2011;82(3):117–26.
9. Kurita T, Nakamura H. Embryology of the uterus. In: Aplin JD, Fazleabas AT, Glasser SR, Giudice LC, editors. Endometrium. 2nd ed. London, UK: Informa UK Ltd.; 2008. p. 1–18.
10. Koff AK. Development of the vagina in the human fetus. Contrib Embryol. 1933;24(140):59–91.
11. Hashimoto R. Development of the human Müllerian duct in the sexually undifferentiated stage. Anat Rec A Discov Mol Cell Evol Biol. 2003;272(2):514–9.
12. Rey R, Josso N, Racine C. Sexual differentiation. In: Feingold KR, Anawalt B, Boyce A, Chrousos G, Dungan K, Grossman A, et al., editors. Endotext. South Dartmouth (MA): MDText.com, Inc.; 2000.
13. Foster JW, Dominguez-Steglich MA, Guioli S, Kwok C, Weller PA, Stevanovic M, et al. Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. Nature. 1994;372(6506):525–30.
14. Wagner T, Wirth J, Meyer J, Zabel B, Held M, Zimmer J, et al. Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. Cell. 1994;79(6):1111–20.
15. De Santa BP, Bonneaud N, Boizet B, Desclozeaux M, Moniot B, Sudbeck P, et al. Direct interaction of SRY-related protein SOX9 and steroidogenic factor 1 regulates transcription of the human anti-Mullerian hormone gene. Mol Cell Biol. 1998;18(11):6653–65.
16. Nicol B, Yao HH. Building an ovary: insights into establishment of somatic cell lineages in the mouse. Sex Dev. 2014;8(5):243–51.
17. Pannetier M, Chassot AA, Chaboissier MC, Pailhoux E. Involvement of FOXL2 and RSPO1 in ovarian determination, development, and maintenance in mammals. Sex Dev. 2016;10(4):167–84.
18. Choussein S, Nasioudis D, Schizas D, Economopoulos KP. Mullerian dysgenesis: a critical review of the literature. Arch Gynecol Obstet. 2017;295(6):1369–81.
19. Patnaik SS, Brazile B, Dandolu V, Ryan PL, Liao J. Mayer-Rokitansky-Kuster-Hauser (MRKH) syndrome: a historical perspective. Gene. 2015;555(1):33–40.
20. Fontana L, Gentilin B, Fedele L, Gervasini C, Miozzo M. Genetics of Mayer-Rokitansky-Kuster-Hauser (MRKH) syndrome. Clin Genet. 2017;91(2):233–46.
21. Jacquinet A, Millar D, Lehman A. Etiologies of uterine malformations. Am J Med Genet A. 2016;170(8):2141–72.
22. The American Fertility Society classifications of adnexal adhesions, distal tubal occlusion, tubal occlusion secondary to tubal ligation, tubal pregnancies, mullerian anomalies and intrauterine adhesions. Fertil Steril. 1988;49(6):944–55.
23. Mossman HW. Comparative anatomy. In: Wynn RM, editor. Biology of the uterus. New York: Plenum Press; 1977. p. 19–34.
24. Maltais LJ, Blake JA, Eppig JT, Davisson MT. Rules and guidelines for mouse gene nomenclature: a condensed version. International Committee on Standardized Genetic Nomenclature for Mice. Genomics. 1997;45(2):471–6.
25. White JA, McAlpine PJ, Antonarakis S, Cann H, Eppig JT, Frazer K, et al. Guidelines for human gene nomenclature (1997). HUGO Nomenclature Committee. Genomics. 1997;45(2):468–71.
26. Huang CC, Orvis GD, Kwan KM, Behringer RR. Lhx1 is required in Mullerian duct epithelium for uterine development. Dev Biol. 2014;389(2):124–36.
27. Kobayashi A, Kwan KM, Carroll TJ, McMahon AP, Mendelsohn CL, Behringer RR. Distinct and sequential tissue-specific activities of the LIM-class homeobox gene Lim1 for tubular morphogenesis during kidney development. Development. 2005;132(12):2809–23.
28. Kobayashi A, Shawlot W, Kania A, Behringer RR. Requirement of Lim1 for female reproductive tract development. Development. 2004;131(3):539–49.
29. Ledig S, Brucker S, Barresi G, Schomburg J, Rall K, Wieacker P. Frame shift mutation of LHX1 is associated with Mayer-Rokitansky-Kuster-Hauser (MRKH) syndrome. Hum Reprod. 2012;27(9):2872–5.

30. Davis RJ, Harding M, Moayedi Y, Mardon G. Mouse Dach1 and Dach2 are redundantly required for Mullerian duct development. Genesis. 2008;46(4):205–13.
31. Patel SR, Dressler GR. The genetics and epigenetics of kidney development. Semin Nephrol. 2013;33(4):314–26.
32. Bouchard M, Souabni A, Mandler M, Neubuser A, Busslinger M. Nephric lineage specification by Pax2 and Pax8. Genes Dev. 2002;16(22):2958–70.
33. Torres M, Gomez-Pardo E, Dressler GR, Gruss P. Pax-2 controls multiple steps of urogenital development. Development. 1995;121(12):4057–65.
34. Mansouri A, Chowdhury K, Gruss P. Follicular cells of the thyroid gland require Pax8 gene function. Nat Genet. 1998;19(1):87–90.
35. Boualia SK, Gaitan Y, Tremblay M, Sharma R, Cardin J, Kania A, et al. A core transcriptional network composed of Pax2/8, Gata3 and Lim1 regulates key players of pro/mesonephros morphogenesis. Dev Biol. 2013;382(2):555–66.
36. Barua M, Stellacci E, Stella L, Weins A, Genovese G, Muto V, et al. Mutations in PAX2 associate with adult-onset FSGS. J Am Soc Nephrol. 2014;25(9):1942–53.
37. Schimmenti LA. Renal coloboma syndrome. Eur J Hum Genet: EJHG. 2011;19(12): 1207–12.
38. Sanyanusin P, Schimmenti LA, McNoe LA, Ward TA, Pierpont ME, Sullivan MJ, et al. Mutation of the PAX2 gene in a family with optic nerve colobomas, renal anomalies and vesicoureteral reflux. Nat Genet. 1995;9(4):358–64.
39. Meeus L, Gilbert B, Rydlewski C, Parma J, Roussie AL, Abramowicz M, et al. Characterization of a novel loss of function mutation of PAX8 in a familial case of congenital hypothyroidism with in-place, normal-sized thyroid. J Clin Endocrinol Metab. 2004;89(9):4285–91.
40. Madariaga L, Moriniere V, Jeanpierre C, Bouvier R, Loget P, Martinovic J, et al. Severe prenatal renal anomalies associated with mutations in HNF1B or PAX2 genes. Clin J Am Soc Nephrol. 2013;8(7):1179–87.
41. Miyamoto N, Yoshida M, Kuratani S, Matsuo I, Aizawa S. Defects of urogenital development in mice lacking Emx2. Development. 1997;124(9):1653–64.
42. McGinnis W, Krumlauf R. Homeobox genes and axial patterning. Cell. 1992;68(2):283–302.
43. Dollé P, Izpisua-Belmonte JC, Brown JM, Tickle C, Duboule D. HOX-4 genes and the morphogenesis of mammalian genitalia. Genes Dev. 1991;5(10):1767–75.
44. Ma L, Benson GV, Lim H, Dey SK, Maas RL. Abdominal B (AbdB) Hoxa genes: regulation in adult uterus by estrogen and progesterone and repression in Müllerian duct by the synthetic estrogen diethylstilbestrol (DES). Dev Biol. 1998;197(2):141–54.
45. Warot X, Fromental-Ramain C, Fraulob V, Chambon P, Dolle P. Gene dosage-dependent effects of the Hoxa-13 and Hoxd-13 mutations on morphogenesis of the terminal parts of the digestive and urogenital tracts. Development. 1997;124(23):4781–91.
46. Raines AM, Adam M, Magella B, Meyer SE, Grimes HL, Dey SK, et al. Recombineering-based dissection of flanking and paralogous Hox gene functions in mouse reproductive tracts. Development. 2013;140(14):2942–52.
47. Benson GV, Lim H, Paria BC, Satokata I, Dey SK, Maas RL. Mechanisms of reduced fertility in Hoxa-10 mutant mice: uterine homeosis and loss of maternal Hoxa-10 expression. Development. 1996;122(9):2687–96.
48. Gendron RL, Paradis H, Hsieh-Li HM, Lee DW, Potter SS, Markoff E. Abnormal uterine stromal and glandular function associated with maternal reproductive defects in Hoxa-11 null mice. Biol Reprod. 1997;56(5):1097–105.
49. Branford WW, Benson GV, Ma L, Maas RL, Potter SS. Characterization of Hoxa-10/Hoxa-11 transheterozygotes reveals functional redundancy and regulatory interactions. Dev Biol. 2000;224(2):373–87.
50. Dolle P, Dierich A, LeMeur M, Schimmang T, Schuhbaur B, Chambon P, et al. Disruption of the Hoxd-13 gene induces localized heterochrony leading to mice with neotenic limbs. Cell. 1993;75(3):431–41.
51. Roux M, Bouchard M, Kmita M. Multifaceted Hoxa13 function in urogenital development underlies the Hand-Foot-Genital Syndrome. Hum Mol Genet. 2019;28(10):1671–81.

52. Mortlock DP, Innis JW. Mutation of HOXA13 in hand-foot-genital syndrome. Nat Genet. 1997;15(2):179–80.
53. Innis JW. Hand-foot-genital syndrome. In: Adam MP, Ardinger HH, Pagon RA, Wallace SE, LJH B, Stephens K, et al., editors. GeneReviews((R)). Seattle(WA): University of Washington, Seattle; 1993.
54. Goodman FR, Bacchelli C, Brady AF, Brueton LA, Fryns JP, Mortlock DP, et al. Novel HOXA13 mutations and the phenotypic spectrum of hand-foot-genital syndrome. Am J Hum Genet. 2000;67(1):197–202.
55. Debeer P, Bacchelli C, Scambler PJ, De Smet L, Fryns JP, Goodman FR. Severe digital abnormalities in a patient heterozygous for both a novel missense mutation in HOXD13 and a polyalanine tract expansion in HOXA13. J Med Genet. 2002;39(11):852–6.
56. Ekici AB, Strissel PL, Oppelt PG, Renner SP, Brucker S, Beckmann MW, et al. HOXA10 and HOXA13 sequence variations in human female genital malformations including congenital absence of the uterus and vagina. Gene. 2013;518(2):267–72.
57. Zhu Y, Cheng Z, Wang J, Liu B, Cheng L, Chen B, et al. A novel mutation of HOXA11 in a patient with septate uterus. Orphanet J Rare Dis. 2017;12(1):178.
58. Wilson JG, Warkany J. Malformations in the genito-urinary tract induced by maternal vitamin a deficiency in the rat. Am J Anat. 1948;83(3):357–407.
59. Mendelsohn C, Lohnes D, Decimo D, Lufkin T, LeMeur M, Chambon P, et al. Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. Development. 1994;120(10):2749–71.
60. Kastner P, Mark M, Ghyselinck N, Krezel W, Dupe V, Grondona JM, et al. Genetic evidence that the retinoid signal is transduced by heterodimeric RXR/RAR functional units during mouse development. Development. 1997;124(2):313–26.
61. Marshall H, Morrison A, Studer M, Popperl H, Krumlauf R. Retinoids and Hox genes. FASEB J. 1996;10(9):969–78.
62. Langston AW, Gudas LJ. Retinoic acid and homeobox gene regulation. Curr Opin Genet Dev. 1994;4(4):550–5.
63. DiMartino JF, Selleri L, Traver D, Firpo MT, Rhee J, Warnke R, et al. The Hox cofactor and proto-oncogene Pbx1 is required for maintenance of definitive hematopoiesis in the fetal liver. Blood. 2001;98(3):618–26.
64. Schnabel CA, Selleri L, Jacobs Y, Warnke R, Cleary ML. Expression of Pbx1b during mammalian organogenesis. Mech Dev. 2001;100(1):131–5.
65. Schnabel CA, Selleri L, Cleary ML. Pbx1 is essential for adrenal development and urogenital differentiation. Genesis. 2003;37(3):123–30.
66. Schnabel CA, Godin RE, Cleary ML. Pbx1 regulates nephrogenesis and ureteric branching in the developing kidney. Dev Biol. 2003;254(2):262–76.
67. Clevers H, Nusse R. Wnt/beta-catenin signaling and disease. Cell. 2012;149(6):1192–205.
68. Wang J, Sinha T, Wynshaw-Boris A. Wnt signaling in mammalian development: lessons from mouse genetics. Cold Spring Harb Perspect Biol. 2012;4:a007963.
69. Zhan T, Rindtorff N, Boutros M. Wnt signaling in cancer. Oncogene. 2016;36:1461.
70. Vainio S, Heikkila M, Kispert A, Chin N, McMahon AP. Female development in mammals is regulated by Wnt-4 signalling. Nature. 1999;397(6718):405–9.
71. Prunskaite-Hyyrylainen R, Skovorodkin I, Xu Q, Miinalainen I, Shan J, Vainio SJ. Wnt4 coordinates directional cell migration and extension of the Mullerian duct essential for ontogenesis of the female reproductive tract. Hum Mol Genet. 2016;25(6):1059–73.
72. Jeays-Ward K, Hoyle C, Brennan J, Dandonneau M, Alldus G, Capel B, et al. Endothelial and steroidogenic cell migration are regulated by WNT4 in the developing mammalian gonad. Development. 2003;130(16):3663–70.
73. Philibert P, Biason-Lauber A, Rouzier R, Pienkowski C, Paris F, Konrad D, et al. Identification and functional analysis of a new WNT4 gene mutation among 28 adolescent girls with primary amenorrhea and mullerian duct abnormalities: a French collaborative study. J Clin Endocrinol Metab. 2008;93(3):895–900.

74. Philibert P, Biason-Lauber A, Gueorguieva I, Stuckens C, Pienkowski C, Lebon-Labich B, et al. Molecular analysis of WNT4 gene in four adolescent girls with mullerian duct abnormality and hyperandrogenism (atypical Mayer-Rokitansky-Kuster-Hauser syndrome). Fertil Steril. 2011;95(8):2683–6.
75. Green J, Nusse R, van Amerongen R. The role of Ryk and Ror receptor tyrosine kinases in Wnt signal transduction. Cold Spring Harb Perspect Biol. 2014;6:a009175.
76. Miller C, Pavlova A, Sassoon DA. Differential expression patterns of Wnt genes in the murine female reproductive tract during development and the estrous cycle. Mech Dev. 1998;76(1–2):91–9.
77. Mericskay M, Kitajewski J, Sassoon D. Wnt5a is required for proper epithelial-mesenchymal interactions in the uterus. Development. 2004;131(9):2061–72.
78. St-Jean G, Boyer A, Zamberlam G, Godin P, Paquet M, Boerboom D. Targeted ablation of Wnt4 and Wnt5a in Mullerian duct mesenchyme impedes endometrial gland development and causes partial Mullerian agenesis. Biol Reprod. 2019;100(1):49–60.
79. Person AD, Beiraghi S, Sieben CM, Hermanson S, Neumann AN, Robu ME, et al. WNT5A mutations in patients with autosomal dominant Robinow syndrome. Dev Dyn. 2010;239(1):327–37.
80. Takasato M, Little MH. The origin of the mammalian kidney: implications for recreating the kidney in vitro. Development. 2015;142(11):1937–47.
81. Dressler GR. Advances in early kidney specification, development and patterning. Development. 2009;136(23):3863–74.
82. Carroll TJ, Park JS, Hayashi S, Majumdar A, McMahon AP. Wnt9b plays a central role in the regulation of mesenchymal to transitions underlying organogenesis of the mammalian urogenital. Dev Cell. 2005;9(2):283–92.
83. Waschk DE, Tewes AC, Romer T, Hucke J, Kapczuk K, Schippert C, et al. Mutations in WNT9B are associated with Mayer-Rokitansky-Kuster-Hauser syndrome. Clin Genet. 2016;89(5):590–6.
84. Lokmane L, Heliot C, Garcia-Villalba P, Fabre M, Cereghini S. vHNF1 functions in distinct regulatory circuits to control ureteric bud branching and early nephrogenesis. Development. 2010;137(2):347–57.
85. Coffinier C, Barra J, Babinet C, Yaniv M. Expression of the vHNF1/HNF1beta homeoprotein gene during mouse organogenesis. Mech Dev. 1999;89(1–2):211–3.
86. Schimke RN, King CR. Hereditary urogenital adysplasia. Clin Genet. 1980;18(6):417–20.
87. Verhave JC, Bech AP, Wetzels JF, Nijenhuis T. Hepatocyte nuclear factor 1beta-associated kidney disease: more than renal cysts and diabetes. J Am Soc Nephrol. 2016;27(2):345–53.
88. Lindner TH, Njolstad PR, Horikawa Y, Bostad L, Bell GI, Sovik O. A novel syndrome of diabetes mellitus, renal dysfunction and genital malformation associated with a partial deletion of the pseudo-POU domain of hepatocyte nuclear factor-1beta. Hum Mol Genet. 1999;8(11):2001–8.
89. Iwasaki N, Okabe I, Momoi MY, Ohashi H, Ogata M, Iwamoto Y. Splice site mutation in the hepatocyte nuclear factor-1 beta gene, IVS2nt + 1G > A, associated with maturity-onset diabetes of the young, renal dysplasia and bicornuate uterus. Diabetologia. 2001;44(3):387–8.
90. Bellanne-Chantelot C, Chauveau D, Gautier JF, Dubois-Laforgue D, Clauin S, Beaufils S, et al. Clinical spectrum associated with hepatocyte nuclear factor-1beta mutations. Ann Intern Med. 2004;140(7):510–7.
91. Grote D, Boualia SK, Souabni A, Merkel C, Chi X, Costantini F, et al. Gata3 acts downstream of beta-catenin signaling to prevent ectopic metanephric kidney induction. PLoS Genet. 2008;4(12):e1000316.
92. Belge H, Dahan K, Cambier JF, Benoit V, Morelle J, Bloch J, et al. Clinical and mutational spectrum of hypoparathyroidism, deafness and renal dysplasia syndrome. Nephrol Dial Transplant. 2017;32(5):830–7.
93. Hernandez AM, Villamar M, Rosello L, Moreno-Pelayo MA, Moreno F, Del Castillo I. Novel mutation in the gene encoding the GATA3 transcription factor in a Spanish familial case of

hypoparathyroidism, deafness, and renal dysplasia (HDR) syndrome with female genital tract malformations. Am J Med Genet A. 2007;143A(7):757–62.
94. Iizuka-Kogo A, Ishidao T, Akiyama T, Senda T. Abnormal development of urogenital organs in Dlgh1-deficient mice. Development. 2007;134(9):1799–807.
95. Jamin SP, Arango NA, Mishina Y, Hanks MC, Behringer RR. Requirement of Bmpr1a for Müllerian duct regression during male sexual development. Nat Genet. 2002;32(3):408–10.
96. Orvis GD, Jamin SP, Kwan KM, Mishina Y, Kaartinen VM, Huang S, et al. Functional redundancy of tgf-Beta family type I receptors and receptor-smads in mediating anti-mullerian hormone-induced mullerian duct regression in the mouse. Biol Reprod. 2008;78(6):994–1001.
97. Massague J. TGFbeta signalling in context. Nat Rev Mol Cell Biol. 2012;13(10):616–30.
98. Moustakas A, Heldin CH. The regulation of TGF beta signal transduction. Development. 2009;136(22):3699–714.
99. Kobayashi A, Stewart CA, Wang Y, Fujioka K, Thomas NC, Jamin SP, et al. beta-Catenin is essential for Mullerian duct regression during male sexual differentiation. Development. 2011;138(10):1967–75.
100. Parr BA, McMahon AP. Sexually dimorphic development of the mammalian reproductive tract requires Wnt-7a. Nature. 1998;395(6703):707–10.
101. Deutscher E, Hung-Chang YH. Essential roles of mesenchyme-derived beta-catenin in mouse Müllerian duct morphogenesis. Dev Biol. 2007;307(2):227–36.
102. Tanwar PS, Zhang L, Tanaka Y, Taketo MM, Donahoe PK, Teixeira JM. Focal Mullerian duct retention in male mice with constitutively activated beta-catenin expression in the Mullerian duct mesenchyme. Proc Natl Acad Sci U S A. 2010;107(37):16142–7.
103. Park JH, Tanaka Y, Arango NA, Zhang L, Benedict LA, Roh MI, et al. Induction of WNT inhibitory factor 1 expression by Mullerian inhibiting substance/anti-Mullerian hormone in the Mullerian duct mesenchyme is linked to Mullerian duct regression. Dev Biol. 2014;386(1):227–36.
104. Mullen RD, Wang Y, Liu B, Moore EL, Behringer RR. Osterix functions downstream of anti-Mullerian hormone signaling to regulate Mullerian duct regression. Proc Natl Acad Sci U S A. 2018;115(33):8382–7.
105. Josso N, Belville C, di Clemente N, Picard JY. AMH and AMH receptor defects in persistent Mullerian duct syndrome. Hum Reprod Update. 2005;11(4):351–6.
106. Knebelmann B, Boussin L, Guerrier D, Legeai L, Kahn A, Josso N, et al. Anti-Mullerian hormone Bruxelles: a nonsense mutation associated with the persistent Mullerian duct syndrome. Proc Natl Acad Sci U S A. 1991;88(9):3767–71.
107. Imbeaud S, Faure E, Lamarre I, Mattei MG, di Clemente N, Tizard R, et al. Insensitivity to anti-mullerian hormone due to a mutation in the human anti-mullerian hormone receptor. Nat Genet. 1995;11(4):382–8.
108. Laronda MM, Unno K, Ishi K, Serna VA, Butler LM, Mills AA, et al. Diethylstilbestrol induces vaginal adenosis by disrupting SMAD/RUNX1-mediated cell fate decision in the Mullerian duct epithelium. Dev Biol. 2013;381(1):5–16.
109. Kurita T, Mills AA, Cunha GR. Roles of p63 in the diethylstilbestrol-induced cervicovaginal adenosis. Development. 2004;131(7):1639–49.
110. Kurita T, Cunha GR, Robboy SJ, Mills AA, Medina RT. Differential expression of p63 isoforms in female reproductive organs. Mech Dev. 2005;122(9):1043–55.
111. Cunha GR, Kurita T, Cao M, Shen J, Robboy S, Baskin L. Molecular mechanisms of development of the human fetal female reproductive tract. Differentiation. 2017;97:54–72.
112. Valdés-Dapena MA. The development of the uterus in late fetal life, infancy, and childhood. In: Norris HJ, Hertig AT, Abell MR, editors. The uterus. Baltimore: The Williams & Wilkins Company; 1973.
113. Haber HP, Mayer EI. Ultrasound evaluation of uterine and ovarian size from birth to puberty. Pediatr Radiol. 1994;24(1):11–3.
114. Salardi S, Orsini LF, Cacciari E, Bovicelli L, Tassoni P, Reggiani A. Pelvic ultrasonography in premenarcheal girls: relation to puberty and sex hormone concentrations. Arch Dis Child. 1985;60(2):120–5.

115. Hagen CP, Mouritsen A, Mieritz MG, Tinggaard J, Wohlfahrt-Veje C, Fallentin E, et al. Uterine volume and endometrial thickness in healthy girls evaluated by ultrasound (3-dimensional) and magnetic resonance imaging. Fertil Steril. 2015;104(2):452–9.e2.
116. Holm K, Laursen EM, Brocks V, Muller J. Pubertal maturation of the internal genitalia: an ultrasound evaluation of 166 healthy girls. Ultrasound Obstet Gynecol. 1995;6(3):175–81.
117. Ivarsson SA, Nilsson KO, Persson PH. Ultrasonography of the pelvic organs in prepubertal and postpubertal girls. Arch Dis Child. 1983;58(5):352–4.
118. Maybin JA, Critchley HO. Menstrual physiology: implications for endometrial pathology and beyond. Hum Reprod Update. 2015;21(6):748–61.
119. Noyes RW, Hertig AT, Rock J. Dating the endometrial biopsy. Am J Obstet Gynecol. 1975;122(2):262–3.
120. Duggan MA, Brashert P, Ostor A, Scurry J, Billson V, Kneafsey P, et al. The accuracy and interobserver reproducibility of endometrial dating. Pathology. 2001;33(3):292–7.
121. Li TC, Dockery P, Rogers AW, Cooke ID. How precise is histologic dating of endometrium using the standard dating criteria? Fertil Steril. 1989;51(5):759–63.
122. Brosens JJ, Gellersen B. Cyclic decidualization of the human endometrium in reproductive health and failure. Endocr Rev. 2014;35(6):851–905.
123. Rock J, Bartlett MK. Biopsy studies of human endometrium: criteria of dating and information about amenorrhea, menorrhagia and time of ovulation. J Am Med Assoc. 1937;108(24):2022–8.
124. Maslar IA, Riddick DH. Prolactin production by human endometrium during the normal menstrual cycle. Am J Obstet Gynecol. 1979;135(6):751–4.
125. Rutanen EM, Koistinen R, Sjoberg J, Julkunen M, Wahlstrom T, Bohn H, et al. Synthesis of placental protein 12 by human endometrium. Endocrinology. 1986;118(3):1067–71.
126. van der Horst CJ. The placentation of elephantulus. Transactions of the Royal Society of South Africa. 1949;32(5):435–629.
127. Carter AM. Classics revisited: C. J. van der Horst on pregnancy and menstruation in elephant shrews. Placenta. 2018;67:24–30.
128. Bellofiore N, Ellery SJ, Mamrot J, Walker DW, Temple-Smith P, Dickinson H. First evidence of a menstruating rodent: the spiny mouse (Acomys cahirinus). Am J Obstet Gynecol. 2017;216(1):40 e1–e11.
129. Rasweiler JJ, Badwaik NK. 5 - Anatomy and physiology of the female reproductive tract. In: Crichton EG, Krutzsch PH, editors. Reproductive biology of bats. London: Academic Press; 2000. p. 157–219.
130. Sato T, Fukazawa Y, Kojima H, Enari M, Iguchi T, Ohta Y. Apoptotic cell death during the estrous cycle in the rat uterus and vagina. Anat Rec. 1997;248(1):76–83.
131. Kurita T, Wang YZ, Donjacour AA, Zhao C, Lydon JP, O'Malley BW, et al. Paracrine regulation of apoptosis by steroid hormones in the male and female reproductive system. Cell Death Differ. 2001;8(2):192–200.
132. Cheung TH, Rando TA. Molecular regulation of stem cell quiescence. Nat Rev Mol Cell Biol. 2013;14(6):329–40.
133. Rezza A, Sennett R, Rendl M. Adult stem cell niches: cellular and molecular components. Curr Top Dev Biol. 2014;107:333–72.
134. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. Exp Cell Res. 1961;25:585–621.
135. Shay JW. Telomeres and aging. Curr Opin Cell Biol. 2018;52:1–7.
136. Greider CW, Blackburn EH. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell. 1985;43(2 Pt 1):405–13.
137. Olovnikov AM. A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. J Theor Biol. 1973;41(1):181–90.
138. Gire V, Dulic V. Senescence from G2 arrest, revisited. Cell cycle (Georgetown, Tex.). 2015;14(3):297–304.

139. Jabbour HN, Kelly RW, Fraser HM, Critchley HO. Endocrine regulation of menstruation. Endocr Rev. 2006;27(1):17–46.
140. Padykula HA. Regeneration in the primate uterus: the role of stem cells. Ann N Y Acad Sci. 1991;622:47–56.
141. Tempest N, Maclean A, Hapangama DK. Endometrial stem cell markers: current concepts and unresolved questions. Int J Mol Sci. 2018;19(10):3240.
142. Gargett CE, Chan RW, Schwab KE. Endometrial stem cells. Curr Opin Obstet Gynecol. 2007;19(4):377–83.
143. Suda K, Nakaoka H, Yoshihara K, Ishiguro T, Tamura R, Mori Y, et al. Clonal Expansion and Diversification of Cancer-Associated Mutations in Endometriosis and Normal Endometrium. Cell Rep. 2018;24(7):1777–89.
144. Valentijn AJ, Saretzki G, Tempest N, Critchley HO, Hapangama DK. Human endometrial epithelial telomerase is important for epithelial proliferation and glandular formation with potential implications in endometriosis. Hum Reprod. 2015;30(12):2816–28.
145. Tanaka M, Kyo S, Takakura M, Kanaya T, Sagawa T, Yamashita K, et al. Expression of telomerase activity in human endometrium is localized to epithelial glandular cells and regulated in a menstrual phase-dependent manner correlated with cell proliferation. Am J Pathol. 1998;153(6):1985–91.
146. Yokoyama Y, Takahashi Y, Shinohara A, Lian Z, Xiaoyun W, Niwa K, et al. Telomerase activity is found in the epithelial cells but not in the stromal cells in human endometrial cell culture. Mol Hum Reprod. 1998;4(10):985–9.
147. Vue Z, Gonzalez G, Stewart CA, Mehra S, Behringer RR. Volumetric imaging of the developing prepubertal mouse uterine epithelium using light sheet microscopy. Mol Reprod Dev. 2018;85(5):397–405.
148. Gray CA, Bartol FF, Tarleton BJ, Wiley AA, Johnson GA, Bazer FW, et al. Developmental biology of uterine glands. Biol Reprod. 2001;65(5):1311–23.
149. Yuan J, Deng W, Cha J, Sun X, Borg JP, Dey SK. Tridimensional visualization reveals direct communication between the embryo and glands critical for implantation. Nat Commun. 2018;9(1):603.
150. Tanaka M, Kyo S, Kanaya T, Yatabe N, Nakamura M, Maida Y, et al. Evidence of the monoclonal composition of human endometrial epithelial glands and mosaic pattern of clonal distribution in luminal epithelium. Am J Pathol. 2003;163(1):295–301.
151. Bhatt H, Brunet LJ, Stewart CL. Uterine expression of leukemia inhibitory factor coincides with the onset of blastocyst implantation. Proc Natl Acad Sci U S A. 1991;88(24):11408–12.
152. Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Kontgen F, et al. Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. Nature. 1992;359(6390):76–9.
153. Chen JR, Cheng JG, Shatzer T, Sewell L, Hernandez L, Stewart CL. Leukemia inhibitory factor can substitute for nidatory estrogen and is essential to inducing a receptive uterus for implantation but is not essential for subsequent embryogenesis. Endocrinology. 2000;141(12):4365–72.
154. Kobayashi R, Terakawa J, Kato Y, Azimi S, Inoue N, Ohmori Y, et al. The contribution of leukemia inhibitory factor (LIF) for embryo implantation differs among strains of mice. Immunobiology. 2014;219(7):512–21.
155. Jeong JW, Lee HS, Franco HL, Broaddus RR, Taketo MM, Tsai SY, et al. beta-catenin mediates glandular formation and dysregulation of beta-catenin induces hyperplasia formation in the murine uterus. Oncogene. 2009;28(1):31–40.
156. Franco HL, Dai D, Lee KY, Rubel CA, Roop D, Boerboom D, et al. WNT4 is a key regulator of normal postnatal uterine development and progesterone signaling during embryo implantation and decidualization in the mouse. FASEB J. 2011;25(4):1176–87.
157. Miller C, Sassoon DA. Wnt-7a maintains appropriate uterine patterning during the development of the mouse female reproductive tract. Development. 1998;125(16):3201–11.

158. Dunlap KA, Filant J, Hayashi K, Rucker EB, 3rd, Song G, Deng JM, et al. Postnatal deletion of Wnt7a inhibits uterine gland morphogenesis and compromises adult fertility in mice. Biol Reprod. 2011;85(2):386–96.
159. Goad J, Ko YA, Kumar M, Syed SM, Tanwar PS. Differential Wnt signaling activity limits epithelial gland development to the anti-mesometrial side of the mouse uterus. Dev Biol. 2017;423(2):138–51.
160. Shelton DN, Fornalik H, Neff T, Park SY, Bender D, DeGeest K, et al. The role of LEF1 in endometrial gland formation and carcinogenesis. PLoS One. 2012;7(7):e40312.
161. Terakawa J, Serna VA, Taketo MM, Daikoku T, Suarez A, Kurita T. Ovarian-insufficiency and CTNNB1 mutations drive malignant transformation of endometrial hyperplasia with altered PTEN/PI3K activities. Proc Natl Acad Sci U S A. 2019;116(10):4528–37.
162. Oh SJ, Shin JH, Kim TH, Lee HS, Yoo JY, Ahn JY, et al. beta-Catenin activation contributes to the pathogenesis of adenomyosis through epithelial-mesenchymal transition. J Pathol. 2013;231(2):210–22.
163. Farah O, Biechele S, Rossant J, Dufort D. Porcupine-dependent Wnt signaling controls stromal proliferation and endometrial gland maintenance through the action of distinct WNTs. Dev Biol. 2017;422(1):58–69.
164. Glinka A, Dolde C, Kirsch N, Huang YL, Kazanskaya O, Ingelfinger D, et al. LGR4 and LGR5 are R-spondin receptors mediating Wnt/beta-catenin and Wnt/PCP signalling. EMBO Rep. 2011;12(10):1055–61.
165. Sone M, Oyama K, Mohri Y, Hayashi R, Clevers H, Nishimori K. LGR4 expressed in uterine epithelium is necessary for uterine gland development and contributes to decidualization in mice. FASEB J. 2013;27(12):4917–28.
166. Hirate Y, Suzuki H, Kawasumi M, Takase HM, Igarashi H, Naquet P, et al. Mouse Sox17 haploinsufficiency leads to female subfertility due to impaired implantation. Sci Rep. 2016;6:24171.
167. Guimaraes-Young A, Neff T, Dupuy AJ, Goodheart MJ. Conditional deletion of Sox17 reveals complex effects on uterine adenogenesis and function. Dev Biol. 2016;414(2):219–27.
168. Kurita T, Cooke PS, Cunha GR. Epithelial-stromal tissue interaction in paramesonephric (Mullerian) epithelial differentiation. Dev Biol. 2001;240(1):194–211.
169. Kurita T, Lee KJ, Cooke PS, Taylor JA, Lubahn DB, Cunha GR. Paracrine regulation of epithelial progesterone receptor by estradiol in the mouse female reproductive tract. Biol Reprod. 2000;62(4):821–30.
170. Engert S, Burtscher I, Liao WP, Dulev S, Schotta G, Lickert H. Wnt/beta-catenin signalling regulates Sox17 expression and is essential for organizer and endoderm formation in the mouse. Development. 2013;140(15):3128–38.
171. Sinner D, Rankin S, Lee M, Zorn AM. Sox17 and beta-catenin cooperate to regulate the transcription of endodermal genes. Development. 2004;131(13):3069–80.
172. Robledo RF, Rajan L, Li X, Lufkin T. The Dlx5 and Dlx6 homeobox genes are essential for craniofacial, axial, and appendicular skeletal development. Genes Dev. 2002;16(9):1089–101.
173. Bellessort B, Le Cardinal M, Bachelot A, Narboux-Neme N, Garagnani P, Pirazzini C, et al. Dlx5 and Dlx6 control uterine adenogenesis during post-natal maturation: possible consequences for endometriosis. Hum Mol Genet. 2016;25(1):97–108.
174. Rakowiecki S, Epstein DJ. Divergent roles for Wnt/beta-catenin signaling in epithelial maintenance and breakdown during semicircular canal formation. Development. 2013;140(8):1730–9.
175. Cancer Genome Atlas Research N, Kandoth C, Schultz N, Cherniack AD, Akbani R, Liu Y, et al. Integrated genomic characterization of endometrial carcinoma. Nature. 2013;497(7447):67–73.
176. Chang HJ, Shin HS, Kim TH, Yoo JY, Teasley HE, Zhao JJ, et al. Pik3ca is required for mouse uterine gland development and pregnancy. PLoS One. 2018;13(1):e0191433.
177. Fehon RG, McClatchey AI, Bretscher A. Organizing the cell cortex: the role of ERM proteins. Nat Rev. 2010;11(4):276–87.

178. McClatchey AI, Giovannini M. Membrane organization and tumorigenesis--the NF2 tumor suppressor, Merlin. Genes Develop. 2005;19(19):2265–77.
179. Lopez EW, Vue Z, Broaddus RR, Behringer RR, Gladden AB. The ERM family member Merlin is required for endometrial gland morphogenesis. Dev Biol. 2018;442(2):301–14.
180. Cooke PS, Spencer TE, Bartol FF, Hayashi K. Uterine glands: development, function and experimental model systems. Mol Hum Reprod. 2013;19(9):547–58.
181. Hou X, Tan Y, Li M, Dey SK, Das SK. Canonical Wnt signaling is critical to estrogen-mediated uterine growth. Mol Endocrinol. 2004;18(12):3035–49.
182. Hayashi K, Erikson DW, Tilford SA, Bany BM, Maclean JA 2nd, Rucker EB 3rd, et al. Wnt genes in the mouse uterus: potential regulation of implantation. Biol Reprod. 2009;80(5):989–1000.
183. Cooke PS, Buchanan DL, Young P, Setiawan T, Brody J, Korach KS, et al. Stromal estrogen receptors mediate mitogenic effects of estradiol on uterine epithelium. Proc Natl Acad Sci U S A. 1997;94(12):6535–40.
184. Kurita T, Young P, Brody JR, Lydon JP, O'Malley BW, Cunha GR. Stromal progesterone receptors mediate the inhibitory effects of progesterone on estrogen-induced uterine epithelial cell deoxyribonucleic acid synthesis. Endocrinology. 1998;139(11):4708–13.
185. Kurita T, Lee KJ, Cooke PS, Lydon JP, Cunha GR. Paracrine regulation of epithelial progesterone receptor and lactoferrin by progesterone in the mouse uterus. Biol Reprod. 2000;62(4):831–8.
186. Buchanan DL, Setiawan T, Lubahn DB, Taylor JA, Kurita T, Cunha GR, et al. Tissue compartment-specific estrogen receptor-alpha participation in the mouse uterine epithelial secretory response. Endocrinology. 1999;140(1):484–91.
187. Winuthayanon W, Hewitt SC, Orvis GD, Behringer RR, Korach KS. Uterine epithelial estrogen receptor alpha is dispensable for proliferation but essential for complete biological and biochemical responses. Proc Natl Acad Sci U S A. 2010;107(45):19272–7.
188. Mehta FF, Son J, Hewitt SC, Jang E, Lydon JP, Korach KS, et al. Distinct functions and regulation of epithelial progesterone receptor in the mouse cervix, vagina, and uterus. Oncotarget. 2016;7(14):17455–67.
189. Kaufman RH, Adam E, Binder GL, Gerthoffer E. Upper genital tract changes and pregnancy outcome in offspring exposed in utero to diethylstilbestrol. Am J Obstet Gynecol. 1980;137(3):299–308.
190. Kaufman RH, Binder GL, Gray PM Jr, Adam E. Upper genital tract changes associated with exposure in utero to diethylstilbestrol. Am J Obstet Gynecol. 1977;128(1):51–9.
191. Dodds EC, Golberg L, Lawson W, Robinson R. synthetic oestrogenic compounds related to stilbene and diphenylethane. Part I Proceedings of the Royal Society of London Series B, Biological Sciences. 1939;127(847):140–67.
192. Dodds EC, Goldberg L, Lawson W, Robinson R. OEstrogenic activity of certain synthetic compounds. Nature. 1938;141(3562):247–8.
193. Berger MJ, Goldstein DP. Impaired reproductive performance in DES-exposed women. Obstet Gynecol. 1980;55(1):25–7.
194. Fernandez H, Garbin O, Castaigne V, Gervaise A, Levaillant JM. Surgical approach to and reproductive outcome after surgical correction of a T-shaped uterus. Hum Reprod (Oxford, England). 2011;26(7):1730–4.
195. Dieckmann WJ, Davis ME, Rynkiewicz LM, Pottinger RE. Does the administration of diethylstilbestrol during pregnancy have therapeutic value? Am J Obstet Gynecol. 1953;66(5):1062–81.
196. Herbst AL, Ulfelder H, Poskanzer DC. Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women. N Engl J Med. 1971;284(15):878–81.
197. Greenwald P, Barlow JJ, Nasca PC, Burnett WS. Vaginal cancer after maternal treatment with synthetic estrogens. N Engl J Med. 1971;285(7):390–2.
198. Giusti RM, Iwamoto K, Hatch EE. Diethylstilbestrol revisited: a review of the long-term health effects. Ann Intern Med. 1995;122(10):778–88.

199. Robboy SJ, Szyfelbein WM, Goellner JR, Kaufman RH, Taft PD, Richard RM, et al. Dysplasia and cytologic findings in 4,589 young women enrolled in diethylstilbestrol-adenosis (DESAD) project. Am J Obstet Gynecol. 1981;140(5):579–86.
200. Robboy SJ, Young RH, Welch WR, Truslow GY, Prat J, Herbst AL, et al. Atypical vaginal adenosis and cervical ectropion. Association with clear cell adenocarcinoma in diethylstilbestrol-exposed offspring. Cancer. 1984;54(5):869–75.
201. Laronda MM, Unno K, Butler LM, Kurita T. The development of cervical and vaginal adenosis as a result of diethylstilbestrol exposure in utero. Differentiation. 2012;84(3):252–60.
202. Terakawa J, Rocchi A, Serna VA, Bottinger EP, Graff JM, Kurita T. FGFR2IIIb-MAPK activity is required for epithelial cell fate decision in the lower Mullerian duct. Mol Endocrinol. 2016;30(7):783–95.
203. Kurita T, Cunha GR. Roles of p63 in differentiation of Mullerian duct epithelial cells. Ann N Y Acad Sci. 2001;948:9–12.
204. Miller C, Degenhardt K, Sassoon DA. Fetal exposure to DES results in de-regulation of Wnt7a during uterine morphogenesis. Nat Genet. 1998;20(3):228–30.
205. Couse JF, Dixon D, Yates M, Moore AB, Ma L, Maas R, et al. Estrogen receptor-alpha knockout mice exhibit resistance to the developmental effects of neonatal diethylstilbestrol exposure on the female reproductive tract. Dev Biol. 2001;238(2):224–38.
206. Kurita T, Medina R, Schabel AB, Young P, Gama P, Parekh TV, et al. The activation function-1 domain of estrogen receptor alpha in uterine stromal cells is required for mouse but not human uterine epithelial response to estrogen. Differentiation. 2005;73(6):313–22.
207. Liu S, Gao X, Qin Y, Liu W, Huang T, Ma J, et al. Nonsense mutation of EMX2 is potential causative for uterus didelphysis: first molecular explanation for isolated incomplete mullerian fusion. Fertil Steril. 2015;103(3):769–74.e2.
208. Slavotinek A, Risolino M, Losa M, Cho MT, Monaghan KG, Schneidman-Duhovny D, et al. De novo, deleterious sequence variants that alter the transcriptional activity of the homeoprotein PBX1 are associated with intellectual disability and pleiotropic developmental defects. Hum Mol Genet. 2017;26(24):4849–60.
209. Biason-Lauber A, Konrad D, Navratil F, Schoenle EJ. A WNT4 mutation associated with Müllerian-duct regression and virilization in a 46,XX woman. N Engl J Med. 2004;351(8):792–8.
210. Biason-Lauber A, De Filippo G, Konrad D, Scarano G, Nazzaro A, Schoenle EJ. WNT4 deficiency--a clinical phenotype distinct from the classic Mayer-Rokitansky-Kuster-Hauser syndrome: a case report. Hum Reprod. 2007;22(1):224–9.

Chapter 2
Molecular Biology Techniques for Endometrial Gene Expression: Recent Technological Advances

Ke Ni and Lijia Ma

Background

The endometrium provides an optimal environment for embryo implantation and fetal development, which ensure the accurate assessment of endometrium is of great clinical value. However, the assessment of endometrium is complicated. The endometrium is a dynamic tissue that undergoes a cyclic change that is driven by estrogen, progesterone, as well as other biochemical factors [1, 2]. The human endometrium has complex structures including stratum compactum, stratum spongiosum, and stratum basalis, which contain different cell types and involve in various biological processes [1]. Current assessments focus on endometrial morphology including thickness, surface, and endocytosis abundance, which involve traditional medical tests like imaging and histology. The rapid development of the next-generation sequencing (NGS) technology provides new sets of tools to physicians. One of the major applications of the NGS technology in the clinic is the expression profiling of samples of interests. The gene expression in different cell types and different physiological conditions is highly regulated, dynamic, and closely related to cellular function, and researchers and physicians have started to use it as a molecular signature for disease prediction, evaluation, and diagnosis.

The transcriptome is the sum of all the messenger RNA (mRNA) molecules and noncoding RNA produced by an organism. In this review, we discussed the recent advances in the characterization of different RNA species, including mRNA, long noncoding RNA (lncRNA), and microRNA (miRNA). We also discussed some clinical conditions that are related to abnormal gene expression in the endometrium. Finally, we discussed the potential application of newly developed gene profiling technologies in the field of endometrial research and clinical practice.

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Technology Introduction

The main applications of high-throughput RNA profiling include techniques that are identifying diverse RNA species (e.g., mRNA, microRNA, and lncRNA), as well as those derivatives that are focusing on characterizing RNA-relevant regulatory events (e.g., Gro-seq (global run-on sequencing), Ribo-seq (ribosome profiling), and RIP-seq (RNA immunoprecipitation sequencing)).

Besides, single-cell RNA sequencing (scRNA-seq) emerged in the past 5 years as a major approach to identify not only RNA profiles in bulk cells but also transcriptome in the individual cell as well as expression heterogeneity of a cell population.

In this part, we will review major applications of RNA profiling, RNA sequencing derivatives, and scRNA-seq.

Techniques in mRNA Profiling

There are three major approaches to quantify RNA abundance, quantitative polymerase reaction (qPCR), microarray, and high-throughput sequencing.

The qPCR is the most accessible approach in a molecular biology laboratory. RNA molecules will firstly be reverse transcribed into complementary DNA (cDNA) using DNA primers that are complementary to the RNA sequence. The cDNA signal will then be quantified on commercially available quantitative PCR system, using primers labeled with fluorescence or with the addition of SYBR Green.

Microarray uses probes to simultaneously analyze the expression of thousands of the known genes, with each probe targeting the RNA transcript of interest [3]. To perform a microarray analysis, mRNAs are typically collected from the samples and converted into complementary DNA (cDNA), with each sample labeled with a fluorescent probe of a different color. Then, the cDNA samples are mixed together and allowed to bind to the microarray slide, which is called hybridization. Following hybridization, the microarray is scanned to measure the expression of each gene printed on the slide.

RNA-seq is another high-throughput method to profile RNA abundance and has been applied widely in various researches and clinics (Fig. 2.1a) [4]. Like qPCR, RNA molecules of interest are converted into cDNA and followed by multiple steps to modify fragment ends, add Illumina adapters, and amplify. The entire procedure is usually called "library preparation," and the final product is called "library," which can be read directly on the Illumina Sequencer.

The major differences between these three methods are throughput and whether the sequence of the interested RNA molecule is known.

The qPCR is suitable for quantifying RNA molecules with known sequences and in low throughput, typically ten to hundreds of transcripts. The microarray and RNA-seq can both quantify tens of thousands of transcripts in a single experiment. However, the microarray technique uses the fluorescent signal as a proxy of RNA abundances. Instead, the RNA-seq provides "digital" signals, which are the absolute

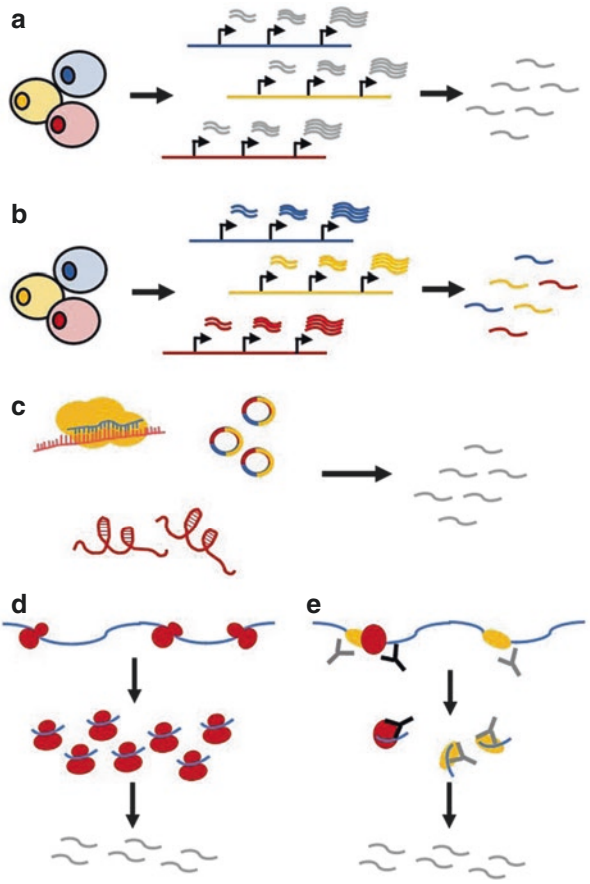

Fig. 2.1 (**a**) Bulk RNA-seq reveals the quantity of RNA from a group of cells at a given moment. (**b**) Single-cell RNA-seq reveals the quantity of RNA from a group of cells at the single-cell resolution at a given moment. RNAs from different cells can be distinguished through molecular barcodes added to each cell during library preparation. (**c**) The quantities of microRNA, IncRNA (long intergenic noncoding RNA), and circRNA can also be detected through RNA-seq. (**d**) Ribo-seq monitors the RNA that is being processed by the ribosome at a given time. (**e**) RIP-seq maps the sites at which proteins are bound to the RNA within RNA-protein complexes at a given time

counts of DNA molecules (from the RNA as a template). Furthermore, since microarray requires probe design prior to the experiment, it can only quantify transcripts with known sequences, while RNA-seq can be applied for transcript quantification as well as novel transcript discovery.

Recent studies have indicated the significant heterogeneities among the cells. Correspondingly, scRNA-seq opens a new avenue to address the biological or pathological features at the resolution of a single cell (Fig. 2.1b) [5]. The critical steps of scRNA-seq are to isolate cells physically either in microwells or in droplets and label individual cell with a sequence barcode before cell lysis. To avoid the biases produced by cDNA amplification, the unique molecular identifiers (UMIs) that tag individual mRNA molecule are incorporated to identify the original molecules and the amplified replicates. The Drop-seq (droplet-sequencing) technique and its commercial derivate 10x genomics single-cell 3' RNA-seq are two widely used platforms for this purpose and give an exciting insight into cell biology at a high resolution [6]. In another circumstance, people need to quantify RNA abundance from a very limited number of cells, typically between 1 and 100, which is far below the detection threshold of bulk

cell RNA-seq approaches. The single-cell approaches (e.g., switching mechanism at 5' end of RNA template sequencing (SMART-seq), cell expression by linear amplification and sequencing (CEL-seq)) are specifically designed to address this issue by introducing a template switch oligo and an additional step of cDNA amplification before the library preparation.

Techniques in Noncoding RNA Profiling

Although mRNAs are the critical part of the transcriptome, transcriptome analyses have suggested that only 1–2% of the mammalian genome is protein-coding, whereas 70–90% is transcriptionally active [7, 8]. This fact emphasizes the importance of researches focusing on noncoding RNA. Noncoding RNAs are functional RNAs transcribed from DNA, but not translated into proteins. Ranging from 100 nt to >100,000 nt, most of these noncoding RNAs are functionally unknown and may originate within or between genes [9]. Some noncoding RNA species have been discovered to be essential for gene regulation, and we will briefly discuss three of them in the following paragraphs.

MicroRNAs are highly conserved and with 19–22 nt in length that regulate gene expression at the posttranscriptional level. MicroRNAs function via base-pairing with complementary sequences within mRNA molecules. MicroRNAs have been associated with human pathologies [10], which give rise to the development of novel diagnostic tools and could serve as drug targets. The major challenge to make sequencing library for microRNAs is their length. A typical strategy is conducting size-selection on high concentration polyacrylamide gel electrophoresis (PAGE), and 143~146 bp products will be retained. The microRNA-seq queries thousands of miRNA sequences with unprecedented sensitivity and dynamic range.

lncRNAs are defined as transcripts longer than 200 nt that are not translated into protein. Increasing evidence has been accumulated to suggest that changes in expression levels of many lncRNAs have medical implications [11]. However, the majority of lncRNAs have not yet been studied in mechanistic details. Starting from RNA with ribosomal RNA (rRNA) depletion, RNA-seq is a powerful tool for the detection of lncRNAs, which provides a genome-wide view of the location and abundance of lncRNAs (Fig. 2.1c).

Circulating RNAs (circRNAs), a group of noncoding RNAs that can absorb microRNAs and enhance mRNA translation, have provided novel insights into gene regulation. circRNA molecules typically comprise exonic sequences and are spliced at canonical splice sites [12]. They can be sequenced by digesting away linear RNAs using exonuclease R, followed by hybridization-based microarray or RNA-seq (Fig. 2.1c) [13]. Recent evidence has revealed that circRNAs are involved in a wide range of biological processes including human fetal development [14], carcinogenesis [15, 16], and diseases [17, 18]. Also, there are an increasing number of studies exploring the relationship between circRNAs and endometrial disorders via RNA-seq. However, the crucial roles, functions, and working mechanism of circRNAs have not been well recognized.

Techniques to Profile RNAs Involved in the Specific Biological Context

Along and after the transcription, the mRNA molecules keep undergoing complex regulations, which are together named as "posttranscriptional regulation." Naturally, besides the entire pool of transcribed RNA molecules, people are also interested in subsets of them that are involved in various regulatory events. However, more dedicated library preparation would be required to enrich or isolate them. For example, Ribo-seq can specifically measure the ribosome-associated RNA and identify the ribosome location on the transcript, which can be further interpreted into the translation efficiency. RNA-binding protein is another attracting topic that involves identification of RNA molecules protected by proteins. The RIP-seq approach was established to serve this purpose.

Ribo-seq

Ribo-seq is used to detect the RNA that is being processed by the ribosome (Fig. 2.1d) [19]. The ribosome-protected RNA undergoes digestion and is followed with rRNA depletion to enrich transcripts. The postdepleted RNAs are then converted to cDNA, and an additional enrichment step is usually required to eliminate contaminations from other RNA species. Ribo-seq exhibits the advantages in identifying protein-coding regions, indicating the precise location of ribosomes on the RNA, and more closely reflecting the protein synthesis than RNA-seq.

RIP-seq

This technique maps the RNA sites at which proteins are bound to the RNA within RNA-protein complexes [20] (Fig. 2.1e). First, RNA-protein complexes are immunoprecipitated with antibodies targeted to the RNA-binding protein of interest. After RNase digestion, RNA protected by protein binding is extracted and converted to cDNA. Then, the deep sequencing of cDNA identifies the protein-bound RNA. The locations of RNA and protein association can then be mapped back to the genome.

Applications

Transcriptome characterization provides comprehensive information of the molecular heterogeneity of a cell population or a piece of tissue, which shows great value in clinical studies. Numerous research groups have tried to apply the transcriptome

profiling in endometrial characterization. This part is framed with four sections covering the healthy endometrial characterization, gynecological disorders, reproductive disorders, and endometrial cancer, in which the brief summary about the application of transcriptome profiling will be made separately.

The Characterization of Global Gene Expression for Healthy Endometrium

Endometrium exhibits cyclically dynamic change under the control of estrogen and progesterone, which subsequently changes its gene expression level during the menstrual cycle. The characterization of endometrium in the menstrual cycle is of great importance in the understanding of the endometrial physiology.

Based on the microarray, the transcriptome of the endometrium in the whole menstrual cycle has been defined [21, 22]. Ponnampalam et al. firstly tried to predict the endometrial cycle stage based on the global gene expression profile [21]. They investigated the changing global gene expression profile of human endometrium during the menstrual cycle using microarray and determined the correlation between histopathological evaluation and molecular profile of the samples. The study identified 1452 genes that showed significant changes in expression across the menstrual cycle. Furthermore, they identified a small subset of genes whose expression profile could be used to classify nearly all the biopsies into their correct cycle stage. To understand the molecular signatures of the endometrium, Talbi and his colleagues investigated 54,600 genes of endometrium sampled across the cycle in 28 normal ovulatory women via microarrays [22]. According to the unsupervised principal component analysis, samples self-clustered into four groups consistent with histological phenotypes of proliferative (PE), early-secretory (ESE), mid-secretory (MSE), and late-secretory (LSE) endometrium. Their results demonstrated that the endometrial samples from the common subjects could be classified by their molecular signatures and corresponded to the known phases of the menstrual cycle via microarray.

Meanwhile, studies have tried to describe the endometrial features via RNA-seq. In the study conducted by Sigurgeirsson and his colleagues [23], the transcriptome of the human endometrium by comparing endometrial biopsies at day 7 to day 9 after ovulation was comprehensively presented. The authors reported in detail the shift of RNA profile from the proliferative to receptive endometrium with the aim to coordinate the gene transcription with different endometrial phases for successful embryo implantation. Consequently, a total of 3297 mRNAs, 516 lncRNAs, and 102 small noncoding RNAs were identified as statistically differentially expressed at different time points. A total of 34 molecules that have not been reported before were found to be involved in the physiological changes in the human endometrium. This study provided the complete description of the transcriptome profile after ovulation in human endometrium via RNA-seq.

The attempt to introduce scRNA-seq in the endometrial characterization has also been conducted. The complete pipeline from clinical sampling to statistical data

analysis has been exploited in one study [24]. In this study, the uncultured and cultured stromal single cells derived from the same endometrial biopsy were comparatively sequenced and analyzed. The authors evaluate the possible impact of the in vitro culture on the transcriptome of a single cell. Besides, they performed a gene ontology analysis of the differentially expressed genes between uncultured and cultured cells and found that these genes are mainly related to cell cycle, translational processes, and metabolism. Bingbing Wu and colleagues have tried to dissect the cell heterogeneities of the full-thickness human uterus from 2735 single cells by scRNA-seq [25]. They defined numerous clusters including epithelial cells, stromal cells, endothelial cells, smooth muscle cells, myofibroblasts, and immune cells. Furthermore, they identified a unique ciliated epithelial cell cluster showing characteristics of stem/progenitors with properties of epithelial-mesenchymal transition (EMT). Another study focused on characterizing the transcriptomic transformation of human endometrium through the menstrual cycle at a single-cell resolution [26]. They identified endometrial cell types, including a previously uncharacterized ciliated epithelial cell type, during four major phases of endometrial transformation. They described characteristic signatures for each cell type and phase. All these studies are insightful explorations that link clinical diagnostic needs and modern laboratory and bioinformatic solutions.

In the field of noncoding RNAs, one research group tried to find a menstrual blood biomarker through microarray screening for microRNAs. They firstly selected 3 miRNAs (miR-141-3p, miR-497-5p, and miR-143-5p) from menstrual blood, of which expression levels exhibited significant difference compared to the level in peripheral blood, based on the microarray and quantitative PCR validation [27]. Then, the authors evaluated the dynamic expression changes of the 3 miRNAs in menstrual blood samples collected from different menstrual cycle stages. Another study focused on determining the plasma microRNA profile of healthy women during the menstrual cycle by microarray [28]. They found circulating microRNA expression levels in healthy women were not significantly altered during the menstrual cycle.

The Application of Endometrial Gene Expression Profiling in Gynecological Disorders

In the past decade, much effort has been made in learning the genetic etiology of gynecological disorders, especially endometriosis. Endometriosis is a benign gynecological disorder characterized by the abnormal location of endometrial tissue outside the uterine cavity [29]. Despite its significant impact on the quality of life, its etiology remains elusive and lacks the effective biomarker for diagnosis [30]. There is a hypothesis that the eutopic endometrium in the patients with endometriosis contained aberrant gene expression that governs its abnormal implantation, invasion, and migration. Therefore, studies that detect the transcriptome profile of eutopic endometrium in women with endometriosis compared with the normal endometrium from healthy control subjects have been conducted.

The endometrial microarray has been applied to the studies of endometriosis. In the study conducted by Colón-Caraballo and his colleagues, the ovarian hormone receptor expression status in the women with endometriosis was investigated [31]. They found the expression pattern of ovarian hormone receptors varied among different types of endometriotic lesions and eutopic endometrium from women with endometriosis and controls, which could potentially predict the individual responses to hormone therapies. In another study, researchers identified differentially expressed genes in endometriosis via microarray and further analyzed the molecular mechanism implicated in the pathogenesis [32]. A total of 2255 up- and 408 downregulated genes were identified in the patients with endometriosis compared with the control, among which focal adhesion regulation of actin cytoskeleton, mitogen-activated protein kinase (MAPK), and TGFB/SMAD signaling pathway might be the important molecular mechanism underlying the pathogenesis of endometriosis.

RNA-seq has also been performed to detect the transcriptome profile of eutopic endometrium in women with endometriosis. In the study conducted by Zhao and colleagues [33], eight eutopic and five normal endometrial tissues were collected for RNA-seq. A total of 72 differently expressed genes (66 upregulated and 6 downregulated) were identified in the samples from women with endometriosis compared with those from control subjects. The annotation of the differently expressed genes found the extracellular matrix (ECM) remodeling, angiogenesis, cell proliferation, and differentiation are involved. This study provided a preliminary insight for elucidating the molecular mechanisms of endometriosis.

Multiple studies have shown that microRNA expression is altered in eutopic endometrium [34–36]. In a representative study of microRNA profiling via microarray, a series of microRNA expression in the ectopic and the eutopic endometrium in women with/without endometriosis were determined [37]. The researchers found 156 mature microRNAs that were differentially expressed in the ovarian endometrioma or the eutopic endometrium compared with the healthy tissue. After bioinformatic analysis, they selected 12 microRNAs for quantitative real-time polymerase chain reaction (qRT-PCR) validation in a larger cohort. Among the selected microRNAs, six microRNAs (miR-29c-3p, miR-138, miR-202-3p, miR-411-5p, miR-411-3p, miR-424-5p) were found significantly upregulated, and six miRNAs (miR-16, miR-373-3p, miR-449b-3p, miR-556-3p, miR-636, miR-935) were found significantly downregulated in ovarian endometrioma or eutopic endometrium compared with the control endometrium. The endometrium of the patients showed higher vascular endothelial growth factor A (VEGF-A) levels and lower expression of miR-202-3p and miR-449b-3p compared with control endometrium. In addition, the ovarian ectopic endometrioma showed significantly higher expression of the angiogenic inhibitor thrombospondin 1 (TSP-1) and lower expression of miR-449b-3p than the control endometrium. Moreover, miR-29c-3p and miR-202-3p were more abundant in endometrial tissues than those of control. This study pointed out that these microRNAs were potential candidates in the search for novel diagnostic biomarkers to guide the therapeutic interventions to endometriosis.

circRNAs are a naturally occurring group of noncoding RNA that may regulate gene expression. They are more stable than mRNAs due to their resistance to RNA exonuclease, which qualify them as a good biomarker candidate. Recently, one study aimed to profile the circRNAs expressed in eutopic endometrium from patients with ovarian endometriosis has been conducted [38]. In this study, a total of 63 clinical samples, including control endometrium and eutopic endometrium, were collected. Through the circRNA microarray, a total of 4 upregulated circRNAs were screened out for qRT-PCR validation. Based on target prediction, they constructed a circRNA-microRNA-mRNA network and depicted the relationship between the identified circRNAs and their dominant target microRNAs. The researchers found circRNAs were differentially expressed between eutopic and normal endometrium, which suggested that circRNAs could become candidate factors in the activation of endometriosis. Besides, they found circ_0002198 and circ_0004712 might be the potential biomarkers for the diagnosis of ovarian endometriosis.

The Application of Endometrial Gene Expression Profiling in Reproductive Disorders

In vitro fertilization (IVF) is the most effective assisted reproductive technology that treats reproductive disorders. Although embryos now are considered as the major limiting factor in IVF success, it can still happen that serial transfers of the embryos with high quality often fail to result in a pregnancy. It has been recognized that the assessment of endometrial receptivity is critical for the successful embryo implantation. However, no clinically relevant morphological, histological, or molecular marker that is capable of indicating the endometrial receptivity has been identified. The eager need for the intervention to address recurrent implantation failure (RIF) successfully leads to the introduction of the high-throughput sequencing techniques into this field.

The microarray is the earliest technique applied in the identification of a transcriptomic signature of the patients with RIF. In the study conducted by Koot and his colleagues, the gene signature was defined by microarray in a discovery set including 31 RIF patients and 50 controls and validated in 12 RIF patients and 22 controls [39]. Finally, a 303-gene expression signature was selected to distinguish RIF patients from the controls with high sensitivity and accuracy. According to annotation analysis, the genes involved in cell cycle regulation, cell division, cytoskeleton, and cilia formation were found downregulated in RIF patients. Another study on a smaller scale has been reported by another group [40]. In this study, a total of 17 volunteers, including 5 patients with RIF, 6 patients with a final successful cycle, and 6 controls with normal fertility, were recruited. They found compromised progesterone signaling might be the underlying mechanism for the deregulation of endometrial gene expression in women with RIF. Similar studies have been conducted by several research groups. Although very few consensus genes have

been identified across the studies, they demonstrate that a multitude of genes are associated with the endometrial transcriptome.

In the studies based on RNA-seq, the scientists compared the endometrial gene expression profiles in the window of implantation among women with unexplained RIF and RPL [41]. In this study, 9 patients with RIF and 11 patients with RPL were recruited. Firstly, the transcriptomes of the two groups were determined by RNA-seq, and then the differentially expressed genes between RIF and RPL were validated by qRT-PCR. According to the principal component analysis (PCA), complementary and coagulation cascades pathway was significantly upregulated in RIF while downregulated in RPL. In another study, researchers tried to identify novel molecular markers for the assessment of endometrial receptivity based on the endometrial biopsies from 12 recruited volunteers with normal menstrual cycles [42]. The endometrial transcriptomes were determined by RNA-seq, and the expression of selected differentially expressed genes was validated by qRT-PCR. A total of 2372 differently expressed genes were identified by RNA-seq. The authors found metallothionein (MT) family members MT1E, F, G, H, M, X, and 2A, and four novel transcripts, Huntingtin-associated protein 1 (HAP1), zinc finger CCHC domain-containing protein 12 (ZCCHC12), melanocortin 2 receptor accessory protein 2 (MRAP2), and oviductal glycoprotein 1 (OVGP1), exhibited significant expression changes during embryo implantation. Gene co-expression network analysis identified five core regulatory factors including GLI Family Zinc Finger 2 (GLI2), cell division cycle 25A (CDC25A), toll-like receptor 9 precursor (TLR9), metallothionein 1G (MT1G), and solute carrier family 5 member 1 (SLC5A1) are related to the endometrial receptivity during embryo implantation. Examination of the promoter regions of the differently expressed genes identified AP2 and SP1 binding sites, suggesting a potential regulatory role of the two transcription factors in the endometrial gene expression. Through these studies, the candidate gene markers found by RNA-seq provide valuable information for the assessment of endometrial receptivity, which may contribute to improving the clinical outcome of IVF.

MicroRNAs have been implicated as causal factors in implantation failure during IVF. A microRNA microarray study of mid-secretory endometrium from women with RIF identified a panel of 13 dysregulated miRNAs. The overexpression of miR-23b and miR-145 with the target as a group of cell adhesion molecules involved in implantation was found in samples from an independent cohort of patients with implantation defects [43].

The Application of Endometrial Gene Expression Profiling in Tumor

Precision medicine calls for the better characterization of cancer tissue. Endometrial cancer of the uterus is the most common gynecological malignancy, which is responsible for tens of thousands of deaths each year worldwide [44]. As the golden

standard, histopathologic diagnosis shows obvious limitations, such as low sensitivity, difficult to practice, etc. Therefore, it is necessary to apply the latest transcriptome profiling techniques to better characterize the molecular details of endometrial cancer.

Gene Expression Profile as a Biomarker in Cancer Diagnosis

Global gene expression analysis is recognized as an effective strategy to discover a criterion that classifies cancer tissues into clinically meaningful subgroups. Since the early 2000s, microarray has been applied to characterize the gene expression profiles among different types of endometrial cancer [45–47]. However, background noises of hybridization in microarray limit the accuracy of expression measurements, particularly for transcripts with a low amount, which reduces the reproductivity among different studies. In addition, although microarray can identify gene expression differences among samples, comparability of the results from different studies is still in doubt. To overcome the discrepancy and low reproducibility of individual microarray studies of endometrial cancer, a meta-analysis of gene expression profile in endometrial cancer investigated 12 microarray results, in which 121 genes were found to be associated with poor outcome among endometrioid endometrial cancer (EEC) patients [48].

Along with the progress of sequencing technologies, RNA-seq is becoming an established and effective screening method in identifying new biomarkers and molecular targets for chemotherapy. In the field of endometrial cancer, numerous studies via RNA-seq have generated tremendous amounts of new data and new insights for the characterization of the cancer tissues.

In one study, the RNA-seq was performed on an Illumina platform for seven patients with EEC [49]. A sum of five genes, including VSIG2, PTPRT, PRTG, IGSF9, and PTPRF, were found to share a functional similarity and form the only enriched cluster from the list of top 66 genes that were taken from the differentially expressed genes cutoff. Among them, IGSF9 is the biomarker with the maximum increase, which is validated by immunohistochemistry staining in human endometrial cancer tissues.

In another study, RNA-seq analysis was utilized to define the biological processes that govern the clinical behavior of endometrial cancers [50]. The researchers collected RNA-seq data from 323 cases of endometrial cancer in The Cancer Genome Atlas (TCGA) to determine the transcription module of each prototype gene. The expression of prototype genes and modules and their association with the clinical outcome were assessed in univariate and multivariate survival analysis. The author found that the clinical behavior of endometrial cancers was associated with hormone receptor signaling, phosphatidylinositol 3-kinase (PI3K) pathway signaling, and DNA mismatch repair processes. In univariate analysis, hormone receptor, PI3K, and DNA mismatch repair modules were significantly associated with the clinical outcome, whereas the clinical behavior of endometrial cancers was likely governed by apoptosis and Wnt signaling. Multivariate survival analysis revealed

that MSH6 gene expression was associated with clinical outcome of endometrial cancer independently from the traditional prognostic clinicopathologic parameters.

With the goal of identifying diagnostic and prognostic biomarkers for endometrial cancer, microRNA profiling has been carried out in numerous laboratories [51]. The microRNAs involved in oncogenesis, invasion, and metastasis have been widely reported. In addition, the microRNAs involved in DNA methylation during the progression of endometrial cancer have also been discovered. Most of these studies are conducted based on the microarray. Their results suggested the link of microRNA with cancer classification, cancer grade, recurrence, and lymph node metastasis.

lncRNA expression patterns in endometrial cancer as the biomarker have also been investigated. In a recent study, the lncRNA transcriptome of endometrial cancers and adjacent normal endometrium from the same patients was compared with those of other gynecologic malignancies [52]. In detail, RNA was isolated from malignant and adjacent nonaffected endometrial tissue from six patients with low-grade and stage Type I endometrial cancer. Subsequently, RNA-seq was performed to determine different transcriptome patterns. The author found LINC00958 was upregulated in all cancers, and lncRNAs including LINC01480, LINC00645, LINC00891, and LINC00702 specifically expressed in malignant endometrium, but not normal endometrium.

Gene Expression Profile as a Biomarker in Cancer Therapy

A subset of patients experienced a recurrence of endometrial cancer for reasons that remain unclear. Recurrence with chemoresistance to carboplatin and paclitaxel was related to the high mortality. Understanding the pathways involved in endometrial cancer chemoresistance is paramount for the identification of novel molecular targets for this disease. In the study conducted by Hellweg and his group, the matched pairs of carboplatin-sensitive/resistant and paclitaxel-sensitive/resistant endometrial cancer cells are subjected to RNA-seq [53]. They found that the expression of 45 genes is commonly upregulated in carboplatin- and paclitaxel-resistant cells as compared to controls. In these genes, LIF, PTP4A3, and TGFB1 showed a highly significant correlation between the expression level and cancer cell survival. Additionally, four upregulated chemoresistance-associated genes including ADAMTS5, MICAL2, STAT5A, and PTP4A3 were responsible for the expression of the proteins for which small-molecule inhibitors had already existed. This study underlines the utility of RNA-seq in the assessment of chemoresistance, which will benefit the precise medicine for patients with relapse of endometrial cancer.

Besides the mRNA profiling, the changes of microRNA associated with therapeutic resistance in patients with endometrial cancer have been tried to be defined. In one study, the researchers investigated the changes in microRNA profiles in samples derived from a cohort of the patients with endometrial cancer [54]. In the samples from patients who had progressive disease during or shortly following chemotherapy and patients who remained without disease recurrence, the expression profiles of microRNAs were analyzed via a cancer-specific 84-microRNA

microarray. They identified the upregulation of miR-141-3p and miR-96-5p along with a downregulation of miR-26, miR-126-3p, miR-23b, miR-195-5p, miR-374a, and let-7 family of miRNAs in endometrial cancer. Differential analysis of microRNA profile between recurrent and nonrecurrent patients indicated that the upregulation of the tumor suppressor miR-142-3p, miR-142-5p, and miR-15a-5p along with a drastically increased expression of miR-96-5p was observed in the samples derived from patients who showed a progression-free survival (PFS) of more than 21 months. In contrast, the samples derived from patients with PFS of less than 21 months showed little or no expression of miR-142-3p, miR-142-5p, and miR-15a-5p and relatively weaker expression of miR-96-5p.

The Exploration in Heterogeneity Characterization of Endometrial Cancer

Along with the appearance of scRNA-seq, the studies focusing on the heterogeneity of tumor cells increased rapidly. Last year, Shinichi Hashimoto et al. performed scRNA-seq on the endometrioid adenocarcinoma (EA), which is the most common type of endometrial cancer [55]. They demonstrated that established endometrioid adenocarcinoma subtype classifiers were variably expressed across individual cells within the cancer tissue.

Conclusion and Perspectives

The endometrium is of great biological importance because it provides the microenvironment for the embryo implantation. However, a comprehensive endometrial characterization is still a big challenge due to its complex structure and dynamic cycling changes. Over the years, numerous efforts have been made on the endometrial characterization. Along with the rapid progress of molecular biological technology, endometrial gene expression profiling has provided a tremendous amount of information for the endometrial characterization. Nowadays, RNA-seq and even scRNA-seq are replaying microarray increasingly, which could provide a gene expression profile at a higher resolution. Besides expression of protein-coding genes, more regulatory RNA species have been identified, including microRNA, lncRNA, and circRNA. RNA fragments that reflect RNA-protein binding or ribosome binding could also be selectively sequenced and characterized by various library preparation methods. These assays collectively depict a comprehensive overview of the mRNA molecules and other RNA species involved in transcriptional or posttranscriptional regulation.

The liquid biopsy has attracted huge attention in its application in the prediction and diagnosis of various diseases. In addition to the local RNA profiling in the pathogenic endometrium, the circulating cell-free RNA is also considered as a good candidate of biomarkers because of the convenient operation and low cost. Studies found endogenous circulating RNA have a half-life reported to be from several

minutes to hours [56]. Their enhanced stability is attributed to their association with proteins, lipoproteins, and shielding by extracellular vesicles, such as exosomes, microvesicles, and apoptotic bodies [57]. Clearance kinetics of RNA-containing complexes are determined by the nature of production and accumulation, which is associated with the cell origin and other molecules present in the biological fluid (Fig. 2.2). Consequently, similar to circulating DNA, the circulating RNA can also

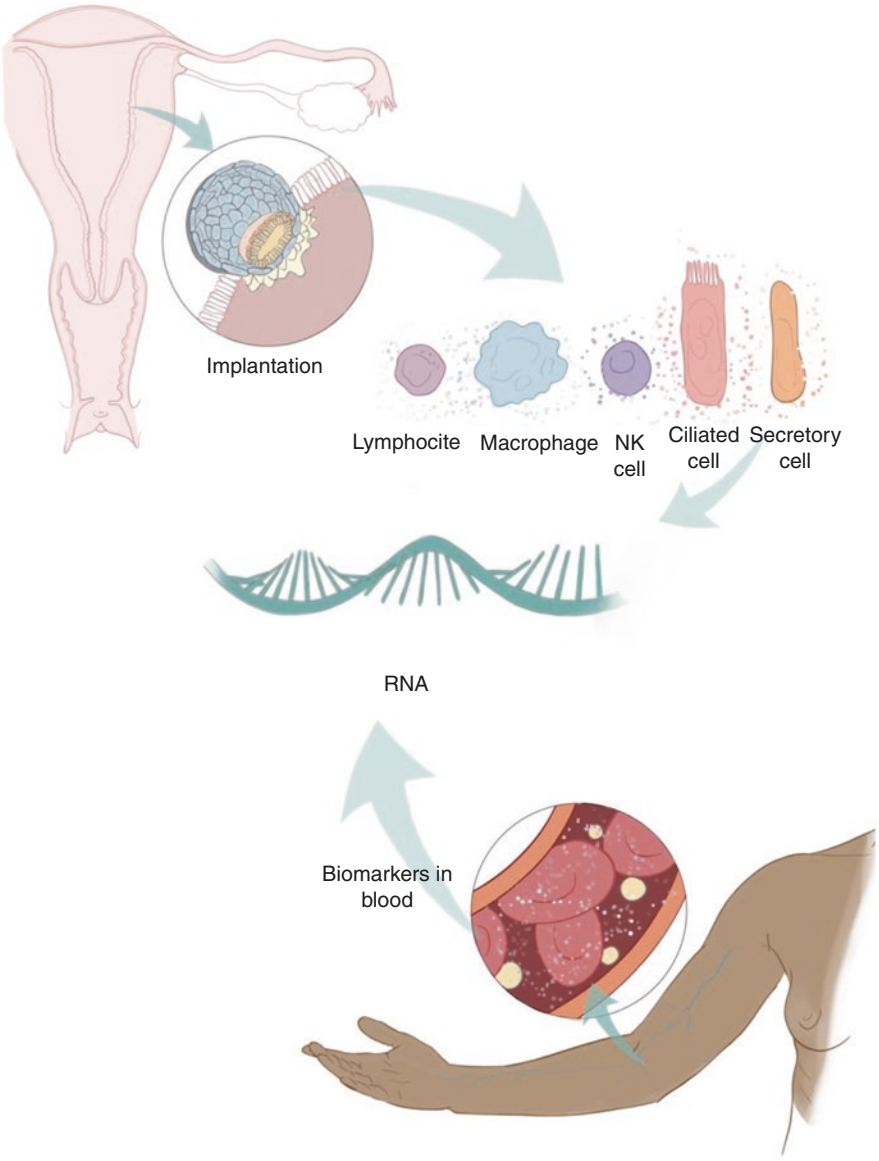

Fig. 2.2 Circulating RNAs are lucrative biomarker candidates for endometrial characterization (Photo credits: Isabel Romero Calvo)

reflect the current state of the microenvironment in the human body. Moreover, it will be more sensitive than circulating DNA if the signal exhibits significant timeliness. Although increased successes have revolutionized our view that circulating RNA is lucrative as the clinical biomarker [58], the circulating RNA-based biomarker is still less investigated comparing with the local RNA in the pathogenic tissue. Considering the cost and operation of circulating RNA detection, more efforts should be made in this direction for the endothelial characterization. Therefore, more sensitive molecular techniques are waiting to be explored. We hope the new prospective method for endometrial characterization based on future findings will streamline the clinical diagnosis and therapy.

References

1. Guyton AC, Hall JE. Chapter 81 Female physiology before pregnancy and female hormones. In: Textbook of medical physiology. 11th ed. W.B. Saunder Co.; 2006. p. 1018ff.
2. Strassmann BI. The evolution of endometrial cycles and menstruation. Q Rev Biol. 1996;71(2):181–220.
3. Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science. 1995;270(5235):467–70.
4. Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet. 2009;10(1):57–63.
5. Nawy T. Single-cell sequencing. Nat Methods. 2014;11(1):18.
6. Macosko EZ, Basu A, Satija R, Nemesh J, Shekhar K, Goldman M, et al. Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. Cell. 2015;161(5):1202–14.
7. Carninci P, Kasukawa T, Katayama S, Gough J, Frith MC, Maeda N, et al. The transcriptional landscape of the mammalian genome. Science. 2005;309(5740):1559–63.
8. Kapranov P, Cheng J, Dike S, Nix DA, Duttagupta R, Willingham AT, et al. RNA maps reveal new RNA classes and a possible function for pervasive transcription. Science. 2007;316(5830):1484–8.
9. Guttman M, Amit I, Garber M, French C, Lin MF, Feldser D, et al. Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature. 2009;458(7235):223–7.
10. Hesse M, Arenz C. MicroRNA maturation and human disease. Methods Mol Biol. 2014;1095:11–25.
11. Mercer TR, Dinger ME, Mattick JS. Long non-coding RNAs: insights into functions. Nat Rev Genet. 2009;10(3):155–9.
12. Salzman J. Circular RNA expression: its potential regulation and function. Trends Genet. 2016;32(5):309–16.
13. Hrdlickova R, Toloue M, Tian B. RNA-Seq methods for transcriptome analysis. Wiley Interdiscip Rev RNA. 2017;8(1):e1364.
14. Szabo L, Morey R, Palpant NJ, Wang PL, Afari N, Jiang C, et al. Statistically based splicing detection reveals neural enrichment and tissue-specific induction of circular RNA during human fetal development. Genome Biol. 2015;16:126.
15. Hansen TB, Kjems J, Damgaard CK. Circular RNA and miR-7 in cancer. Cancer Res. 2013;73(18):5609–12.
16. Zhao ZJ, Shen J. Circular RNA participates in the carcinogenesis and the malignant behavior of cancer. RNA Biol. 2017;14(5):514–21.
17. Han B, Chao J, Yao H. Circular RNA and its mechanisms in disease: from the bench to the clinic. Pharmacol Ther. 2018;187:31–44.

18. Holdt LM, Kohlmaier A, Teupser D. Molecular functions and specific roles of circRNAs in the cardiovascular system. Noncoding RNA Res. 2018;3(2):75–98.
19. Ingolia NT, Ghaemmaghami S, Newman JR, Weissman JS. Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling. Science. 2009;324(5924):218–23.
20. McFadden EJ, Hargrove AE. Biochemical methods to investigate lncRNA and the influence of lncRNA:protein complexes on chromatin. Biochemistry. 2016;55(11):1615–30.
21. Ponnampalam AP, Weston GC, Trajstman AC, Susil B, Rogers PA. Molecular classification of human endometrial cycle stages by transcriptional profiling. Mol Hum Reprod. 2004;10(12):879–93.
22. Talbi S, Hamilton AE, Vo KC, Tulac S, Overgaard MT, Dosiou C, et al. Molecular phenotyping of human endometrium distinguishes menstrual cycle phases and underlying biological processes in normo-ovulatory women. Endocrinology. 2006;147(3):1097–121.
23. Sigurgeirsson B, Amark H, Jemt A, Ujvari D, Westgren M, Lundeberg J, et al. Comprehensive RNA sequencing of healthy human endometrium at two time points of the menstrual cycle. Biol Reprod. 2017;96(1):24–33.
24. Krjutskov K, Katayama S, Saare M, Vera-Rodriguez M, Lubenets D, Samuel K, et al. Single-cell transcriptome analysis of endometrial tissue. Hum Reprod. 2016;31(4):844–53.
25. Wu B, Li Y, Liu Y, Jin K, Zhao K, An C, et al. Cell atlas of human uterus. bioRxiv. 2018.
26. Wang W, Vilella F, Moreno I, Pan W, Simon C, Quake SR. Single cell RNAseq provides a molecular and cellular cartography of changes to the human endometrium through the menstrual cycle. bioRxiv. 2018.
27. Li Z, Bai P, Peng D, Wang H, Guo Y, Jiang Y, et al. Screening and confirmation of microRNA markers for distinguishing between menstrual and peripheral blood. Forensic Sci Int Genet. 2017;30:24–33.
28. Rekker K, Saare M, Roost AM, Salumets A, Peters M. Circulating microRNA profile throughout the menstrual cycle. PLoS One. 2013;8(11):e81166.
29. Kennedy S, Bergqvist A, Chapron C, D'Hooghe T, Dunselman G, Greb R, et al. ESHRE guideline for the diagnosis and treatment of endometriosis. Hum Reprod. 2005;20(10):2698–704.
30. Sourial S, Tempest N, Hapangama DK. Theories on the pathogenesis of endometriosis. Int J Reprod Med. 2014;2014:179515.
31. Colon-Caraballo M, Garcia M, Mendoza A, Flores I. Human endometriosis tissue microarray reveals site-specific expression of estrogen receptors, progesterone receptor, and Ki67. Appl Immunohistochem Mol Morphol. 2019;27(7):491–500.
32. Ping S, Ma C, Liu P, Yang L, Yang X, Wu Q, et al. Molecular mechanisms underlying endometriosis pathogenesis revealed by bioinformatics analysis of microarray data. Arch Gynecol Obstet. 2016;293(4):797–804.
33. Zhao L, Gu C, Ye M, Zhang Z, Han W, Fan W, et al. Identification of global transcriptome abnormalities and potential biomarkers in eutopic endometria of women with endometriosis: a preliminary study. Biomed Rep. 2017;6(6):654–62.
34. Gilabert-Estelles J, Braza-Boils A, Ramon LA, Zorio E, Medina P, Espana F, et al. Role of microRNAs in gynecological pathology. Curr Med Chem. 2012;19(15):2406–13.
35. Hawkins SM, Creighton CJ, Han DY, Zariff A, Anderson ML, Gunaratne PH, et al. Functional microRNA involved in endometriosis. Mol Endocrinol. 2011;25(5):821–32.
36. Ohlsson Teague EM, Van der Hoek KH, Van der Hoek MB, Perry N, Wagaarachchi P, Robertson SA, et al. MicroRNA-regulated pathways associated with endometriosis. Mol Endocrinol. 2009;23(2):265–75.
37. Braza-Boils A, Mari-Alexandre J, Gilabert J, Sanchez-Izquierdo D, Espana F, Estelles A, et al. MicroRNA expression profile in endometriosis: its relation to angiogenesis and fibrinolytic factors. Hum Reprod. 2014;29(5):978–88.

38. Xu XX, Jia SZ, Dai Y, Zhang JJ, Li XY, Shi JH, et al. Identification of circular RNAs as a novel biomarker for ovarian endometriosis. Chin Med J. 2018;131(5):559–66.
39. Koot YE, van Hooff SR, Boomsma CM, van Leenen D, Groot Koerkamp MJ, Goddijn M, et al. An endometrial gene expression signature accurately predicts recurrent implantation failure after IVF. Sci Rep. 2016;6:19411.
40. Tapia-Pizarro A, Figueroa P, Brito J, Marin JC, Munroe DJ, Croxatto HB. Endometrial gene expression reveals compromised progesterone signaling in women refractory to embryo implantation. Reprod Biol Endocrinol. 2014;12:92.
41. Huang J, Qin H, Yang Y, Chen X, Zhang J, Laird S, et al. A comparison of transcriptomic profiles in endometrium during window of implantation between women with unexplained recurrent implantation failure and recurrent miscarriage. Reproduction. 2017;153(6):749–58.
42. Hu S, Yao G, Wang Y, Xu H, Ji X, He Y, et al. Transcriptomic changes during the pre-receptive to receptive transition in human endometrium detected by RNA-Seq. J Clin Endocrinol Metab. 2014;99(12):E2744–53.
43. Revel A, Achache H, Stevens J, Smith Y, Reich R. MicroRNAs are associated with human embryo implantation defects. Hum Reprod. 2011;26(10):2830–40.
44. Arend RC, Jones BA, Martinez A, Goodfellow P. Endometrial cancer: molecular markers and management of advanced stage disease. Gynecol Oncol. 2018;150(3):569–80.
45. Risinger JI, Maxwell GL, Chandramouli GV, Jazaeri A, Aprelikova O, Patterson T, et al. Microarray analysis reveals distinct gene expression profiles among different histologic types of endometrial cancer. Cancer Res. 2003;63(1):6–11.
46. Risinger JI, Allard J, Chandran U, Day R, Chandramouli GV, Miller C, et al. Gene expression analysis of early stage endometrial cancers reveals unique transcripts associated with grade and histology but not depth of invasion. Front Oncol. 2013;3:139.
47. Maxwell GL, Chandramouli GV, Dainty L, Litzi TJ, Berchuck A, Barrett JC, et al. Microarray analysis of endometrial carcinomas and mixed mullerian tumors reveals distinct gene expression profiles associated with different histologic types of uterine cancer. Clin Cancer Res. 2005;11(11):4056–66.
48. O'Mara TA, Zhao M, Spurdle AB. Meta-analysis of gene expression studies in endometrial cancer identifies gene expression profiles associated with aggressive disease and patient outcome. Sci Rep. 2016;6:36677.
49. Shi Z, Li C, Tarwater L, Li J, Li Y, Kaliney W, et al. RNA-seq reveals the overexpression of IGSF9 in endometrial Cancer. J Oncol. 2018;2018:2439527.
50. Lemetre C, Vieites B, Ng CK, Piscuoglio S, Schultheis AM, Marchio C, et al. RNASeq analysis reveals biological processes governing the clinical behaviour of endometrioid and serous endometrial cancers. Eur J Cancer. 2016;64:149–58.
51. Yanokura M, Banno K, Iida M, Irie H, Umene K, Masuda K, et al. MicroRNAS in endometrial cancer: recent advances and potential clinical applications. EXCLI J. 2015;14:190–8.
52. Chen BJ, Byrne FL, Takenaka K, Modesitt SC, Olzomer EM, Mills JD, et al. Transcriptome landscape of long intergenic non-coding RNAs in endometrial cancer. Gynecol Oncol. 2017;147(3):654–62.
53. Hellweg R, Mooneyham A, Chang Z, Shetty M, Emmings E, Iizuka Y, et al. RNA sequencing of carboplatin- and paclitaxel-resistant endometrial cancer cells reveals new stratification markers and molecular targets for cancer treatment. Horm Cancer. 2018;9(5):326–37.
54. Jayaraman M, Radhakrishnan R, Mathews CA, Yan M, Husain S, Moxley KM, et al. Identification of novel diagnostic and prognostic miRNA signatures in endometrial cancer. Genes Cancer. 2017;8(5–6):566–76.
55. Hashimoto S, Tabuchi Y, Yurino H, Hirohashi Y, Deshimaru S, Asano T, et al. Comprehensive single-cell transcriptome analysis reveals heterogeneity in endometrioid adenocarcinoma tissues. Sci Rep. 2017;7(1):14225.

56. Fleischhacker M, Schmidt B. Circulating nucleic acids (CNAs) and cancer--a survey. Biochim Biophys Acta. 2007;1775(1):181–232.
57. Crescitelli R, Lasser C, Szabo TG, Kittel A, Eldh M, Dianzani I, et al. Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosomes. J Extracell Vesicles. 2013;2.
58. Zaporozhchenko IA, Ponomaryova AA, Rykova EY, Laktionov PP. The potential of circulating cell-free RNA as a cancer biomarker: challenges and opportunities. Expert Rev Mol Diagn. 2018;18(2):133–45.

Chapter 3
T Cell-Related Endometrial Gene Expression in Normal and Complicated Pregnancies

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Introduction

T cells, including $CD4^+$ and $CD8^+$ T cells, play a central role in immune regulation and induction of tolerance during pregnancy, which allows the successful implantation and maintenance of pregnancy. $CD4^+$ T cells can be classified into T helper (Th) 1, Th2, T regulatory (Treg), and Th17 cells. Naïve $CD4^+$ T cells under the stimulus of cytokines, such as interleukin (IL)-12 and IFN-γ, usually differentiate into Th1 cells, characterized by producing interferon (IFN)-γ and tumor necrosis factor (TNF)-α [1]. The Th1 subset is generally involved in macrophage activation and cell-mediated immunity. IL-4 stimulates naïve $CD4^+$ T cells to differentiate into a Th2 phenotype

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[2], while IL-12 inhibits Th2 cell differentiation [3]. Th2 cells express type 2 cytokines such as IL-4, IL-5, IL-6, and IL-13 [4] and are responsible for antibody production and inhibition of macrophage activation. Treg cells release transforming growth factor-β (TGF-β) and IL-10 and are responsible for immune tolerance and pregnancy success [5] by suppressing Th1 and natural killer (NK) cell activation. TGF-β stimulates naïve $CD4^+$ T cells to differentiate into Treg cells [5], while IL-6 represses Treg development [6]. Th17 cells are responsible for pregnancy losses and autoimmunities such as rheumatoid arthritis and multiple sclerosis by producing IL-17, IL-21, and IL-22 [7]. TGF-β and IL-6 promote naïve $CD4^+$ T cells to differentiate into Th17 cells. In contrast, IL-4 and IFN-γ have been reported to suppress the differentiation of naïve $CD4^+$ T cell into Th17 cell as a negative feedback mechanism [8]. Interestingly, Th17 and Treg cells have some degree of plasticity, and Th17 cells can transdifferentiate into Treg cells and vice versa. The micromilieu around these cells determines their destiny [9]. Compared with $CD4^+$ T cells, $CD8^+$ T cells, which produce lower levels of cytokines than $CD4^+$ T cells, have correspondingly classified subsets defined by their cytokine producing patterns and involve into T cell immunity [1].

T cells are infiltrated in the endometrium and decidua throughout pregnancy and contribute to the establishment of a proper immunological micromilieu by producing various cytokines and chemokines [10]. The paradigms of the Th1/Th2 balance toward Th2 bias and the Th17/Treg balance toward Treg bias have been suggested to be beneficial for the maintenance of normal pregnancy [11, 12]. In contrast, aberrant T cell immunity, such as Th1 and Th17 shifts in T cell immunity, has been associated with various reproductive disorders, such as recurrent implantation failure (RIF), recurrent pregnancy losses (RPL), preterm labor, and preeclampsia (PE) [13–18]. Hence, targeting the balance of endometrial and decidual T cell subsets might be an effective strategy for sustaining maternal-fetal immune homeostasis, and maintaining pregnancy. The comprehensive understanding of T cell subsets and their functions by investigating endometrial gene expressions may provide new insights into understanding the immunopathology of various reproductive disorders and pregnancy complications and develop a future therapeutic strategy. In this chapter, we aim to review the possible role of peripheral and endometrial/decidual T cell immunities in normal pregnancy, and complicated pregnancies particularly focused on endometrial/decidual gene expression patterns.

Regulation of T Cell Responses During Pregnancy

The embryo is considered as a semi-allograft. Paternal antigens, which are expressed by the trophoblast cells, cause the maternal immune system to respond; hence, a successful pregnancy is a result of persistent immune regulation which induces maternal tolerance to fetal alloantigens [19, 20]. In contrast, inadequate maternal immune responses lead to the failure of the trophoblast invasion and development of the feto-placental unit, resulting in various reproductive disorders, including RPL, RIF, PE, and preterm labor [13, 21–23].

Multiple immunological mechanisms regulating maternal immune responses and inducing immune tolerance at the maternal-fetal interface during pregnancy have been reported [24]. Particularly, the regulation of T cell immunity has been explored at the time of implantation and early pregnancy. It has been demonstrated that the decidua prevents decidual dendritic cells (DCs) stationed at the maternal-fetal interface from migrating to the lymphatic vessels of the uterus, and thus is essential in preventing immune rejection of the fetus [25]. DCs are trapped within the decidua which curtails immunogenic T cell activation by fetal/placental antigens. Hence, it is plausible that impaired development or function of the human decidua may lead to pathological T cell activation during pregnancy [25]. Since tissue-resident DCs have negligible input, local T cell response to the feto-placental unit is controlled by passive antigen transport, and thus a tolerogenic mode of antigen presentation is secured [25]. In addition, during early pregnancy, gradual and low level of antigen presentation to T cells leads to the induction of Treg cells and the deletion of effector T cells [26]. Uterine NK (uNK) cells were reported to interact with human $CD14^+$ DCs and improve DC ability to induce Treg cells [27].

At the time of implantation and early pregnancy, the presence of immune effectors, cytokines, and growth factors, extensive vascularization, angiogenesis, tissue implantation, and cellular infiltration are the common features at the maternal-fetal interface [28–30]. However, T cell density in human decidua is relatively low (10% of endometrial stromal leukocyte population) [31], suggesting a presence of regulatory mechanism. Effector T cells express C-X-C motif chemokine receptor 3 (CXCR3) which guides T cells to decidua during T cell trafficking process [32], whereas effector T cell trafficking is regulated by limited expression of Th1/T cytotoxic (Tc) 1-attracting chemokine CXCL9 (CXCR3 ligand) in the decidua, consequently preventing the Th1/Tc1 cell accumulation in the decidua. Impaired accumulation of T cells was partly attributable to the epigenetic silencing of key T cell-attracting inflammatory chemokine genes in decidual stromal cells. Trimethylation at lysine 27 of histone H3 (H3K27me3), known for shutting down transcription, was reported to modify *Cxcl9/10* promoters when endometrial stromal cells transformed into decidual stromal cells, influencing the tissue's capacity for T cell accumulation [33]. Hence, the developmental program of decidual chemokine silencing is implicated as a potentially conserved mechanism of maternal-fetal tolerance [33].

Treg and memory Treg cells may reinforce fetal tolerance during pregnancy and promote reproductive success by dampening cell-mediated immunity and producing IL-10 and TGF-β. The majority of decidual T cells are $CD45RO^+$ effector cells which are more differentiated effector memory (EM) phenotype than in blood and have increased capacity to produce IL-4 and IFN-γ [10]. Depletion of Treg cells promoted the proliferation of fetal antigen-specific decidual T cells [10]. During pregnancy, maternal forkhead box protein 3^+ ($Foxp3^+$) Treg cells with defined fetal specificity are expanded systematically and then progressively diminish after delivery. In a subsequent pregnancy, previously primed memory Treg cells rapidly expand when they are reexposed to the same fetal antigens in accelerated kinetics compared to the first pregnancy [34]. Indeed, parous women carry a higher number

of $CD4^+$ EM, central memory, and activated memory T cells compared to nulligravid women, suggesting memory cells are generated during pregnancy and involved in the pregnancy outcome in human [35]. In a subsequent pregnancy, fetus-specific Treg cells are highly enriched than those in the first pregnancy, conferring immune-protective properties against immune deregulation in fetal tolerance [36].

CD4+ T Cells and Pregnancy

Normal pregnancy is associated with increased Th2-type immunity and decreased Th1-type pro-inflammatory immunity which is more evident at the maternal-fetal interface [19]. Previously, Th2-type-cytokine-knockout mice showed normal pregnancy, suggesting that predominant Th2-type immunity might not be essential for a successful pregnancy [37], while the administration of an excessive amount of Th1-type cytokines, such as TNF-α and IFN-γ coadministration, induced abortion in mice [38]. However, IFN-γ also plays a crucial role in vascular remodeling at the early stage of murine pregnancy [39]. In the decidua, DCs regulate Th1/Th2 immune balance and maintain a Th2-dominant status [40]. During early pregnancy, myeloid DCs are the major DC population and secrete significantly lower level of IL-12 compared to peripheral blood. Decidual myeloid DCs lead the differentiation of naïve CD4+ T cells to a higher percentage of Th2 cells than with peripheral myeloid DCs, contributing to the maintenance of pregnancy [40].

Treg cells have a critical role in maternal-fetal tolerance during pregnancy. Trophoblast cells constitutively secrete high levels of TGF-β and induce T-cell differentiation into a Treg cell phenotype characterized by Foxp3 expression [41]. Adoptive transfer of Treg cells, purified from normal pregnant mice, elevated decidual Foxp3 mRNA levels and prevented fetal loss in abortion-prone CBA/J mice mated with DBA/2J males [42]. Treg cells operate to inhibit effector immunity, contain the inflammation, and support maternal vascular adaptations, thereby facilitating trophoblast invasion and placental access to the maternal blood supply [43]. Insufficient Treg numbers or inadequate functional competence are implicated in implantation failures [43]. During the implantation phase, decidual Th1 cell densities are moderately elevated, while Th17 and Th2 cells are generally not enriched, indicating a mild inflammatory environment controlled by Treg cells [43]. Treg cells present a certain level of plasticity under some circumstances and can transdifferentiate into Th17 cells [9]. The pro-inflammatory role of Th17 cells at the maternal-fetal junction might lead to RPL [14], while downregulation of Th17 cells by Treg during early pregnancy potentially exacerbated bacterial infection in a mouse study [44]. Hence, the balance between Treg and Th17 immunity determines the pregnancy outcome. Th17 cells secrete IL-17, IL-21, and IL-22. IL-22 is involved in allograft rejection, and recently an exclusive presence of Th17/Th2/IL-22^+ and Th17/Th0/IL-22^+ cells at the embryo implantation site has been reported, where IL-4, GATA-binding protein 3 (GATA-3), IL-17A, retinoic acid related orphan receptor C (ROR-C),

IL-22, and aryl hydrocarbon receptor (AHR) mRNAs are expressed. IL-22 did not have a pathogenic role when IL-4 was also produced by decidual $CD4^+$ T cells [45]. The potential role of Th22 cells at the implantation site should be explored further.

$CD8^+$ Tc Cell Subsets Involved in Pregnancy

In the peripheral blood, fetal antigen-specific $CD8^+$ T cells are detectable during and after pregnancy [46, 47]. In the decidua, $CD8^+$ T cells are the most common T cell subsets, which promote trophoblast invasion and maintain maternal tolerance [48]. During pregnancy, decidual $CD8^+$ ($dCD8^+$) T cells have different transcriptional and alternative splicing landscapes as compared with peripheral blood $CD8^+$ T cells in a study using high-throughput mRNA sequencing [49]. $dCD8^+$ T cells have subsets including $dCD8^+$ Treg and EM cells. $dCD8^+$ EM cells express significantly lower levels of perforin and granzyme B compared to peripheral blood $CD8^+$ EM T cells demonstrated by quantitative polymerase chain reaction (PCR) analysis [50]. Contrarily, perforin and granzyme B mRNA expression was significantly increased in decidual EM $CD8^+$ T cells compared to peripheral blood $CD8^+$ EM T cells, suggesting $CD8^+$ T cells may adopt alternative means of EM cell differentiation which may include blockage of perforin and granzyme B mRNA translation [50]. Contrarily, the recent study reported that human $dCD8^+$ T cells had upregulated gene expression involved in M phase of mitotic cell cycle and immune system process and downregulated gene expressions related to the metabolic process. $dCD8^+$ T cells, which displayed $CD8^+$ Treg and EM phenotypes, demonstrated increased activation and proliferation, and enhanced functionality in degranulation and cytokine production on a per-cell base [49]. $dCD8^+$ T cells have upregulated expression of CD69, CD38, CD122, CD276, and inducible T-cell costimulator (ICOS) [49]. Upregulated CD69 and CD103 expressions in $dCD8^+$ T cells suggest that $dCD8^+$ T cells are mucosal memory T cells rather than circulating memory T cells [17]. Mucosal memory T cells provide not only the first line of defense against subsequent infection [51] but immune tolerance to subsequent pregnancy.

Alteration of Endometrial Gene Expression

Various factors dynamically change endometrial gene expressions. Aging plays a role in changes in the endometrial function and its related gene expression. In a cow model, aging is related to the activation of endometrial inflammatory and interferon-signaling pathways [52]. Using RNAseq analysis, endometrial expression of inflammation-related molecules, such as *interleukin 1 alpha (IL1A)*, *complement component 1q, serum* (*C1QS)*, *DExD/H-Box Helicase 58* (*DDX58)*, *nuclear factor – kappa B cells* (*NFKB)*, and *chemokine (C-C motif) ligand 5* (*CCL5)*, was

significantly increased in aged animals than in young cows [52]. In addition, expression of interferon-signaling predictor molecules, such as *interferon regulatory factors (IRFs), interferon-induced proteins with tetratricopeptide repeats (IFITs), signal transducers and activators of transcription (STATs),* and *IFNs*, was significantly increased in aged cows than in young cows. Aged endometrium also has activation of DNA damage checkpoint regulation and inhibition of mitotic mechanisms [52]. Therefore, inflammatory- and IFN-signaling and dysfunction of cell division are increased with the aging process.

There are several genes altered during early pregnancy, suggesting additional conceptus-derived proteins may be involved in the alteration of gene expression [53]. In a bovine study using RNAseq, 459 differentially expressed genes were detected in pregnant endometrium as compared with that of cycle day 16 endometrium [53]. Notably, the expression of *PARP12, ZNFX1, HERC6, IFI16, RNF213,* and *DDX58* was increased in the decidua of pregnant animal compared with that of cycle day 16 endometrium, which was directly upregulated by interferon-tau (INFT) in the bovine endometrium in vivo. RNAseq also identified the altered expression of several genes detected as early as cycle day 13, which was not directly regulated by INFT in vivo [53].

In human, decidual T cells demonstrated a unique transcriptional gene profile with elevated expression of proteins associated with the response to interferon signaling [10, 46]. IFN-α, type 1 IFN is an antiangiogenic factor which downregulates proangiogenic factor such as vascular endothelial growth factor (VEGF), resulting in abnormal placentation and obstetrical complications. Taken together, decidual T cells have a critical role in the modulation of vascular development with advances in pregnancy [10]. In addition, $CD4^+$ effector T cells have upregulated *CLIC1* and *LGALS1* gene expression [10]. Hypoxia increases *CLIC1* gene expression which acts to regulate cell function through the reactive oxygen species (ROS)-mediated p38 mitogen-activated protein kinase (MAPK) signaling pathway [10, 54]. *LGALS1* gene encodes Galectin 1 [10]. Activated T cells have intracellular Galectin 1 expression [55], which activates downstream apoptosis [56]. Contrarily, $CD8^+$ T cells have upregulated expression of Galectin 9, a ligand of T cell immunoglobulin mucin-3 (Tim-3). Interaction with Tim-3 induces chemoattraction, apoptosis, and suppression of chronic inflammation [57, 58]. Expression of Tim-3 on Treg cells promotes the sustainable phenotype of immunosuppression. Therefore, fetal-specific recognition is carefully modulated by multiple checkpoint proteins and Treg cells [10].

T Cell Abnormalities and Reproductive Disorders

Implantation Failures

Successful implantation is dependent on the development of a high-quality embryo and the acquisition of endometrial receptivity. Implantation phase can be described as four steps: the first step is apposition, which is a loose interaction between the

blastocyst and the epithelium of the endometrium; the second step is attachment, which is a much stronger interaction between the blastocyst and the epithelium. The adhesion molecules play a significant role in this phase. The third step is an invasion. At this phase, trophoblast cells proliferate and penetrate the endometrium and differentiate into syncytiotrophoblast cells. The fourth step is an inflammatory response which is observed between endometrial immune cells and blastocyst implantation [59]. Inflammation at the implantation site supports angiogenesis, tissue remodeling, and the recruitment of macrophages for the removal of apoptotic cells. Inflammation is necessary for embryo implantation; however, excessive inflammation often results in implantation failure and embryo resorption [19]. In women with RIF, endometrial biopsies taken during the proliferation phase of the menstrual cycle substantially increase the amount of HLA-DR^{+}CD11c^{+} macrophages/dendritic cells, and expression of TNF-α, growth-regulated oncogene-alpha (GRO-α), IL-15, and macrophage inflammatory protein 1 beta (MIP-1β) protein on cycle day 21. The implantation and clinical pregnancy rates were positively correlated with the number of macrophages, MIP-1β and TNF-α. Hence, the local injury induces an inflammatory response, including elevated pro-inflammatory cytokines such as TNF-α [60]. TNF-α stimulates primary endometrial stromal cells to express cytokines that attract monocytes and induce their differentiation into DCs [60]. Collectively, these findings emphasize the importance of inflammation for the receptivity of the maternal decidua and determining the success of implantation and early placentation.

During the implantation window, endometrial gene expression profiles of women with infertility and RPL are different prominently from those of normal fertile women. Women with RIF were reported to have 2126 differentially expressed endometrial genes compared to fertile women using Affymetrix chips (GeneChip Human Genome U133 Plus 2.0 Array) [61]. Majority of the differentially expressed genes are attributable to DNA transcription and expression. Others are related to cell morphology, development, cycle, and assembly. Interestingly, women with RIF have deregulated genes responsible for cellular immune response include abnormal T-cell development, mainly Th-2 differentiation and development, Th1 cell transmigration, and the NK cell migration. Additionally, genes associated with integrin patterns, such as *ITGA6, ITGB3, ITGAL,* and *ITGAL2B*, were downregulated in women with RIF, which seems to be associated with expression of genes related to coagulation disorder, such as coagulation factor II thrombin receptor (F2R), urokinase plasminogen activator receptor (PLAUR or u-PAR), and coagulation factor XII (F12). It has been reported that the canonical pathways involved in T cell differentiation (inducible T cell costimulator ligand (ICOSLG), CD40, IL-18, CD86) may affect the communication between DCs and uNK cells by integrins, colony stimulating factor 2 (CSF-2), IL-18, CD40, CD86. Therefore, women with RIF have intricate endometrial gene deregulation including immune deregulation, integrin pattern, and coagulation disorders [61].

Several genes have been reported to play a critical role in implantation. TNF Weak inducer of Apoptosis (TWEAK) is fundamental to protect an invading embryo against the pro-inflammatory effects of a Th1-dominant (TNF-rich) environment

during implantation and help embryo survival. Fibroblast growth factor-inducible molecule 14 (Fn-14) is a receptor for TWEAK which acts as an immune regulator of the Th1/Th2 cytokine balance in the human endometrium [62–64]. IL-18 is a Th2-promoting cytokine that affects the crucial destabilization of spiral arteries through the action of angiopoietin-2. Interestingly, a high level of IL-18 acts as pro-inflammatory Th1-type cytokine and induces the production of Th1 cytokine including IFN-γ and TNF-α [62, 64]. Ratios between these gene expressions have been reported to predict pregnancy outcome. Increased ratios of IL-18/TWEAK which reflect local angiogenesis and possibly a Th1 deviation, and IL-15/Fn-14 mRNA expressions were documented in women with RIF as compared with those of fertile controls [65]. Abnormal signaling of the colony-stimulating factor family expression (CSF-2 or granulocyte-macrophage colony-stimulating factor (GM-CSF)) and its receptor colony stimulating factor 3 Receptor (CSF3R) as well as abnormal chemokine expression (CXCR3) was reported, suggesting deregulated vascular remodeling. Interestingly, IL-18 and CSF3R gene expressions were not downregulated in women with RPL [61].

Clinical data also support a concept that Treg cells play an essential role in implantation. For example, expression of mRNA for master transcription factor for Treg, Foxp3, is reduced in endometrial tissue of women with infertility. Endometrial samples from women with unexplained infertility after experiencing repeated failures in in vitro fertilization (IVF) (Infertile women = 10, controls = 12), expression of Foxp3 mRNA was reduced by approximately twofold in the tissue of infertile women. In contrast, mRNA expressions of T-bet and GATA3, which are associated with the differentiation of Th1 and Th2 cells, TGF-beta-1, -2 and -3 mRNAs and other cytokine gene expressions including IL-2, -4, -5, -10, -12p40 (Th1, Th2 cell differentiation), IL-1α, IL-1β, IL-6, leukemia inhibitory factor (LIF), GM-CSF, and TNF-α (DC regulating cytokines), were not different between infertile women and normal fertile women [66]. There are limited data regarding decidual Th17 immunity and implantation failures. Controlling the pro-inflammatory role of Th17 cells would benefit implantation and will be a new therapeutic strategy for women with RIF. The precise mechanism of Th17 immunity at the maternal-fetal interface is still unclear and needs to be studied further.

Recurrent Pregnancy Losses

RPL is one of the common pregnancy complications before 12 weeks of gestation, occurring in 1–2% of human pregnancies and defined as two or more consecutive pregnancy losses [67]. In RPL women with unknown etiology, immunological and thrombophilic etiologies have been reported in over 50% of cases [67, 68]. Excessive Th1-type immunity and/or predominant Th17-type immunity have been reported in the peripheral blood of women with RPL [13, 14, 17]. Therefore, adequate balance for Th1/Th2 and Treg/Th17 immunities may be suitable for the maintenance of a healthy pregnancy [69]. In a recent study that investigated the effect of lymphocyte immunotherapy on Th17 and Treg cells in women with RPL, upregulation of Treg

cells and downregulation of Th17 cells were reported to contribute to the successful outcome of pregnancy [70]. Moreover, intravenous immunoglobulin G (IVIg) treatment modulates the imbalance between Th17 and Treg cells in pregnant women with RPL and cellular immune abnormalities, by upregulating Treg cells and downregulating Th17 cells [71, 72].

Furthermore, in the decidua of RPL patients, expressions of Th17-related cytokines and the transcription factor, such as IL-17, IL-23, and retinoic acid-related orphan receptor gamma t (RORγt), were significantly increased compared to those of normal early pregnant women [17]. Meanwhile, expressions of Treg-cell-related cytokines and transcription factors, such as IL-10, TGF-β, and Foxp3, were significantly downregulated [69]. Hence, the excessive Th17 cells or deficiency of Treg cells may induce spontaneous abortion, while the balance between these two cells is beneficial to pregnancy.

IL-7 plays a central role in the proliferation and survival of pathogenic Th17 cells and promotes the development of autoimmune diseases [73]. In a mouse study, the Th17 transcription factor, RORγt mRNA, was significantly increased, and Treg cell transcription factor, Foxp3 mRNA expression, was significantly decreased in both normal and abortion-prone mice treated with IL-7 when compared to matching controls. IL-7R antagonist treatment showed a significantly decreased RORγt mRNA and increased Foxp3 mRNA in the decidua of abortion-prone mice as compared to normal pregnancy controls. Therefore, blockade of IL-7/IL-7R signaling pathway may contribute to maintaining pregnancy by shifting Th17/Treg cell ratios [74].

The expression of mRNA encoding IL-6 and IL-1β in the endometrium was decreased in women with RPL as compared with normal fertile women [75]. However, the endometrial expression of pro-inflammatory cytokines, such as IL-1β, TNF-α, IFN-γ, and TGF-β1, was reported to be upregulated in women with RPL of unknown etiology as compared with controls [76]. Recently, abnormal activation of the endometrial inflammasome, NACHT, LRR and PYD domains-containing protein 3 (Nalp-3) was reported. Nalp-3 represents the first line of defense against cellular stress, and it is a crucial component of innate immunity. After Nalp-3 activation, an apoptosis-associated speck-like protein containing a CARD (ASC) and caspase-1 are assembled, to form a multiprotein complex which enables the caspase-1-mediated proteolytic processing of the pro-inflammatory cytokines (IL-1β and IL-18) and generates their respective mature secretory forms. Women with RPL have increased expression and activation of Nalp-3 inflammasome proteins, along with increased caspase-1 activation and secretion of IL-1β in the endometrium [77]. The innate immunity-related Nalp3 inflammasome and IL-1β expression need further study to explore their roles in implantation and RPL.

Prostaglandins, lipid autocoids, maintain homeostasis between Th1 and Th2 cell responses [78] and suppress lymphocyte alloreactivity of decidual cells during early pregnancy [79]. Prostaglandin E2 (PGE_2) stimulates inflammatory reaction via PGE_2 receptor 3 (EP3). It has been demonstrated via immunohistochemical study, the expression of cyclooxygenase-2, EP3, and G protein alpha inhibitor 1 (G_{i1}) was enhanced in the placenta of the women with unexplained RPL in comparison to the controls. Elevated activation of EP3 signaling in first-trimester placentas plays a vital role in regulating the inflammatory microenvironment, the hormone secretion

of extravillous trophoblasts, and the remodeling of the extracellular matrix in the feto-maternal interface [80]. Therefore, the placenta also contributes to the decidual homeostasis between Th1/Th2 immune responses during pregnancy via enhanced expression of EP3 signaling.

Preeclampsia

Preeclampsia (PE) is a hypertensive disorder that occurs after 20 weeks of gestation. Trophoblast invasion and placentation are impaired, and blood flow to the fetus, which is tightly regulated by immune cells in the decidua during normal pregnancy, is significantly reduced [81]. In women with PE, placenta presents the elevated levels of TNF-α and IL-6 which are Th1-type cytokines, while the main Th2 anti-inflammatory cytokines such as IL-4 and IL-10 are decreased at the maternal-fetal interface [2, 82]. Therefore, increased effector Th1 cells within the circulation and placentas compromise the immunomodulatory functions of Th2 cells in women with PE. It has been reported that Th17/Treg cell ratios in early pregnancy are associated with the occurrence of PE [83, 84]. Decreased Treg cells and increased Th17 cells in the decidua and circulation in women with PE lead to a failure of the maternal immune tolerance, which in turn induces placental ischemia and oxidative stress which are the major pathophysiological factors of PE [83, 85, 86]. Adoptive transfer of Treg cells from normal pregnancy could decrease blood pressure and vasoactive factors in a rat model of PE [81]. The cause of Treg/Th17 imbalance in PE is unknown. Emerging evidence proved that altered programmed death (PD)-1/PD-1 ligand (PD-L) 1 signaling pathway contributed to Treg/Th17 in PE [87]. Th22 cells, which were correlated with Th17 cells also, increased in severe PE patients [88]. Although most studies focus on $CD4^+$ T cells, emerging evidence also proved a lower frequency of regulatory $CD8^+CD28^-$ T cells in women with PE, which can suppress $CD4^+$ T cell proliferation and memory $CD4^+$ T cell responses [89].

Th1/Th2 and Th17/Treg Paradigms in Preterm Labor

Subclinical infection and the intrauterine inflammatory responses are often associated with preterm labor and delivery [90]. The concentration of pro-inflammatory cytokines such as IL-1, IL-6, IL-8, and TNF-α is increased in amniotic fluid, decidual tissue, and chorionic tissue in these patients [91]. IL-6 was reported to promote the mild increase of decidual $CD4^+$ T, a notable increase of $CD8^+$ T including $CD8^+CD25^+Foxp3^+$ Treg, and marked reduction of $CD4^+CD9^+$ Th-9 cells [92]. IL-6 deficiency delays parturition. The aberrant profile of the Th1/Th2 dichotomy has been reported to be associated with preterm labor. Placentas from women following preterm delivery demonstrated a Th1 bias with significantly higher levels of IFN-γ and IL-2, along with the Th1-inducing cytokine IL-12 as compared with term

delivery [93, 94]. In contrast, term placentas exhibit comparatively higher levels of the Th2 cytokines, IL-4, and IL-10. At term pregnancy, Treg cells are found at the fetal-maternal interface with suppressive function. Women with preterm labor had a reduced proportion of Treg, and the suppressive activity of Treg cells is significantly reduced in term and preterm labor [95]. Chorioamnionitis (CAM) is a major reason for preterm delivery. Inflammatory cytokines and chemokines play crucial roles in the pathogenesis of preterm delivery. IL-17 is a key cytokine which induces inflammation and is critical to host defense. IL-17 levels in the amniotic fluid of severe CAM (stage III) preterm delivery cases were significantly higher than those of CAM-negative preterm delivery cases. In addition, IL-17 levels were positively correlated with IL-8 levels in amniotic fluid. These findings showed that Th17 cells promoted inflammation at the maternal-fetal interface in preterm delivery [96]. Overall, the collaboration between the innate and adaptive limbs of the immune system is required to sustain the pregnancy until term. Disruption of either limb at term may lead to physiological labor, and an untimely disruption could result in pathological preterm labor. Researches targeting the immune cells involved in the process of labor might reveal new strategies to prevent preterm labor and consequently preterm birth.

Complement and coagulation cascade have been reported to be one of the top enriched pathways in preterm labor by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis [97–99]. Another upregulated pathway in preterm labor was cytokine-cytokine receptor interaction, suggesting a presence of pro-inflammatory signaling [99]. In term labor, *IL-6, PTGS2, ATF3, IER3,* and *TNFAIP3* genes were upregulated in decidua, while in preterm labor, *CXCL8, MARCO, LILRA3,* and *PLAU* genes were upregulated by qRT-PCR, suggesting that parturition is associated with extensive changes in decidual gene expressions but, particular genes are involved in pathological onset of labor [99].

Conclusion

Overall, abnormal T cell response, which is demonstrated by the presence of unbalanced Th1/Th2 and Th17/Treg immunities, is keenly associated with immune-inflammatory obstetrical complications, such as RIF, RPL, PE, and preterm labor. Therefore, systemic and local immune modulation to restore abnormal Th1/Th2 and Th17/Treg immune balances can be a therapeutic strategy for women with T cell inflammatory consequences of obstetrical complications. Abnormal T cell responses can be detected in the peripheral blood and the endometrium/decidua. Endometrial and decidual gene expression patterns reflecting abnormal T cell immune responses may serve as biological markers to predict pregnancy outcome in women with a history of RIF and RPL undergoing reproductive cycles. Although abnormal systemic T cell immunity has been well documented in women with PE and preterm labor, prepregnancy endometrial and early-pregnancy decidual gene expressions have not been elucidated well. Detection of abnormal gene expressions in preconception endometrium and early decidua may enable us to identify high-risk women for the second/

third-trimester obstetrical complications and afford early prevention and management of these conditions. Studies focused on preconception, and early pregnancy T cell immune abnormalities including endometrial/decidual gene expressions are urgently needed.

References

1. Mosmann TR, Li L, Hengartner H, Kagi D, Fu W, Sad S. Differentiation and functions of T cell subsets. Ciba Found Symp. 1997;204:148–54; discussion 54–8.
2. Chatterjee P, Chiasson VL, Bounds KR, Mitchell BM. Regulation of the anti-inflammatory cytokines interleukin-4 and interleukin-10 during pregnancy. Front Immunol. 2014;5:253.
3. Saito S, Nakashima A, Shima T, Ito M. Th1/Th2/Th17 and regulatory T-cell paradigm in pregnancy. Am J Reprod Immunol. 2010;63(6):601–10.
4. Berger A. Th1 and Th2 responses: what are they? BMJ. 2000;321(7258):424.
5. Aluvihare VR, Betz AG. The role of regulatory T cells in alloantigen tolerance. Immunol Rev. 2006;212:330–43.
6. Li X, Wang B, Li Y, Wang L, Zhao X, Zhou X, et al. The Th1/Th2/Th17/Treg paradigm induced by stachydrine hydrochloride reduces uterine bleeding in RU486-induced abortion mice. J Ethnopharmacol. 2013;145(1):241–53.
7. Figueiredo AS, Schumacher A. The T helper type 17/regulatory T cell paradigm in pregnancy. Immunology. 2016;148(1):13–21.
8. Martinez GJ, Nurieva RI, Yang XO, Dong C. Regulation and function of proinflammatory TH17 cells. Ann N Y Acad Sci. 2008;1143:188–211.
9. Liu ZZ, Sun GQ, Hu XH, Kwak-Kim J, Liao AH. The transdifferentiation of regulatory T and Th17 cells in autoimmune/inflammatory diseases and its potential implications in pregnancy complications. Am J Reprod Immunol. 2017;78:e12657.
10. Powell RM, Lissauer D, Tamblyn J, Beggs A, Cox P, Moss P, et al. Decidual T cells exhibit a highly differentiated phenotype and demonstrate potential fetal specificity and a strong transcriptional response to IFN. J Immunol. 2017;199(10):3406–17.
11. Wegmann TG, Lin H, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? Immunol Today. 1993;14(7):353–6.
12. Clark DA, Chaouat G. Regulatory T cells and reproduction: how do they do it? J Reprod Immunol. 2012;96(1–2):1–7.
13. Kwak-Kim JY, Chung-Bang HS, Ng SC, Ntrivalas EI, Mangubat CP, Beaman KD, et al. Increased T helper 1 cytokine responses by circulating T cells are present in women with recurrent pregnancy losses and in infertile women with multiple implantation failures after IVF. Hum Reprod. 2003;18(4):767–73.
14. Lee SK, Kim JY, Hur SE, Kim CJ, Na BJ, Lee M, et al. An imbalance in interleukin-17-producing T and Foxp3(+) regulatory T cells in women with idiopathic recurrent pregnancy loss. Hum Reprod. 2011;26(11):2964–71.
15. Santner-Nanan B, Peek MJ, Khanam R, Richarts L, Zhu E, Fazekas de St Groth B, et al. Systemic increase in the ratio between Foxp3+ and IL-17-producing CD4+ T cells in healthy pregnancy but not in preeclampsia. J Immunol. 2009;183(11):7023–30.
16. Zahran AM, Zharan KM, Hetta HF. Significant correlation between regulatory T cells and vitamin D status in term and preterm labor. J Reprod Immunol. 2018;129:15–22.
17. Wang WJ, Hao CF, Yi L, Yin GJ, Bao SH, Qiu LH, et al. Increased prevalence of T helper 17 (Th17) cells in peripheral blood and decidua in unexplained recurrent spontaneous abortion patients. J Reprod Immunol. 2010;84(2):164–70.
18. Koucky M, Malickova K, Cindrova-Davies T, Germanova A, Parizek A, Kalousova M, et al. Low levels of circulating T-regulatory lymphocytes and short cervical length are associated with preterm labor. J Reprod Immunol. 2014;106:110–7.

19. Kwak-Kim J, Bao S, Lee SK, Kim JW, Gilman-Sachs A. Immunological modes of pregnancy loss: inflammation, immune effectors, and stress. Am J Reprod Immunol. 2014;72(2):129–40.
20. Gleicher N, Kushnir VA, Barad DH. Redirecting reproductive immunology research toward pregnancy as a period of temporary immune tolerance. J Assist Reprod Genet. 2017;34(4):425–30.
21. Erlebacher A. Why isn't the fetus rejected? Curr Opin Immunol. 2001;13(5):590–3.
22. Thellin O, Coumans B, Zorzi W, Igout A, Heinen E. Tolerance to the foeto-placental 'graft': ten ways to support a child for nine months. Curr Opin Immunol. 2000;12(6):731–7.
23. Gaunt G, Ramin K. Immunological tolerance of the human fetus. Am J Perinatol. 2001;18(6):299–312.
24. Bonney EA. Alternative theories: pregnancy and immune tolerance. J Reprod Immunol. 2017;123:65–71.
25. Collins MK, Tay CS, Erlebacher A. Dendritic cell entrapment within the pregnant uterus inhibits immune surveillance of the maternal/fetal interface in mice. J Clin Invest. 2009;119(7):2062–73.
26. Lin WW, Karin M. A cytokine-mediated link between innate immunity, inflammation, and cancer. J Clin Invest. 2007;117(5):1175–83.
27. Vacca P, Cantoni C, Vitale M, Prato C, Canegallo F, Fenoglio D, et al. Crosstalk between decidual NK and CD14+ myelomonocytic cells results in induction of Tregs and immunosuppression. Proc Natl Acad Sci. 2010;107(26):11918–23.
28. Torry DS, Hinrichs M, Torry RJ. Determinants of placental vascularity. Am J Reprod Immunol. 2004;51(4):257–68.
29. Vacca P, Montaldo E, Croxatto D, Loiacono F, Canegallo F, Venturini PL, et al. Identification of diverse innate lymphoid cells in human decidua. Mucosal Immunol. 2015;8(2):254–64.
30. Lima PD, Zhang J, Dunk C, Lye SJ, Croy BA. Leukocyte driven-decidual angiogenesis in early pregnancy. Cell Mol Immunol. 2014;11(6):522–37.
31. Bulmer JN, Williams PJ, Lash GE. Immune cells in the placental bed. Int J Dev Biol. 2010;54(2–3):281–94.
32. Lissauer D, Kilby MD, Moss P. Maternal effector T cells within decidua: the adaptive immune response to pregnancy? Placenta. 2017;60:140–4.
33. Nancy P, Tagliani E, Tay CS, Asp P, Levy DE, Erlebacher A. Chemokine gene silencing in decidual stromal cells limits T cell access to the maternal-fetal interface. Science. 2012;336(6086):1317–21.
34. Rosenblum MD, Way SS, Abbas AK. Regulatory T cell memory. Nat Rev Immunol. 2016;16(2):90–101.
35. Kieffer TE, Faas MM, Scherjon SA, Prins JR. Pregnancy persistently affects memory T cell populations. J Reprod Immunol. 2017;119:1–8.
36. Rowe JH, Ertelt JM, Xin L, Way SS. Pregnancy imprints regulatory memory that sustains anergy to fetal antigen. Nature. 2012;490(7418):102–6.
37. Bonney EA. Maternal tolerance is not critically dependent on interleukin-4. Immunology. 2001;103(3):382–9.
38. Clark DA, Chaouat G, Arck PC, Mittruecker HW, Levy GA. Cutting edge: cytokine-dependent abortion in CBA × DBA/2 mice is mediated by the procoagulant fgl2 prothombinase. J Immunol. 1998;160(2):545–9.
39. Murphy SP, Tayade C, Ashkar AA, Hatta K, Zhang J, Croy BA. Interferon gamma in successful pregnancies. Biol Reprod. 2009;80(5):848–59.
40. Miyazaki S, Tsuda H, Sakai M, Hori S, Sasaki Y, Futatani T, et al. Predominance of Th2-promoting dendritic cells in early human pregnancy decidua. J Leukoc Biol. 2003;74(4):514–22.
41. Ramhorst R, Fraccaroli L, Aldo P, Alvero AB, Cardenas I, Leirós CP, et al. Modulation and recruitment of inducible regulatory T cells by first trimester trophoblast cells. Am J Reprod Immunol. 2012;67(1):17–27.
42. Zenclussen AC, Gerlof K, Zenclussen ML, Sollwedel A, Bertoja AZ, Ritter T, et al. Abnormal T-cell reactivity against paternal antigens in spontaneous abortion: adoptive transfer of pregnancy-induced CD4+CD25+ T regulatory cells prevents fetal rejection in a murine abortion model. Am J Pathol. 2005;166(3):811–22.

43. Robertson SA, Care AS, Moldenhauer LM. Regulatory T cells in embryo implantation and the immune response to pregnancy. J Clin Invest. 2018;128(10):4224–35.
44. Agbayani G, Wachholz K, Chattopadhyay A, Gurnani K, Murphy SP, Krishnan L. Modulation of Th17 and regulatory T-cell responses during murine pregnancy contributes to increased maternal susceptibility to Salmonella Typhimurium infection. Am J Reprod Immunol. 2017;78:e12742.
45. Logiodice F, Lombardelli L, Kullolli O, Haller H, Maggi E, Rukavina D, et al. Decidual interleukin-22-producing CD4+ T cells (Th17/Th0/IL-22+ and Th17/Th2/IL-22+, Th2/IL-22+, Th0/IL-22+), which also produce IL-4, are involved in the success of pregnancy. Int J Mol Sci. 2019;20(2):E428.
46. Lissauer D, Piper K, Goodyear O, Kilby MD, Moss PA. Fetal-specific CD8+ cytotoxic T cell responses develop during normal human pregnancy and exhibit broad functional capacity. J Immunol. 2012;189(2):1072–80.
47. Piper KP, McLarnon A, Arrazi J, Horlock C, Ainsworth J, Kilby MD, et al. Functional HY-specific CD8+ T cells are found in a high proportion of women following pregnancy with a male fetus. Biol Reprod. 2007;76(1):96–101.
48. Tilburgs T, Strominger JL. CD8+ effector T cells at the fetal-maternal interface, balancing fetal tolerance and antiviral immunity. Am J Reprod Immunol. 2013;69(4):395–407.
49. Zeng W, Liu X, Liu Z, Zheng Y, Yu T, Fu S, et al. Deep surveying of the transcriptional and alternative splicing signatures for decidual CD8+ T cells at the first trimester of human healthy pregnancy. Front Immunol. 2018;9:937.
50. Tilburgs T, Schonkeren D, Eikmans M, Nagtzaam NM, Datema G, Swings GM, et al. Human decidual tissue contains differentiated CD8+ effector-memory T cells with unique properties. J Immunol. 2010;185(7):4470–7.
51. Sheridan BS, Lefrançois L. Regional and mucosal memory T cells. Nat Immunol. 2011;12(6):485–91.
52. Tanikawa N, Ohtsu A, Kawahara-Miki R, Kimura K, Matsuyama S, Iwata H, et al. Age-associated mRNA expression changes in bovine endometrial cells in vitro. Reprod Biol Endocrinol. 2017;15(1):63.
53. Forde N, Duffy GB, McGettigan PA, Browne JA, Mehta JP, Kelly AK, et al. Evidence for an early endometrial response to pregnancy in cattle: both dependent upon and independent of interferon tau. Physiol Genomics. 2012;44(16):799–810.
54. Averaimo S, Milton RH, Duchen MR, Mazzanti M. Chloride intracellular channel 1 (CLIC1): sensor and effector during oxidative stress. FEBS Lett. 2010;584(10):2076–84.
55. Fuertes MB, Molinero LL, Toscano MA, Ilarregui JM, Rubinstein N, Fainboim L, et al. Regulated expression of galectin-1 during T-cell activation involves Lck and Fyn kinases and signaling through MEK1/ERK, p38 MAP kinase and p70S6 kinase. Mol Cell Biochem. 2004;267(1–2):177–85.
56. Deak M, Hornung A, Novak J, Demydenko D, Szabo E, Czibula A, et al. Novel role for galectin-1 in T-cells under physiological and pathological conditions. Immunobiology. 2015;220(4):483–9.
57. Rabinovich GA, Toscano MA. Turning 'sweet' on immunity: galectin-glycan interactions in immune tolerance and inflammation. Nat Rev Immunol. 2009;9(5):338–52.
58. Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, et al. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. Nat Immunol. 2005;6(12):1245–52.
59. Mor G, Cardenas I, Abrahams V, Guller S. Inflammation and pregnancy: the role of the immune system at the implantation site. Ann N Y Acad Sci. 2011;1221(1):80–7.
60. Gnainsky Y, Granot I, Aldo P, Barash A, Or Y, Mor G, et al. Biopsy-induced inflammatory conditions improve endometrial receptivity: the mechanism of action. Reproduction. 2015;149(1):75–85.
61. Ledee N, Munaut C, Aubert J, Serazin V, Rahmati M, Chaouat G, et al. Specific and extensive endometrial deregulation is present before conception in IVF/ICSI repeated implantation failures (IF) or recurrent miscarriages. J Pathol. 2011;225(4):554–64.

62. Ledee-Bataille N, Bonnet-Chea K, Hosny G, Dubanchet S, Frydman R, Chaouat G. Role of the endometrial tripod interleukin-18, -15, and -12 in inadequate uterine receptivity in patients with a history of repeated in vitro fertilization-embryo transfer failure. Fertil Steril. 2005;83(3):598–605.
63. Petitbarat M, Rahmati M, Serazin V, Dubanchet S, Morvan C, Wainer R, et al. TWEAK appears as a modulator of endometrial IL-18 related cytotoxic activity of uterine natural killers. PLoS One. 2011;6(1):e14497.
64. Lei MZ, Qin LJ, Zhao DD, Wang AH, Zhao XJ, Jin YP, et al. Tumor necrosis factor-like weak inducer of apoptosis regulates the phenotype and cytotoxic activity of goat uterine natural killer cells. J Anim Sci. 2015;93(2):589–97.
65. Ledee N, Petitbarat M, Chevrier L, Vitoux D, Vezmar K, Rahmati M, et al. The uterine immune profile may help women with repeated unexplained embryo implantation failure after in vitro fertilization. Am J Reprod Immunol. 2016;75(3):388–401.
66. Jasper MJ, Tremellen KP, Robertson SA. Primary unexplained infertility is associated with reduced expression of the T-regulatory cell transcription factor Foxp3 in endometrial tissue. Mol Hum Reprod. 2006;12(5):301–8.
67. Practice Committee of American Society for Reproductive Medicine. Definitions of infertility and recurrent pregnancy loss: a committee opinion. Fertil Steril. 2013;99(1):63.
68. Kwak-Kim J, Skariah A, Wu L, Salazar D, Sung N, Ota K. Humoral and cellular autoimmunity in women with recurrent pregnancy losses and repeated implantation failures: a possible role of vitamin D. Autoimmun Rev. 2016;15(10):943–7.
69. Liu Y-S, Wu L, Tong X-H, Wu L-M, He G-P, Zhou G-X, et al. Study on the relationship between Th17 cells and unexplained recurrent spontaneous abortion. Am J Reprod Immunol. 2011;65(5):503–11.
70. Wu L, Luo LH, Zhang YX, Li Q, Xu B, Zhou GX, et al. Alteration of Th17 and Treg cells in patients with unexplained recurrent spontaneous abortion before and after lymphocyte immunization therapy. Reprod Biol Endocrinol. 2014;12:74.
71. Lee SK, Kim JY, Han AR, Hur SE, Kim CJ, Kim TH, et al. Intravenous immunoglobulin G improves pregnancy outcome in women with recurrent pregnancy losses with cellular immune abnormalities. Am J Reprod Immunol. 2016;75(1):59–68.
72. Muyayalo KP, Li ZH, Mor G, Liao AH. Modulatory effect of intravenous immunoglobulin on Th17/Treg cell balance in women with unexplained recurrent spontaneous abortion. Am J Reprod Immunol. 2018;80(4):e13018.
73. Haas J, Korporal M, Schwarz A, Balint B, Wildemann B. The interleukin-7 receptor alpha chain contributes to altered homeostasis of regulatory T cells in multiple sclerosis. Eur J Immunol. 2011;41(3):845–53.
74. Wu L, Li J, Xu HL, Xu B, Tong XH, Kwak-Kim J, et al. IL-7/IL-7R signaling pathway might play a role in recurrent pregnancy losses by increasing inflammatory Th17 cells and decreasing Treg cells. Am J Reprod Immunol. 2016;76(6):454–64.
75. von Wolff M, Thaler CJ, Strowitzki T, Broome J, Stolz W, Tabibzadeh S. Regulated expression of cytokines in human endometrium throughout the menstrual cycle: dysregulation in habitual abortion. Mol Hum Reprod. 2000;6(7):627–34.
76. Banerjee P, Ghosh S, Dutta M, Subramani E, Khalpada J, Roychoudhury S, et al. Identification of key contributory factors responsible for vascular dysfunction in idiopathic recurrent spontaneous miscarriage. PLoS One. 2013;8(11):e80940.
77. Tersigni C, D'Ippolito S, Di Nicuolo F, Marana R, Valenza V, Masciullo V, et al. Recurrent pregnancy loss is associated to leaky gut: a novel pathogenic model of endometrium inflammation. J Transl Med. 2018;16(1):102.
78. Kalinski P. Regulation of immune responses by prostaglandin E2. J Immunol. 2012;188(1):21–8.
79. Lala PK, Kennedy TG, Parhar RS. Suppression of lymphocyte alloreactivity by early gestational human decidua. II. Characterization of the suppressor mechanisms. Cell Immunol. 1988;116(2):411–22.

80. Ye Y, Vattai A, Ditsch N, Kuhn C, Rahmeh M, Mahner S, et al. Prostaglandin E2 receptor 3 signaling is induced in placentas with unexplained recurrent pregnancy losses. Endocr Connect. 2018;7(5):749–61.
81. Cornelius DC, Cottrell J, Amaral LM, LaMarca B. Inflammatory mediators: a causal link to hypertension during preeclampsia. Br J Pharmacol. 2019;176(12):1914–21.
82. Amaral LM, Kiprono L, Cornelius DC, Shoemaker C, Wallace K, Moseley J, et al. Progesterone supplementation attenuates hypertension and the autoantibody to the angiotensin II type I receptor in response to elevated interleukin-6 during pregnancy. Am J Obstet Gynecol. 2014;211(2):158 e1–6.
83. Darmochwal-Kolarz D, Saito S, Tabarkiewicz J, Kolarz B, Rolinski J, Leszczynska-Gorzelak B, et al. Apoptosis signaling is altered in $CD4^+CD25^+FoxP3^+$ T regulatory lymphocytes in pre-eclampsia. Int J Mol Sci. 2012;13(6):6548–60.
84. Salazar Garcia MD, Mobley Y, Henson J, Davies M, Skariah A, Dambaeva S, et al. Early pregnancy immune biomarkers in peripheral blood may predict preeclampsia. J Reprod Immunol. 2018;125:25–31.
85. Wallukat G, Homuth V, Fischer T, Lindschau C, Horstkamp B, Jupner A, et al. Patients with preeclampsia develop agonistic autoantibodies against the angiotensin AT1 receptor. J Clin Invest. 1999;103(7):945–52.
86. Platten M, Youssef S, Hur EM, Ho PP, Han MH, Lanz TV, et al. Blocking angiotensin-converting enzyme induces potent regulatory T cells and modulates TH1- and TH17-mediated autoimmunity. Proc Natl Acad Sci U S A. 2009;106(35):14948–53.
87. Tian M, Zhang Y, Liu Z, Sun G, Mor G, Liao A. The PD-1/PD-L1 inhibitory pathway is altered in pre-eclampsia and regulates T cell responses in pre-eclamptic rats. Sci Rep. 2016;6:27683.
88. Zhang Z, Liu H, Shi Y, Xu N, Wang Y, Li A, et al. Increased circulating Th22 cells correlated with Th17 cells in patients with severe preeclampsia. Hypertens Pregnancy. 2017;36(1):100–7.
89. Vianna P, Mondadori AG, Bauer ME, Dornfeld D, Chies JA. HLA-G and CD8+ regulatory T cells in the inflammatory environment of pre-eclampsia. Reproduction. 2016;152(6):741–51.
90. Vrachnis N, Vitoratos N, Iliodromiti Z, Sifakis S, Deligeoroglou E, Creatsas G. Intrauterine inflammation and preterm delivery. Ann N Y Acad Sci. 2010;1205:118–22.
91. Park JC, Kim DJ, Kwak-Kim J. Upregulated amniotic fluid cytokines and chemokines in emergency cerclage with protruding membranes. Am J Reprod Immunol. 2011;66(4):310–9.
92. Gomez-Lopez N, Olson DM, Robertson SA. Interleukin-6 controls uterine Th9 cells and CD8(+) T regulatory cells to accelerate parturition in mice. Immunol Cell Biol. 2016;94(1):79–89.
93. Makhseed M, Raghupathy R, El-Shazly S, Azizieh F, Al-Harmi JA, Al-Azemi MM. Pro-inflammatory maternal cytokine profile in preterm delivery. Am J Reprod Immunol. 2003;49(5):308–18.
94. Gargano JW, Holzman C, Senagore P, Thorsen P, Skogstrand K, Hougaard DM, et al. Mid-pregnancy circulating cytokine levels, histologic chorioamnionitis and spontaneous preterm birth. J Reprod Immunol. 2008;79(1):100–10.
95. Gomez-Lopez N, Laresgoiti-Servitje E. T regulatory cells: regulating both term and preterm labor? Immunol Cell Biol. 2012;90(10):919–20.
96. Ito M, Nakashima A, Hidaka T, Okabe M, Bac ND, Ina S, et al. A role for IL-17 in induction of an inflammation at the fetomaternal interface in preterm labour. J Reprod Immunol. 2010;84(1):75–85.
97. Elovitz MA, Baron J, Phillippe M. The role of thrombin in preterm parturition. Am J Obstet Gynecol. 2001;185(5):1059–63.
98. Shankar R, Johnson MP, Williamson NA, Cullinane F, Purcell AW, Moses EK, et al. Molecular markers of preterm labor in the choriodecidua. Reprod Sci. 2010;17(3):297–310.
99. Rinaldi SF, Makieva S, Saunders PT, Rossi AG, Norman JE. Immune cell and transcriptomic analysis of the human decidua in term and preterm parturition. Mol Hum Reprod. 2017;23(10):708–24.

Chapter 4
Role of Immunoregulatory Cytokine IL-15 in the Endometrium

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Overview

IL-15 is a key immunoregulatory cytokine that was first discovered due to its ability to mimic IL-2-related activity, namely, stimulation of T cell proliferation [1]. Further studies on IL-15 revealed that this cytokine exhibits rather pleiotropic functions and plays an important role in the development and homeostasis of certain T cell subsets including intestinal intraepithelial T cells and memory CD8+ T cells. But the most prominent role of IL-15 is related to the development of NK cells [2]. Indeed, mice with targeted mutations in the IL-15 gene lack NK cells [3]. IL-15 is evenly important for survival, proliferation, and activation of NK cells. The similarity in IL-15 and IL-2 biological effects is someway commenced from shared subunits in their receptors. The receptor complexes for both IL-2 and IL-15 include the same beta (IL-2/15Rß) and gamma (known as common gamma, γ_c) chains. The common gamma chain, in addition to these two cytokines, is also shared by receptors for several cytokines, including those for IL-4, IL-7, IL-9, and IL-21. JAK Janus kinase (JAK) proteins on cytoplasmic ends of the beta and the gamma chains allow for the downstream phosphorylation of transcription factor STAT5 and/or STAT3. The action of IL-15 is mediated via the activation of these transcription factors [2]. Besides STATs, IL-15 also induces mTOR (mechanistic target of rapamycin) activity in NK cells. The activation of mTOR in NK cells, however, requires high concentrations of IL-15 [4]. This activation results in a substantial increase in glycolysis and respiration in NK cells and supports enhanced metabolic demands that are associated with cellular activation [4, 5].

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The specificity of IL-15 signaling as well as the listed above cytokines is ensured by an exclusive alpha chain in their receptors [6]. IL-15R alpha (IL-15Rα) is unique among the other alpha chains; it is characterized with an extremely high affinity for its cytokine and an unusual broad non-leukocyte expression pattern in various tissues including endometrium, liver, intestine, and brain [7–9]. The IL-15Rα on a surface of IL-15-responsive lymphoid cells forms a heterotrimeric complex with IL-2/15Rß and γ_c chains and mediates the signal from soluble IL-15 (cis-presentation). However, a trans-presentation mechanism prevails in IL-15 signaling. IL-15Rα, when it is expressed as a monomeric protein, binds IL-15 and "presents" it (trans-presentation) to the responding cells expressing two other chains, IL-2/15Rβ and γ_c [9]. While the IL-15 cis-presentation could cause fast and transient response and is found being optimal for NK cell activation during the acute inflammatory response, the IL-15 trans-presentation results in prolonged activation of NK cells [10, 11]. Signaling from a membrane-bound IL-15/IL-15Rα complex is essential in peripheral tissues for the development and survival of NK cells [12]. Moreover, the IL-15/IL-15Rα complex is currently under investigation as an immunotherapeutic agent for the treatment of cancer because of its strong potential to generate more powerful effector NK cells [13, 14].

The production of IL-15 is reported in multiple tissues by various cell types. Monocytes, macrophages, and dendritic cells represent an important source of soluble and/or membrane-bound IL-15 in the context of the immune response [2]. IL-15 is also produced by IL-15Rα bearing stromal (bone marrow mesenchymal cells, synovial and spleen fibroblasts) and epithelial cells (thymic epithelium, renal tubular epithelial cells, intestinal epithelial cells) [15–20]. Membrane-bound presentation of IL-15 by these cells is essential for the regulation of NK cell development and homeostasis in tissues.

IL-15 in Endometrium

High levels of IL-15 mRNA were detected in the human first-trimester decidua, where endothelial and stromal cells revealed positive IL-15 staining [21–23]. Membrane-bound IL-15 molecule was detected on the surface of the first-trimester decidual cells by flow cytometry [21].

The analysis of IL-15 in nonpregnant human endometrium revealed its differential expression throughout the menstrual cycle. The levels of IL-15 mRNA are low in the menstrual and proliferative phases of the cycle. However, abundant IL-15 expression is observed in the secretory phase [21, 22, 24]. Immunohistochemical assessment of the endometrium confirmed the higher immunoreactivity for IL-15 in samples obtained during the secretory phase. Positive staining was demonstrated on stromal cells, especially of perivascular localization, and on glandular epithelial cells, indicating that stromal and epithelial cells are the main source of this cytokine in human endometrium [21].

In vitro experiments with primary endometrial stromal cells demonstrated that IL-15 mRNA expression is induced during medroxyprogesterone acetate/cAMP-regulated decidualization [22]. While the secretion of IL-15 as measured by ELISA in supernatant from undifferentiated primary endometrial stromal cells was shown to be below the level of detection, the levels of IL-15 were found markedly increased after 48 h of decidualization [25]. On the contrary, the prevention of progesterone signaling with asoprisnil, the progesterone receptor modulating drug, demonstrated a strong down-regulation of endometrial IL-15 mRNA levels [23]. Gene expression analysis of endometrium from women treated with asoprisnil due to abnormal bleeding associated with uterine fibroids in comparison with normal controls demonstrated a significant reduction of genes in the IL-15 pathway as well as a striking reduction in a number of uterine NK cells [23]. The expression of IL-15 is also inducible in uterine endothelial cells. Under ovarian steroid stimulation of human uterine microvascular endothelial cells, IL-15 became detectable on a cell surface, but not in the supernatant of cultured endothelial cells [26].

Besides the role of ovarian hormones in the regulation of IL-15, it was shown that the exposure of endometrial stromal cells to prostaglandin E2 leads likewise to the increased expression of IL-15 mRNA [27]. As for negative regulators, a proinflammatory cytokine, IL-1β, was reported to significantly inhibit the progesterone-induced IL-15 production and mRNA expression in long-term culture of endometrial stromal cells [28].

Expression of the exclusive chain of the IL-15 receptor, IL-15Rα, was also reported for endometrium. It was detected in isolated uterine NK cells [29] and endometrial tissue. Immunohistochemical analysis of endometrial samples revealed the localization of IL-15Rα to stromal and epithelial cells [30]. Co-expression of IL-15 and IL-15Rα means that endometrial stromal cells are capable of trans-presenting the cytokine to neighboring uterine NK cells, thus ensuring prolonged activation of these immune cells.

IL-15 Analysis in Endometrium from Women with Reproductive Failures

Endometrial IL-15 expression was compared between women with a history of recurrent spontaneous abortion and fertile controls using qRT-PCR analysis of IL-15 mRNA, protein analysis by ELISA, and immunohistochemistry [24]. The data showed elevated levels of IL-15 in women with unexplained recurrent spontaneous abortion in comparison with fertile controls at the same phase of the menstrual cycle.

Increased levels of IL-15 expression (mRNA and protein) were also reported in placental tissues from recurrent pregnancy losses of unknown etiology where a uterine curettage was performed within 24 h since the clinical presentation of spontaneous abortion. The analysis was performed in comparison with IL-15 expression in

normal control tissues obtained from elective termination of pregnancy at a corresponding gestational age that were ranged from week 7 to week 12 [31].

In contrary, in women with repeated implantation failures after in vitro fertilization (IVF) and embryo transfer procedures, a median endometrial expression of IL-15 mRNA was reported to be significantly lower than in fertile controls [32].

Abnormal endometrium, such as in endometriotic lesions, revealed significantly lower levels of IL-15 expression when compared to samples from eutopic stroma [33]. However, there are other studies reporting opposite results with increased IL-15 expression in ectopic lesions. The study from Yu JJ et al. showed that endometriotic lesions have a high expression of IL-15, which promotes growth and invasion of endometrial stromal cells [34]. IL-15 was detected in peritoneal fluid from women with endometriosis. Interestingly, the levels of IL-15 in the peritoneal fluid were inversely correlated with the depth of endometriotic implants and disease stage, suggesting a possible role for this cytokine in the early pathogenesis of endometriosis [35].

Role of IL-15 in the Endometrium

As the effects of IL-15 are strongly tied to NK cells, an association between the level of IL-15 expression in the endometrium and the number or functional activity of NK cells in the tissue could be foreseen. Indeed, the study by Ledee et al. (2008) demonstrated a positive correlation between endometrial IL-15 mRNA expression and a number of CD56-positive cells determined by immunohistochemistry. The authors also showed that IL-15 expression correlates with sub-endometrial vascular flow index, measured using ultrasonographic vascular imaging, which might indicate IL-15 involvement in the regulation of endometrial angiogenesis [32].

The changing levels of IL-15 in the endometrium have a different impact on NK cells. Low levels of IL-15, which are characteristic for proliferative endometrium, signal via STAT5 pathway and are important for NK cell survival [36]. In the secretory phase endometrium, the NK cells are exposed to increased concentrations of IL-15. Continuous exposure to high levels of IL-15 triggers mTOR signaling in NK cells. In vitro study with mouse NK cells demonstrated that no other cytokines (IL-7, IFNβ, TGFβ, IL-12) with the exception of IL-18 (although its effect was weaker) or signaling via activating or inhibitory receptors (NKp46, NK1.1, Ly49D, Ly49C, Ly49I, NKG2A) were able to upregulate mTOR activity of NK cells [4]. The activation via mTOR mediates metabolic reprogramming in NK cells [5]. Namely, mTOR signaling in NK cells leads to increased glucose uptake and an elevated rate of glycolysis. The glucose is metabolized primarily to lactate (aerobic glycolysis) as the substantial increase in extracellular acidification rate could be recorded using Seahorse technology. Oxygen consumption which reflects a level of oxidative phosphorylation in a cell is also elevated in IL-15-exposed NK cells [36, 37]. However, it is the aerobic glycolysis that prevails substantially as a metabolic pathway to produce energy when NK cells are under control of mTOR activation. These NK

cells demonstrate increased effector functions, including secretion of IFNγ and production of granzyme B, which is a critical component of NK cell granules. Activated NK cells are also distinguished by increased cell size. This can be verified by flow cytometry analysis because large NK cells have higher forward and side scatter characteristics in comparison with the total lymphocyte population [4, 5]. Endometrial NK cells, especially during pregnancy, are long described as large granular lymphocytes [38]. Continuous exposure to the high levels of IL-15 is one possible explanation for this phenomenon. Moreover, the IL-15-exposed NK cells become less susceptible to glucose deprivation and able to keep superior functionality in case of metabolic stress [36].

IL-15 and uterine NK cells play an important role in the process of immune clearance of senescent cells in endometrial stroma. During each menstrual cycle, acute senescence is induced in a fraction of endometrial stromal cells [25]. This takes place during the mid-luteal phase of the cycle upon increasing levels of circulating progesterone simultaneously with decidual transformation in the rest of endometrial stroma. Acute senescence is a regulated biological process that targets a population of cells in the tissue, and it is mostly beneficial by nature, i.e., during wound healing, tissue repair, or embryonic development [39]. Senescent cells lose a proliferative capacity, enter into a permanent cell cycle arrest, and actively secrete proinflammatory cytokines, chemokines, and growth factors. This hyperfunctional phenotype is known as "senescence-associated secretory phenotype" (SASP) [40]. A short period lasting 2–4 days during the mid-luteal phase, known as "a window of implantation," during when the endometrium becomes receptive for embryo implantation, is characterized by an explicit inflammatory reaction in the endometrium with increased expression of prostaglandin E2, IL-6, and TNF [41, 42]. Brighton et al. (2017) showed that the transient inflammatory response is driven by the acute endometrial senescence in a subpopulation of stromal cells. The senescent stromal cells then get cleared by uterine NK cells via exocytosis of granules packed with pore-forming protein perforin and serine protease granzymes [25].

Summary

IL-15-mediated activation is a prerequisite for the effective functioning of uterine NK cells, and it is achieved through a continuous trans-presentation of IL-15 by surrounding decidualized stromal cells (Fig. 4.1). The clearance of senescent cells within the endometrial stroma would ensure successful trophoblast invasion and placental growth and suggest an important role for IL-15 and NK cells in the endometrium during the initial stages of pregnancy. On the opposite side, overexpression of IL-15 at the time of implantation could as well have a detrimental effect on pregnancy development due to excessive activation of uterine NK cells. Endometrial testing for IL-15 is a part of endometrial immune profiling [43]. The endometrial immune profile test is performed to determine if the expression of immune-related factors is skewed toward excessive immune activation or inadequate immune

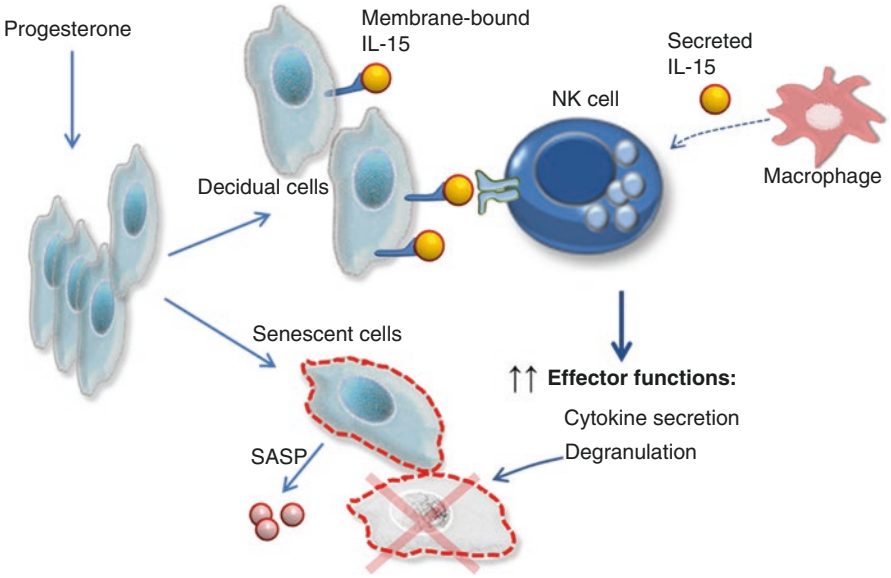

Fig. 4.1 Role of IL-15 in the endometrium. Upon hormonal regulation, a fraction of endometrial stromal cells, which differentiate into decidual cells, produce IL-15 and trans-present it to neighboring uterine NK cells. The membrane-bound IL-15 facilitates effector functions of NK cells such as a release of cytolytic granules (degranulation) and cytokine secretion. A fraction of endometrial stromal cells undergo senescence upon menstrual cycle progression; senescent cells secrete proinflammatory mediators referred to as senescence-associated secretory phenotype (SASP) factors. Membrane-bound IL-15-activated NK cells are essential in the clearance of the senescent cells. Soluble IL-15 secreted by tissue macrophages or dendritic cells could also affect NK cell activity

activation, both of which would not be supportive for implantation and/or proper placental development. IL-15 production in endometrium has to be tightly regulated to achieve an optimal balance in tuning uterine NK cell activity toward supporting embryo implantation and placental development.

References

1. Grabstein KH, Eisenman J, Shanebeck K, Rauch C, Srinivasan S, Fung V, et al. Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor. Science. 1994;264(5161):965–8.
2. Fehniger TA, Caligiuri MA. Interleukin 15: biology and relevance to human disease. Blood. 2001;97(1):14–32.
3. Kennedy MK, Glaccum M, Brown SN, Butz EA, Viney JL, Embers M, et al. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. J Exp Med. 2000;191(5):771–80.
4. Marcais A, Cherfils-Vicini J, Viant C, Degouve S, Viel S, Fenis A, et al. The metabolic checkpoint kinase mTOR is essential for IL-15 signaling during the development and activation of NK cells. Nat Immunol. 2014;15(8):749–57.

5. Donnelly RP, Loftus RM, Keating SE, Liou KT, Biron CA, Gardiner CM, et al. mTORC1-dependent metabolic reprogramming is a prerequisite for NK cell effector function. J Immunol. 2014;193(9):4477–84.
6. Anderson DM, Kumaki S, Ahdieh M, Bertles J, Tometsko M, Loomis A, et al. Functional characterization of the human interleukin-15 receptor alpha chain and close linkage of IL15RA and IL2RA genes. J Biol Chem. 1995;270(50):29862–9.
7. Leonard WJ, Depper JM, Crabtree GR, Rudikoff S, Pumphrey J, Robb RJ, et al. Molecular cloning and expression of cDNAs for the human interleukin-2 receptor. Nature. 1984;311(5987):626–31.
8. Dubois S, Mariner J, Waldmann TA, Tagaya Y. IL-15Ralpha recycles and presents IL-15 in trans to neighboring cells. Immunity. 2002;17(5):537–47.
9. Schluns KS, Stoklasek T, Lefrancois L. The roles of interleukin-15 receptor alpha: trans-presentation, receptor component, or both? Int J Biochem Cell Biol. 2005;37(8):1567–71.
10. Perdreau H, Mortier E, Bouchaud G, Sole V, Boublik Y, Plet A, et al. Different dynamics of IL-15R activation following IL-15 cis- or trans-presentation. Eur Cytokine Netw. 2010;21(4):297–307.
11. Zanoni I, Spreafico R, Bodio C, Di Gioia M, Cigni C, Broggi A, et al. IL-15 cis presentation is required for optimal NK cell activation in lipopolysaccharide-mediated inflammatory conditions. Cell Rep. 2013;4(6):1235–49.
12. Koka R, Burkett PR, Chien M, Chai S, Chan F, Lodolce JP, et al. Interleukin (IL)-15R[alpha]-deficient natural killer cells survive in normal but not IL-15R[alpha]-deficient mice. J Exp Med. 2003;197(8):977–84.
13. Guo Y, Luan L, Patil NK, Sherwood ER. Immunobiology of the IL-15/IL-15Ralpha complex as an antitumor and antiviral agent. Cytokine Growth Factor Rev. 2017;38:10–21.
14. Robinson TO, Schluns KS. The potential and promise of IL-15 in immuno-oncogenic therapies. Immunol Lett. 2017;190:159–68.
15. Cui G, Hara T, Simmons S, Wagatsuma K, Abe A, Miyachi H, et al. Characterization of the IL-15 niche in primary and secondary lymphoid organs in vivo. Proc Natl Acad Sci U S A. 2014;111(5):1915–20.
16. Briard D, Brouty-Boye D, Azzarone B, Jasmin C. Fibroblasts from human spleen regulate NK cell differentiation from blood CD34(+) progenitors via cell surface IL-15. J Immunol. 2002;168(9):4326–32.
17. Kinoshita N, Hiroi T, Ohta N, Fukuyama S, Park EJ, Kiyono H. Autocrine IL-15 mediates intestinal epithelial cell death via the activation of neighboring intraepithelial NK cells. J Immunol. 2002;169(11):6187–92.
18. Miranda-Carus ME, Balsa A, Benito-Miguel M, Perez de Ayala C, Martin-Mola E. IL-15 and the initiation of cell contact-dependent synovial fibroblast-T lymphocyte cross-talk in rheumatoid arthritis: effect of methotrexate. J Immunol. 2004;173(2):1463–76.
19. Giron-Michel J, Azzi S, Khawam K, Mortier E, Caignard A, Devocelle A, et al. Interleukin-15 plays a central role in human kidney physiology and cancer through the gammac signaling pathway. PLoS One. 2012;7(2):e31624.
20. Sandau MM, Schluns KS, Lefrancois L, Jameson SC. Cutting edge: transpresentation of IL-15 by bone marrow-derived cells necessitates expression of IL-15 and IL-15R alpha by the same cells. J Immunol. 2004;173(11):6537–41.
21. Kitaya K, Yasuda J, Yagi I, Tada Y, Fushiki S, Honjo H. IL-15 expression at human endometrium and decidua. Biol Reprod. 2000;63(3):683–7.
22. Okada S, Okada H, Sanezumi M, Nakajima T, Yasuda K, Kanzaki H. Expression of interleukin-15 in human endometrium and decidua. Mol Hum Reprod. 2000;6(1):75–80.
23. Wilkens J, Male V, Ghazal P, Forster T, Gibson DA, Williams AR, et al. Uterine NK cells regulate endometrial bleeding in women and are suppressed by the progesterone receptor modulator asoprisnil. J Immunol. 2013;191(5):2226–35.
24. Chegini N, Ma C, Roberts M, Williams RS, Ripps BA. Differential expression of interleukins (IL) IL-13 and IL-15 throughout the menstrual cycle in endometrium of normal fertile women and women with recurrent spontaneous abortion. J Reprod Immunol. 2002;56(1–2):93–110.

25. Brighton PJ, Maruyama Y, Fishwick K, Vrljicak P, Tewary S, Fujihara R, et al. Clearance of senescent decidual cells by uterine natural killer cells in cycling human endometrium. eLife. 2017;6.
26. Kitaya K, Yasuo T. Regulatory role of membrane-bound form interleukin-15 on human uterine microvascular endothelial cells in circulating CD16(−) natural killer cell extravasation into human endometrium. Biol Reprod. 2013;89(3):70.
27. Dunn CL, Critchley HO, Kelly RW. IL-15 regulation in human endometrial stromal cells. J Clin Endocrinol Metab. 2002;87(4):1898–901.
28. Okada H, Nakajima T, Yasuda K, Kanzaki H. Interleukin-1 inhibits interleukin-15 production by progesterone during in vitro decidualization in human. J Reprod Immunol. 2004;61(1):3–12.
29. Verma S, Hiby SE, Loke YW, King A. Human decidual natural killer cells express the receptor for and respond to the cytokine interleukin 15. Biol Reprod. 2000;62(4):959–68.
30. Franchi A, Zaret J, Zhang X, Bocca S, Oehninger S. Expression of immunomodulatory genes, their protein products and specific ligands/receptors during the window of implantation in the human endometrium. Mol Hum Reprod. 2008;14(7):413–21.
31. Toth B, Haufe T, Scholz C, Kuhn C, Friese K, Karamouti M, et al. Placental interleukin-15 expression in recurrent miscarriage. Am J Reprod Immunol. 2010;64(6):402–10.
32. Ledee N, Chaouat G, Serazin V, Lombroso R, Dubanchet S, Oger P, et al. Endometrial vascularity by three-dimensional power Doppler ultrasound and cytokines: a complementary approach to assess uterine receptivity. J Reprod Immunol. 2008;77(1):57–62.
33. Logan PC, Yango P, Tran ND. Endometrial stromal and epithelial cells exhibit unique aberrant molecular defects in patients with endometriosis. Reprod Sci. 2018;25(1):140–59.
34. Yu JJ, Sun HT, Zhang ZF, Shi RX, Liu LB, Shang WQ, et al. IL15 promotes growth and invasion of endometrial stromal cells and inhibits killing activity of NK cells in endometriosis. Reproduction. 2016;152(2):151–60.
35. Arici A, Matalliotakis I, Goumenou A, Koumantakis G, Vassiliadis S, Selam B, et al. Increased levels of interleukin-15 in the peritoneal fluid of women with endometriosis: inverse correlation with stage and depth of invasion. Hum Reprod. 2003;18(2):429–32.
36. Mah AY, Cooper MA. Metabolic regulation of natural killer cell IFN-gamma production. Crit Rev Immunol. 2016;36(2):131–47.
37. Keating SE, Zaiatz-Bittencourt V, Loftus RM, Keane C, Brennan K, Finlay DK, et al. Metabolic reprogramming supports IFN-gamma production by CD56bright NK cells. J Immunol. 2016;196(6):2552–60.
38. Bulmer JN, Hollings D, Ritson A. Immunocytochemical evidence that endometrial stromal granulocytes are granulated lymphocytes. J Pathol. 1987;153(3):281–8.
39. Childs BG, Baker DJ, Kirkland JL, Campisi J, van Deursen JM. Senescence and apoptosis: dueling or complementary cell fates? EMBO Rep. 2014;15(11):1139–53.
40. van Deursen JM. The role of senescent cells in ageing. Nature. 2014;509(7501):439–46.
41. Griffith OW, Chavan AR, Protopapas S, Maziarz J, Romero R, Wagner GP. Embryo implantation evolved from an ancestral inflammatory attachment reaction. Proc Natl Acad Sci U S A. 2017;114(32):E6566–E75.
42. Vilella F, Ramirez L, Berlanga O, Martinez S, Alama P, Meseguer M, et al. PGE2 and PGF2alpha concentrations in human endometrial fluid as biomarkers for embryonic implantation. J Clin Endocrinol Metab. 2013;98(10):4123–32.
43. Ledee N, Petitbarat M, Chevrier L, Vitoux D, Vezmar K, Rahmati M, et al. The uterine immune profile may help women with repeated unexplained embryo implantation failure after in vitro fertilization. Am J Reprod Immunol. 2016;75(3):388–401.

Chapter 5
Endometrial Immune Profiling: An Emerging Paradigm for Reproductive Disorders

Nathalie LéDée

Introduction

Recent years have seen incredible progress in infertility treatments, such that more women are now able to conceive than ever before. In particular, there have been great advances in assisted conception methods, including in vitro fertilization (IVF) and intra-cytoplasmic sperm injection (ICSI). However, infertility remains a widespread problem, and it can be a very difficult time for those affected physically and emotionally while trying. Despite ongoing progress in the field of embryo implantation, the implantation itself remains the primary factor limiting the success of live birth following assisted conception. Indeed, every seventh couple in Europe and the USA is affected by implantation disorders, and the vast majority of pregnancy losses take place at this early stage. Efficiency for all the parties involved (patients, physicians, and biologists) means to increase the live birth rate in minimum time. The main brake to such an achievement is the still low implantation rate of IVF/ICSI transferred embryos. Only 15–20% of day-3 embryos and 30% of day-5 embryos will effectively lead to livebirth. Embryo quality is mainly related to the maternal age and thus unchangeable except with the oocyte donation program. A complementary strategy would be to optimize the initial dialogue between the local endometrium and the transferred embryo. By documenting the local immune equilibrium, we may personalize the next embryo transfer to promote effective implantation.

Uterine receptivity has been defined and redefined over the years. Histologically, uterine receptivity is established during the mid-luteal phase with the well-described phenomenon of decidualization occurring each cycle independently of the presence of the human embryo [1]. The endometrium can only accommodate the embryo for

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a few days in each cycle – the implantation window – that occurs 5–9 days after ovulation. Extensive transcriptomic studies and innovative tests as the endometrial receptivity array (ERA) test or the Win-test aim to precisely define the signature of the optimal day of uterine receptivity for the embryo transfer [2, 3]. The concept of immune profiling is a distinct concept that relies on the analysis of the local immune reaction occurring within the endometrium during the implantation window. A crucial immune endometrial switch should occur at the time of implantation to not only avoid the rejection of the semi-allogenic embryo but also to promote its growth and nutrition [4]. As embryo implantation is the crux of assisted reproduction, it requires an understanding of the local immune environment and the interplay between the endometrium and the embryo. During this time, important immune cells leave and enter the endometrium, and the newly created immune environment plays a key role in embryo implantation. Indeed, at that crucial time, almost all the immune cells belonging to our adaptive immunity escape from the endometrium while innate immune cells (macrophage, uterine natural killer cells (uNK), and dendritic cells) invade the endometrium [5]. uNK cells are very different from circulating NK cells – by their phenotype, their repertoire of activating and inhibiting receptors, the cytokines they secrete, and their low cytotoxic potential. Regulatory T cells make the link between adaptive and local immune expression.

Experiments with T and NK knockout mice show that the innate immune system may control local uterine vascularization [6, 7]. A wide variety of uterine functions, as well as some facets of the embryo development process, appear to be controlled by locally secreted cytokines (perhaps secreted by immunocytes) that probably play a major role in the development of adequate uterine receptivity [8]. Together they define a complex network, the balance of which may define successful implantation and, later on, adequate placental growth and function; conversely, it may cause placental dysfunction, abnormal uterine development, and eventually real immune rejection. Underactive immune cells fail to create the necessary implantation reaction; conversely, overactive immune cells can lead to the destruction of the endometrium and rejection of the embryo. This unique immune reaction is essential for promoting embryo adhesion, and its disruption is likely to obstruct implantation.

For each of the local immune cells which are present in the endometrium, the choice of their differentiation in a suitable or deleterious pattern regarding the embryo will depend on the local Th-1/Th-2 endometrial equilibrium. The dominance of Th-2 cytokines has been proposed as the local equilibrium promoting local angiogenesis and immune tolerance [9]. Both the absence and the excess of Th-1 immunity have been described as deleterious. In a Th-1-dominant environment, macrophages differentiate into deleterious M-1 macrophages, uterine NK cells into lymphokine-activated killer cells, dendritic cells (DC) into deleterious DC-1, and T cells into deleterious Th-17 cells. All these cells become able to destroy the embryo. On the contrary, in a Th-2-dominant environment, macrophages differentiate into beneficial M-2 macrophages (adhesion), uterine NK cells become angiogenic and immunotropic, dendritic cells differentiate into DC-2 for effective communication, and naïve T helper cells differentiate into T-regulatory cells to promote the local tolerance. All these immune effectors promote effective placentation.

To document the local Th-1/Th-2 equilibrium of cytokines, we chose to quantify the local expression of interleukin-18 because of its bivalence. Interleukin (IL)-18 is mainly expressed by the luminal and glandular epithelial cells, the endothelial cells, and some immune cells scattered in the endometrial stroma in the mid-luteal phase [10]. IL-18 is a Th-2 angiogenic cytokine with an important demonstrated role in the destabilization of spatial arteries [11, 12]. IL-18 is crucial to prepare the future invasion of the spiral arteries by the extravillous cytotrophoblast. However, a local overexpression of IL-18 switches its beneficial role to a deleterious one as IL-18 becomes Th-1 and promotes local cytotoxicity [13]. The level of local immune regulation which characterizes each individual will also be essential to promote or not a Th-1 deviation. To document the local immune regulation, we decided to quantify the local expressions of TWEAK (TNF weak inducer of apoptosis) and its receptor Fn-14 [13, 14]. We previously demonstrated, using a micro-histoculture endometrial model, that high expression of TWEAK was able to neutralize high expression of IL-18 and impairs the transformation of uNK cells to cytotoxic killers cells. On the contrary, low expression of TWEAK was not able to block the transformation of uNK into killers if IL-18 was overexpressed. The ratio IL-18/TWEAK will be therefore used in the uterine immune profiling as a biomarker of the immuno-regulated Th-1/Th-2 local equilibrium [13, 14].

The maturity of uterine NK cells, as well as their state of activation, also seems essential [15]. Uterine natural killer cells are not fully matured and should go through a process of maturation to have effective functions [15]. IL-15 is the central cytokine for their recruitment and maturation in the endometrium [16]. An overexpression of IL-15 is, however, able to activate all the local immune cells in a negative pathway. The ratio IL-15/Fn-14 is used in the immune profiling as a biomarker of the immuno-regulated state of maturation/activation of uNK cells. In complement, we quantify the recruitment of CD56-positive cells (a marker for uNK) initially by immunochemistry, but now by real-time PCR.

Our hypothesis was that the local environment and its immune equilibrium, which was previously poorly investigated, may be crucially deregulated in patients with an unexplained history of repeated embryo implantation failures after in vitro fertilization (IVF)/intra-cytoplasmic sperm injection (ICSI). This is called recurrent implantation failure (RIF). Based on the analysis of the immune endometrial profile and its equilibrium before conception on a first cycle, our objective was to personalize the subsequent assisted reproductive technology treatment to increase live birth rates. This immune endometrial profile method has been patented as a technique for increasing implantation success in assisted fertilization (PCT/EP2013/065355).

In the present chapter, after a short description of the method, we will summarize the results observed in large RIF and controlled RIF cohorts regarding the diagnosis and the subsequent live birth rates after the personalized care based on the immune profile. RIF patients with endometriosis also show some particularities regarding their immune profiles suggesting that some personalization could be beneficial. Regarding specific immunotherapy such as corticoids or slow perfusion of intralipids in some context of implantation failures, we demonstrate that sensitivity to the

drugs should be tested before implantation to attest the normalization of the immune profile under the therapy.

Part I: Method of Endometrial Immune Profiling and Diagnosis in Patients with RIF or Recurrent Miscarriages (RM)

Biopsy and Dating of Samples

As the endometrial study must be performed during the mid-luteal phase, monitoring of ovulation and/or the expected progesterone rise during the mid-luteal phase before sampling is recommended. Biopsies could also be performed during the substituted cycles after 7 days of progesterone (mock cycle). The endometrium was gently aspirated by rotating a Pipelle de Cornier within the endometrial cavity. The pipelle content is emptied onto a gauze compress and divided into two parts, one placed in 4% formaldehyde (QPath Formol 4% buffered, VWR Chemicals, Fontenay-sous-Bois, France) for routine evaluation, endometrial dating, and CD56 immunolabeling. The second part is placed in RNAlater stabilization solution for immunological analyses (MatriceLab Innove, France). Briefly, after histological dating of an endometrial biopsy sample to confirm the mid-luteal phase, RNA was extracted. The RNA was reverse-transcribed for RT-PCR. IL-15/Fn-14 and IL-18/TWEAK mRNA ratios were determined by quantitative RT-PCR with the Light Cycler 480 SYBR Green I Master mix (Roche Diagnostic), and uNK cells were counted after $CD56^+$ immunohistochemistry.

Determination of Uterine Immune Profile

In function of the local equilibrium of IL-18/TWEAK, IL-15/Fn-14, and CD56, we were able to define the local immune profile [17].

Four types of deregulations were diagnosed:

- A regulated immune activation was characterized by IL-18/TWEAK and IL-15/Fn-14 mRNA ratios and $CD56^+$ cell count in the same range defined in the fertile cohort.
- Immune over-activation was characterized by high IL-18/TWEAK and/or IL-15/Fn-14 mRNA ratios, and/or a high $CD56^+$ cell count.
- A low immune activation profile was characterized by low IL-15/Fn-14 mRNA ratios (reflecting immature uNK cells) or the absence of uNK mobilization ($CD56^+$ cell count <10) and/or a very low local IL-18/TWEAK mRNA ratio.
- A mixed profile was characterized by a high ratio of IL-18/TWEAK (excess of Th-1 cytokines) with a simultaneous low IL-15/Fn-14 ratio (reflecting immature NK).

Table 5.1 Repartition of the immune profiles in function of the clinical context

Immune profile	Number of patients	No immune deregulation	Low-immune deregulation	Over-immune deregulation	Mixed immune profile
RIF	1450	19.2% (278)	27.5% (399)	43.3% (628)	10% (145)
RIF-OD	181	15.9% (28)	28% (51)	44.5% (81)	11.5% (21)
RM	180	17.2% (31)	23.9% (43)	45% (81)	13.9% (25)
Good prognosis IVF/ICSI patients	288	29.8%* (68)	25.4% (58)	37.7% (82)	7% (16)

RIF repeated implantation failures, *RIF-OD* repeated implantation failures with oocyte donation, *RM* recurrent miscarriage, *IVF/ICSI* in vitro fertilization and intracytoplasmic sperm injection
*No signiifcant difference between the distinct clinican context

Deregulation in Patients with RIF and/or RM History

Over the last 5 years, large cohorts of patients have been investigated (unpublished results). In the table below, we detail the repartition of observed deregulation in four distinct groups: RIF patients (more than six embryos replaced), RIF patients in oocyte-donation program (more than four embryos replaced), patients with unexplained RM (more than three embryos replaced), and good prognosis IVF patients (less than two embryos replaced) (Table 5.1). We report the same repartition of deregulations in RIF, RIF-oocyte donation (RIF-OD), and RM patients. Seventy-five to seventy-nine percent of the RIF and RM patients showed a local disequilibrium compared to fertile controls. Local disequilibrium was distributed as an over-immune activation in 45%, a too low local immune activation in 25%, and a mixed profile in 10%. A higher percentage of the profile with no immune deregulation was observed in good prognosis patients.

Particularities of the Uterine Immune Profiling in RIF Patients with Endometriosis

Endometriosis affects millions of women and is a major cause of infertility (Practice Committee of the American Society for Reproductive Medicine 2012). Infertility associated with endometriosis can be explained by several non-exclusive mechanisms [18]: a pelvic factor related to chronic local inflammation disrupting natural fertilization [19]; an ovarian factor, related to a poor oocyte quality and/or quantity [20]; and a uterine factor affecting endometrial receptivity and disrupting embryo implantation [21, 22]. The involvement of the endometrium itself as an effector of embryo implantation failures in patients with endometriosis is still a matter of debate [23–25]. The immune profiles of 176 RIF patients with endometriosis and 523 RIF patients with male infertility (RIF-male) were compared. The global repartition of the distinct immune profiles was not significantly different among endometriosis or male RIF patients ($p = 0.07$). However, patients with a low-activated

immune profile were significantly over-represented in the endometriosis RIF group (33% versus 23% respectively, $p = 0.03$). More local depletion of Th-2 cytokines ($p = 0.03$) associated with a higher rate of immature uNK cells ($p = 0.03$) was observed in the RIF endometriosis group when compared to the RIF-male group. Patients with endometriosis and a RIF history exhibit some immune particularities regarding their endometrial immune profile when compared with RIF-male group. These observations are consistent with some previously reported findings as the local perturbation with altered expression of the αvβ3 integrin and its ligand, the L-selectin, in patients with endometriosis described by Lessey et al. [23]. Regarding uNK cells, a decrease in their activity and cytotoxicity has been observed and reported in the early 1990s [26, 27]. Later, several studies have demonstrated a lower mobilization of $CD56^+$ uNK cells and confirmed a defect in uNK cells activity in the eutopic endometrium of women with endometriosis [28, 29].

Part II: Rationale Applied to Set Personalized Care According to the Immune Profile Results – Profile of Immune Over-activation

The hypothesis is that endometrial immune over-activation might induce embryo rejection (Table 5.2). Elevated IL-18/TWEAK mRNA ratios indicate an immune endometrial environment predominantly Th-1 in nature. Such a cytokine environment

Table 5.2 History, immune diagnosis, and outcome at the next embryo transfer in the repeated implantation failure (RIF) cohort of 394 patients

Endometrial immune diagnosis in the RIF cohort	Endometrial immune over-activation	Endometrial immune low activation	No immune dysregulation	*p*-value
Number of RIF patients	223 (56.6%)	99 (25%)	72 (18.3%)	–
Age (mean years)	36.6	37.1	37.0	0.24
Range of attempts (mean no.)	3.3	3.3	3.3	0.29
Years of infertility (mean years)	6.3	6.5	6.7	0.50
Previous ETs (mean no.)	8.8	8.8	9.1	0.89
IR at 3 weeks	29%	36%	19%	0.01
IR at 10 weeks	24.7%	32%	15%	0.009
Clinical PR at 3 weeks	47.1% (105/223)	55.6% (56/99)	30.6% (22/72)	0.005
Ongoing PR	37.7% (84/223)	48.5% (48/99)	20.8% (15/72)	0.001
LBR	36.8% (82/223)	46.5% (46/99)	19.4% (14/72)	0.001
Early miscarriage rate	9% (21/223)	8% (8/99)	9.7% (7/72)	0.42

Statistical analysis was made by ANOVA
IR implantation rate, *PR* pregnancy rate, *LBR* live birth rate

can potentially activate all the endometrial immune cells and possibly engage them in a pathway deleterious for the fetus. The objective of personalized care is to modify the Th-1 environment before the ET to limit activation of local immune cells and thereby avoid early embryo rejection or premature destruction of the endometrium, or both. Following are recommendations.

Modification of the Predominantly Th-1 Endometrial Environment

As first-line treatment, we recommended supplementing treatment from day 3 of ovarian stimulation until the pregnancy test with prednisolone and vitamin E (an antioxidant, 1 g daily). Prednisolone (20 mg/day) was administered to 184 women diagnosed with immune over-activation. Women who became pregnant continued treatment with prednisolone until 8 weeks after ET and then slowly decreased it, stopping it completely at 10 weeks.

Corticoids have been reported

- To decrease levels of Th-1 cytokines, NK cytotoxicity, and hyperactivation in lymphokine-activated killer cells [30].
- To limit the consequence of IL-15 mRNA overexpression [31].
- To modulate the Th1/Th2 balance when it is predominated by Th1 cytokines [32].

Corticotherapy is the leading medication worldwide for RIF, but we still lack precise indications for its use based on objective testing [33–35]. In our hand, only the normalization of the uterine profile under therapy may attest of its efficacy. As the second line of treatment, the efficacy of slow perfusion of intralipids was evaluated. Slow perfusion of diluted Intralipid® has been reported to limit the hyperactivation of circulating NK cells and to regulate a Th-1-predominant cytokine balance [36, 37]. These women also received vitamin E supplementation of the stimulation cycle (1 g daily).

Adaptation of Luteal Hormonal Support After ET

Besides its endocrine role, progesterone can influence the maternal immune system via progesterone-induced blocking factor (PIBF), which inhibits NK cell activity [38] and leads to Th-2-dominant cytokine production by maternal lymphocytes [39]. Progesterone is also an inducer of galectin-1, a progesterone-induced molecule essential for inducing tolerogenic dendritic cells, which in turn promote in vivo expansion of IL-10-secreting Treg cells [40]. If an over-immune activation was diagnosed, we hence recommended high daily vaginal doses of progesterone (1200 mg) for its immunosuppressive properties. Estradiol has been shown to decrease local expression of proinflammatory cytokines, especially for the angiogenic IL-18

system in the endometrium [41]. If IL-18 was elevated, we hence recommended oral estradiol supplementation (4 mg). Treatment began on the day of oocyte retrieval and continued until 8 weeks after ET in women who were pregnant.

Adaptation of Mechanical Local Endometrial Stimulation as a Function of uNK Cell Recruitment and Maturation

If uNK cell mobilization or activation was normal or high, we recommended:

- To avoid any local endometrial injury the cycle before the one during which ET was planned.
- To avoid sexual intercourse after the ET. Seminal plasma has been shown to induce mobilization and activation by local maternal immune cells [42]. If, however, uNK cell mobilization was low (<10 $CD56^+$ cells/field) or if the IL-15/Fn-14 mRNA ratio was low, suggesting uNK immaturity (<0.3), we recommended endometrial scratching the cycle preceding ET and sexual intercourse after ET (see below and mixed profiles).

Profile with Low Immune Activation

A low immune activation profile was characterized by the immaturity of uNK cells (low IL-15/Fn-14 mRNA expression) or the absence of their mobilization ($CD56^+$ cell count <10) and/or a very low local IL-18/TWEAK mRNA ratio, which suggested insufficient preparation (i.e., destabilization) of the spiral arteries for implantation. We interpreted this profile to indicate a disturbance of the molecular mechanisms involved in effective adhesion and adequate angiogenesis. Our hypothesis was that RIF is resulted from the endometrial inability to react appropriately and allow effective embryo apposition and adhesion, and RM is resulted from deficient local angiogenesis to set the placentation. Because the endometrium is spontaneously anti-adhesive, embryo adhesion is an active phenomenon that requires the expression of specific chemokines and adhesion molecules to enable embryo attachment. Expression of these adhesion molecules occurs only during the implantation window and depends on the migration and maturation of innate immune cells [43]. Strategies able to mobilize and activate immune cells might be crucial for adhesion, adequate angiogenesis, and immunotropism in this immune profile.

We therefore recommended:

(a) An endometrial scratching during the mid-luteal phase of the cycle preceding the ET.

An endometrial biopsy (or scratch or another local injury) during the mid-luteal phase of the cycle activates and stimulates subsequent expression of adhesion molecules and, interestingly, IL-15, via toll-like receptor

pathways [44]. This mechanism is specific for the mid-luteal phase, for it is not observed during the proliferative phase. Our objective was to enhance uNK cell maturation, which depends strongly on the adequate expression of IL-15 [45]. IL-15 activates uNK cell maturation for women with a low IL-15/Fn-14 mRNA ratio.

(b) Supplementation of the luteal phase with human chorionic gonadotrophin (hCG).

By activation of the mannose receptor, hCG triggers both proliferation and maturation of uNK cells [46]. Physiologically produced by the embryo, hCG is known to be directly involved in the local reaction that induces immunological tolerance through adequate angiogenesis and activation of uNK cells at the maternal-fetal interface [47]. We hence recommended supplementing the luteal phase with hCG 1500 IU subcutaneously administered 4, 6, and 8 days after oocyte retrieval, which partially overlaps with the implantation window.

(c) Limitation of endometrial exposure to very high estrogen doses.

High concentrations of estrogens decrease the endometrial IL-18 expression, already low in this profile [41]. So we recommended avoiding all the treatment inducing an over-impregnation of estradiol.

(d) Sexual intercourse after the ET.

Studies of seminal plasma have highlighted its role in preparing for acceptance of implantation by inducing expression of pro-inflammatory cytokines and chemokines and the robust recruitment of immune cells [42, 48, 49].

Part III: Clinical Validation of the Efficacy of Personalized Cares in Case of Repeated Implantation Failures

Defining personalized treatments in function of the uterine immune profile has never been evaluated. Evaluating the efficacy of the method is a long and difficult process that needs to be conducted rigorously.

Clinical achievement in a large RIF cohort study with personalized care in case of diagnosed deregulation [17].

From 2012 to 2014, endometrial immune profiling was performed among 394 women with the previous history of RIF. According to their profile, we recommended personalized care to counteract the documented deregulation if diagnosed. One year after the test, the physician was asked to document the live birth rate (LBR) at the next embryo transfer (fresh or thawed) following the immune profile [17]. Endometrial immune profiles appeared to be deregulated in 81.7% of the RIF patients. Over-activation was diagnosed in 56.6% and low activation in 25%. The LBR among these deregulated and treated patients at the first subsequent embryo transfer was 39.8%. History and outcome following the immune profiling are detailed in Table 5.2.

We postulated that the LBR observed in the group with immune deregulation was twice higher than expected. We then launched a control cohort study.

A Control Cohort Study

Between 2012 and 2014, 193 patients (analyzed group) enrolled in the IVF program of the Hospital Les Bluets (Paris 12) benefitted of endometrial immune profiling. Subsequently, they had an effective embryo transfer (ET) with personalization of their treatments if immune deregulation had been diagnosed [50]. Each analyzed patient was paired to the closest patient included in the IVF program according to biological criteria (age, number of mature oocytes, stage and number of the transferred embryo), who had no endometrial immune profiling (193 patients, non-analyzed group). Seventy-eight percent of analyzed patients had a uterine immune dysregulation and therefore received personalized care. Their corresponding live birth rate (LBR) was twice higher than observed in the matched control group with conventional cares (30.5% versus 16.6%; OR, 2.2 [1.27–3.83]; $p = 0.004$) with a simultaneous drastic reduction of miscarriages per initiated pregnancy (17.9% versus 43.2%; OR, 0.29 [0.12–0.71]; $p = 0.005$). Twenty-two percent of analyzed patients had no dysregulation. They did not differ from their matched controls for LBR and miscarriages. This control cohort study then suggested that personalization of treatment according to the woman's uterine immune balance produced a very significantly higher LBR.

An Ongoing Randomized Controlled Trial (NCT-02262117)

A randomized prospective controlled study is ongoing. Results are expected in September 2021. Five hundred patients involved in IVF/ICSI (less than 39 years old, normal ovarian reserve, range of oocyte retrieval below or equal to 3) will benefit from immune endometrial profiling. If deregulation is diagnosed, the randomization process will determine if the next IVF/ICSI treatment would either be personalized or conventional (no specific intervention). The primary outcome is the LBR at the first subsequent fresh or thawed embryo transfer (if freeze all). The hypothesis is that in good prognosis patient, personalization of treatment according to the endometrial immune profile would allow 50% increase of the subsequent LBR.

Uterine Immune Profiling May Also Help to Define Specific Indication for Immunotherapy

The meta-analysis or Cochrane studies exploring the efficacy of immunotherapy (corticoids, IVIG, LMWH, or intralipids) did not report these treatments being effective in RIF patients. But none of these studies defined a sub-group based on precise biological explorations that would be corrected under immunotherapy. We

thus postulated that the immune profiling could help to define which subgroup is responsive to a specific treatment based on the observation of the normalization of the immune profile under therapy. The documentation of the immune environment in RIF patient suggests that distinct immune mechanisms are able to induce an over-immune activation. This diversity of mechanisms suggests that distinct drugs could be used to control the identified pathway of activation.

Corticotherapy would be the leading medication worldwide for patients with a history of RIF after IVF/ICSI even if meta-analysis did not recommend its administration [51]. The local mechanism of action by corticoids could be effective. However, its precise indication is still unknown. We, therefore, documented the impact of prednisone on the endometrial expression of immune biomarkers (CD56 cells count, IL-18/TWEAK, and IL-15/Fn-14 mRNA ratio) at the time of uterine receptivity among 55 RIF patients with an over-immune activation [52]. In 54.5% of the cases, both immune biomarkers were normalized, and in 16.5%, only one was normalized under prednisone. In 29%, we observed a paradoxical increase of both immune biomarkers. The IL-18/TWEAK mRNA ratio reflecting the Th-1/Th-2 local equilibrium was significantly reduced (0.29 versus 0.10, $p = 0.004$), through the very significant increase of TWEAK expression, in patients who were subsequently pregnant under prednisone. This result suggests that corticoids act on the local endometrial expression of immune-regulators to decrease Th-1 cytokines. Our results also suggest that less than half of RIF patients with immune deregulation may be prednisone responders and would benefit from its administration. Testing the response to prednisone in a RIF context may be beneficial.

For patients who did not respond to corticoids, we explored if slow perfusion of intralipids could represent an option to control a diagnosed deregulation with an excess of Th-1 cytokines or hyperactivated NK cells [53]. Intralipid® is a fat emulsion containing soybean oil, glycerin, and egg phospholipids commonly used as a component of parenteral nutrition in patients unable to tolerate an oral diet. While the exact mechanism in which immune modulation is achieved by Intralipid® remains unclear, its active ingredient, soya oil, is reportedly capable of inhibiting pro-inflammatory mediators, specifically Th1 cells [54]. Roussev et al. reported in the context of reproductive failures that Intralipid® has immunosuppressive properties on circulating NK cells [36, 55]. More recently, Meng et al. reported a decrease in the circulating NK cell recruitment and related cytotoxicity under Intralipid® [56].

Ninety-four patients with a history of RIF exhibited an immune profile of endometrial over-immune activation and resistance to corticoids. To get new insight into its mechanism of action, second immune profiling had been performed under Intralipid® before the embryo transfer. They subsequently received slow perfusion of Intralipid® during the IVF/ICSI cycle. The live birth rate of the RIF cohort treated with Intralipid® reached 54% (51/94) at the next embryo transfer. In patients successfully pregnant under Intralipid® who benefitted of a test of sensitivity before the embryo transfer, we observed a significant decrease of the three biomarkers used to diagnose the over-immune endometrial activation (CD56 cells; IL-18/TWEAK, and IL-14/FN-14 mRNA gene expression ratios). Double-blind placebo versus

Intralipid® studies should be conducted in RIF patients who exhibit an over-immune activation of uNK cells.

These two studies suggest that the efficacy of immunotherapy should be verified before the administration of treatment, based on the normalization of the immune profile under the immunotherapy.

Conclusion

The endometrial immune profiling was launched as an innovation to increase the efficacy of assisted reproductive treatment in 2012. ART treatment should be more effective, and embryo implantation is the main limiting factor.

The Endometrial Immune Profiling Aims

- To explore the unexplained failure of embryo implantation by investigating the immunological dialogue at the maternal-fetal interface.
- To understand the mechanisms leading to the absence of embryo implantation.
- To offer personalized fertility treatment to increase pregnancy rates.

Optimizing immune uterine receptivity through a precise personalization may represent the best option to increase the efficacy of ART treatment. It is an unexplored new area in reproductive medicine. In our experience, if a disequilibrium is present, its correction induces an over 75% relative increase of the LBR in a large RIF cohort. No recent innovation reported such an increase. These results should be confirmed in a randomized controlled trial.

References

1. Gellersen B, Brosens IA, Brosens JJ. Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. Semin Reprod Med. 2007;25(6):445–53.
2. Ruiz-Alonso M, Blesa D, Diaz-Gimeno P, Gomez E, Fernandez-Sanchez M, Carranza F, et al. The endometrial receptivity array for diagnosis and personalized embryo transfer as a treatment for patients with repeated implantation failure. Fertil Steril. 2013;100(3):818–24.
3. Haouzi D, Dechaud H, Assou S, De Vos J, Hamamah S. Insights into human endometrial receptivity from transcriptomic and proteomic data. Reprod Biomed Online. 2012;24(1):23–34.
4. Liu S, Diao L, Huang C, Li Y, Zeng Y, Kwak-Kim JYH. The role of decidual immune cells on human pregnancy. J Reprod Immunol. 2017;124:44–53.
5. Lee JY, Lee M, Lee SK. Role of endometrial immune cells in implantation. Clin Exp Reprod Med. 2011;38(3):119–25.
6. Voest EE, Kenyon BM, O'Reilly MS, Truitt G, D'Amato RJ, Folkman J. Inhibition of angiogenesis in vivo by interleukin 12. J Natl Cancer Inst. 1995;87(8):581–6.
7. Croy BA, Esadeg S, Chantakru S, van den Heuvel M, Paffaro VA, He H, et al. Update on pathways regulating the activation of uterine Natural Killer cells, their interactions with decidual spiral arteries and homing of their precursors to the uterus. J Reprod Immunol. 2003;59(2):175–91.

8. Simon C, Moreno C, Remohi J, Pellicer A. Cytokines and embryo implantation. J Reprod Immunol. 1998;39(1–2):117–31.
9. Chaouat G. The Th1/Th2 paradigm: still important in pregnancy? Semin Immunopathol. 2007;29(2):95–113.
10. Ledee-Bataille N, Bonnet-Chea K, Hosny G, Dubanchet S, Frydman R, Chaouat G. Role of the endometrial tripod interleukin-18, -15, and -12 in inadequate uterine receptivity in patients with a history of repeated in vitro fertilization-embryo transfer failure. Fertil Steril. 2005;83(3):598–605.
11. Zhang J, Chen Z, Smith GN, Croy BA. Natural killer cell-triggered vascular transformation: maternal care before birth? Cell Mol Immunol. 2010;8(1):1–11.
12. Goldman-Wohl DS, Ariel I, Greenfield C, Lavy Y, Yagel S. Tie-2 and angiopoietin-2 expression at the fetal-maternal interface: a receptor ligand model for vascular remodelling. Mol Hum Reprod. 2000;6(1):81–7.
13. Petitbarat M, Rahmati M, Serazin V, Dubanchet S, Morvan C, Wainer R, et al. TWEAK appears as a modulator of endometrial IL-18 related cytotoxic activity of uterine natural killers. PLoS One. 2011;6(1):e14497.
14. Petitbarat M, Serazin V, Dubanchet S, Wayner R, de Mazancourt P, Chaouat G, et al. Tumor necrosis factor-like weak inducer of apoptosis (TWEAK)/fibroblast growth factor inducible-14 might regulate the effects of interleukin 18 and 15 in the human endometrium. Fertil Steril. 2009;94(3):1141–3.
15. Manaster I, Mizrahi S, Goldman-Wohl D, Sela HY, Stern-Ginossar N, Lankry D, et al. Endometrial NK cells are special immature cells that await pregnancy. J Immunol. 2008;181(3):1869–76.
16. Blois SM, Barrientos G, Garcia MG, Orsal AS, Tometten M, Cordo-Russo RI, et al. Interaction between dendritic cells and natural killer cells during pregnancy in mice. J Mol Med (Berl). 2008;86(7):837–52.
17. Ledee N, Petitbarat M, Chevrier L, Vitoux D, Vezmar K, Rahmati M, et al. The uterine immune profile may help women with repeated unexplained embryo implantation failure after in vitro fertilization. Am J Reprod Immunol. 2016;75(3):388–401.
18. Borghese B, Santulli P, Marcellin L, Chapron C. Definition, description, clinicopathological features, pathogenesis and natural history of endometriosis: CNGOF-HAS endometriosis guidelines. Gynecol Obstet Fertil Senol. 2018;46(3):156–67.
19. de Ziegler D, Borghese B, Chapron C. Endometriosis and infertility: pathophysiology and management. Lancet. 2010;376(9742):730–8.
20. Simon C, Gutierrez A, Vidal A, de los Santos MJ, Tarin JJ, Remohi J, et al. Outcome of patients with endometriosis in assisted reproduction: results from in-vitro fertilization and oocyte donation. Hum Reprod. 1994;9(4):725–9.
21. Prapas Y, Goudakou M, Matalliotakis I, Kalogeraki A, Matalliotaki C, Panagiotidis Y, et al. History of endometriosis may adversely affect the outcome in menopausal recipients of sibling oocytes. Reprod Biomed Online. 2012;25(5):543–8.
22. Lessey BA, Lebovic DI, Taylor RN. Eutopic endometrium in women with endometriosis: ground zero for the study of implantation defects. Semin Reprod Med. 2013;31(2):109–24.
23. Lessey BA, Kim JJ. Endometrial receptivity in the eutopic endometrium of women with endometriosis: it is affected, and let me show you why. Fertil Steril. 2017;108(1):19–27.
24. Garcia-Velasco JA, Fassbender A, Ruiz-Alonso M, Blesa D, D'Hooghe T, Simon C. Is endometrial receptivity transcriptomics affected in women with endometriosis? A pilot study. Reprod Biomed Online. 2015;31(5):647–54.
25. Miravet-Valenciano J, Ruiz-Alonso M, Gomez E, Garcia-Velasco JA. Endometrial receptivity in eutopic endometrium in patients with endometriosis: it is not affected, and let me show you why. Fertil Steril. 2017;108(1):28–31.
26. Oosterlynck DJ, Cornillie FJ, Waer M, Vandeputte M, Koninckx PR. Women with endometriosis show a defect in natural killer activity resulting in a decreased cytotoxicity to autologous endometrium. Fertil Steril. 1991;56(1):45–51.

27. Tanaka E, Sendo F, Kawagoe S, Hiroi M. Decreased natural killer cell activity in women with endometriosis. Gynecol Obstet Investig. 1992;34(1):27–30.
28. Fernandez-Shaw S, Clarke MT, Hicks B, Naish CE, Barlow DH, Starkey PM. Bone marrow-derived cell populations in uterine and ectopic endometrium. Hum Reprod. 1995;10(9):2285–9.
29. Jones RK, Bulmer JN, Searle RF. Immunohistochemical characterization of stromal leukocytes in ovarian endometriosis: comparison of eutopic and ectopic endometrium with normal endometrium. Fertil Steril. 1996;66(1):81–9.
30. Eddy JL, Krukowski K, Janusek L, Mathews HL. Glucocorticoids regulate natural killer cell function epigenetically. Cell Immunol. 2014;290(1):120–30.
31. Moustaki A, Argyropoulos KV, Baxevanis CN, Papamichail M, Perez SA. Effect of the simultaneous administration of glucocorticoids and IL-15 on human NK cell phenotype, proliferation and function. Cancer Immunol Immunother. 2011;60(12):1683–95.
32. Elenkov IJ. Glucocorticoids and the Th1/Th2 balance. Ann N Y Acad Sci. 2004;1024:138–46.
33. Lunghi L, Pavan B, Biondi C, Paolillo R, Valerio A, Vesce F, et al. Use of glucocorticoids in pregnancy. Curr Pharm Des. 2010;16(32):3616–37.
34. Boomsma CM, Keay SD, Macklon NS. Peri-implantation glucocorticoid administration for assisted reproductive technology cycles. Cochrane Database Syst Rev. 2012;6:CD005996.
35. Boomsma CM, Keay SD, Macklon NS. Peri-implantation glucocorticoid administration for assisted reproductive technology cycles. Cochrane Database Syst Rev. 2007;1:CD005996.
36. Roussev RG, Acacio B, Ng SC, Coulam CB. Duration of intralipid's suppressive effect on NK cell's functional activity. Am J Reprod Immunol. 2008;60(3):258–63.
37. Coulam CB, Acacio B. Does immunotherapy for treatment of reproductive failure enhance live births? Am J Reprod Immunol. 2012;67(4):296–304.
38. Szekeres-Bartho J, Par G, Dombay G, Smart YC, Volgyi Z. The antiabortive effect of progesterone-induced blocking factor in mice is manifested by modulating NK activity. Cell Immunol. 1997;177(2):194–9.
39. Szekeres-Bartho J, Halasz M, Palkovics T. Progesterone in pregnancy; receptor-ligand interaction and signaling pathways. J Reprod Immunol. 2009;83(1–2):60–4.
40. Blois SM, Ilarregui JM, Tometten M, Garcia M, Orsal AS, Cordo-Russo R, et al. A pivotal role for galectin-1 in fetomaternal tolerance. Nat Med. 2007;13(12):1450–7.
41. Ledee N, Dubanchet S, Lombroso R, Ville Y, Chaouat G. Downregulation of human endometrial IL-18 by exogenous ovarian steroids. Am J Reprod Immunol. 2006;56(2):119–23.
42. Robertson SA. Seminal plasma and male factor signalling in the female reproductive tract. Cell Tissue Res. 2005;322(1):43–52.
43. Singh H, Aplin JD. Adhesion molecules in endometrial epithelium: tissue integrity and embryo implantation. J Anat. 2009;215(1):3–13.
44. Gnainsky Y, Granot I, Aldo PB, Barash A, Or Y, Schechtman E, et al. Local injury of the endometrium induces an inflammatory response that promotes successful implantation. Fertil Steril. 2010;94(6):2030–6.
45. Verma S, Hiby SE, Loke YW, King A. Human decidual natural killer cells express the receptor for and respond to the cytokine interleukin 15. Biol Reprod. 2000;62(4):959–68.
46. Kane N, Kelly R, Saunders PT, Critchley HO. Proliferation of uterine natural killer cells is induced by human chorionic gonadotropin and mediated via the mannose receptor. Endocrinology. 2009;150(6):2882–8.
47. Perrier d'Hauterive S, Berndt S, Tsampalas M, Charlet-Renard C, Dubois M, Bourgain C, et al. Dialogue between blastocyst hCG and endometrial LH/hCG receptor: which role in implantation? Gynecol Obstet Investig. 2007;64(3):156–60.
48. Robertson SA, Ingman WV, O'Leary S, Sharkey DJ, Tremellen KP. Transforming growth factor beta—a mediator of immune deviation in seminal plasma. J Reprod Immunol. 2002;57(1–2):109–28.
49. Sharkey DJ, Tremellen KP, Jasper MJ, Gemzell-Danielsson K, Robertson SA. Seminal fluid induces leukocyte recruitment and cytokine and chemokine mRNA expression in the human cervix after coitus. J Immunol. 2012;188(5):2445–54.

50. Ledee N, Prat-Ellenberg L, Chevrier L, Balet R, Simon C, Lenoble C, et al. Uterine immune profiling for increasing live birth rate: a one-to-one matched cohort study. J Reprod Immunol. 2017;119:23–30.
51. Kalampokas T, Pandian Z, Keay SD, Bhattacharya S. Glucocorticoid supplementation during ovarian stimulation for IVF or ICSI. Cochrane Database Syst Rev. 2017;3:CD004752.
52. Ledee N, Prat-Ellenberg L, Petitbarat M, Chevrier L, Simon C, Irani EE, et al. Impact of prednisone in patients with repeated embryo implantation failures: beneficial or deleterious? J Reprod Immunol. 2018;127:11–5.
53. Ledee N, Vasseur C, Petitbarat M, Chevrier L, Vezmar K, Dray G, et al. Intralipid(R) may represent a new hope for patients with reproductive failures and simultaneously an over-immune endometrial activation. J Reprod Immunol. 2018;130:18–22.
54. Granato D, Blum S, Rossle C, Le Boucher J, Malnoe A, Dutot G. Effects of parenteral lipid emulsions with different fatty acid composition on immune cell functions in vitro. JPEN J Parenter Enteral Nutr. 2000;24(2):113–8.
55. Roussev RG, Ng SC, Coulam CB. Natural killer cell functional activity suppression by intravenous immunoglobulin, intralipid and soluble human leukocyte antigen-G. Am J Reprod Immunol. 2007;57(4):262–9.
56. Meng L, Lin J, Chen L, Wang Z, Liu M, Liu Y, et al. Effectiveness and potential mechanisms of intralipid in treating unexplained recurrent spontaneous abortion. Arch Gynecol Obstet. 2016;294(1):29–39.

Chapter 6
Endometrial Receptivity by Endometrial Receptivity Analysis (ERA) for Infertility

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Introduction

Reproduction is one of the main basic functions in life, so the inability of having offspring has been one of the greatest concerns of the human being from the beginning of our history. Along the time, the number of couples with infertility problems has been increasing due to relevant changes in our lifestyle, and science has been involved in solving it. One of the main milestones happened in 1978 when Patrick Steptoe and Robert Edwards developed in vitro fertilization (IVF) [1], an assisted reproduction technique (ART) that has helped millions of couples with fertility disorders. Nevertheless, despite the dramatic evolution of reproductive medicine developing therapeutic interventions and new diagnostic tests, the number of IVF newborns is not as high as expected.

Starting from the first steps of life, the implantation process requires three critical players: a viable embryo, a receptive maternal environment, and a successful endometrial–embryo communication. However, from the beginning of ART, all the researches were focused on the embryo, whose development and quality assessment were thought to be the only issues to care about. Consequently, the

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endometrium has been left aside and not been considered susceptible to be treated in a personalized way. During many decades, unless there was an evident uterine pathology or anomaly, all the women were treated equally at the endometrial level. Fortunately, this approach is changing during the last years to understand better the receptive phenotype of the endometrium and to apply personalized medicine to its assessment.

In this chapter, we present the current knowledge regarding the biological mechanisms underlying endometrial receptivity, how can it be evaluated by the endometrial receptivity analysis (ERA), and the clinical relevance of personalizing the embryo transfer.

Endometrial Receptivity

The hormonal regulation of the endometrium leads to cyclical morphologic and functional changes turning it into a plastic organ. Briefly, once menstruation ends, the estrogen level rises, leading to the proliferation of endometrial cell and increasing the endometrial thickness. Then, ovulation will take place, leaving the corpus luteum which will begin to secrete progesterone. This hormone stimulates the endometrial epithelial differentiation and maturation. In case of no pregnancy, hormonal levels decrease leading to the vasoconstriction of the spiral arteries and tissue breakdown, and then regeneration takes place.

During most of the menstrual cycle, the endometrium is refractory to the embryo and only for a few hours acquires the ability to be adhesive. This short period takes place during the mid-secretory phase, and it is known as window of implantation (WOI). This concept was first suggested in 1956 by Hertig and Rock [2] and subsequently demonstrated by many other authors such as Navot [3] and Lessey [4]. The WOI has been widely studied in the last century from different points of view to understand what happens and how it is achieved. Classically, it has been considered that this ideal condition for the embryo implantation occurs between days 19 and 20 of the cycle in all women.

One of the most relevant changes that the endometrium suffers during the acquisition of receptivity includes plasma membrane transformation from a nonadhesive to adhesive surface encompassing remodeling of the endometrial barrier function and resulting in the replacement of the microvilli in the apical membrane with ectoplasmic projections called pinopodes [5].

In the meanwhile, the developing embryo will go through the fallopian tube to enter the uterine cavity. Once there, it starts to produce several molecules that will mediate implantation through interactions with the endometrium. However, as the endometrium is non-receptive during most of the menstrual cycle, synchronization between the embryo development and the endometrial maturation is needed for the implantation. If both players are properly synchronized, the implantation process will begin sequentially its four stages: apposition, adhesion, penetration, and invasion [6].

There are a fair number of molecules implied in the achievement of the receptive stage:

- Several studies have shown that an inhibition of prostaglandin (PG) production is related to implantation failure [7–9]. In fact, PGE2 and PGF2α exhibit a specific lipidomic signature in endometrial fluid, which was shown by Vilella et al. [10], to distinguish between fertile and infertile women as a preliminary screening.
- Studies by Genbacev et al. [11] and Nejatbakhsh et al. [12] identified the relevance of the selection adhesion system in embryonic implantation. However, their role in receptivity remains unclear.
- Integrins are a group of transmembrane cell adhesion molecules that contribute to endometrial receptivity, and they are proposed as markers to detect the WOI [13], but there is still a debate regarding the utility of integrins as molecular receptivity biomarkers [14–18].
- Mucins provide the endometrium with a physical barrier to implantation to prevent embryo attachment that must be overcome. As an example, the blastocyst induces a local clearance of MUC1 during adhesion to enable its implantation at that site [19].
- Cadherins are responsible for calcium-dependent cell-to-cell adhesion, but conflicting results have been reported especially regarding E-cadherin mRNA expression and protein levels [20–24].
- Cytokines facilitate communication among endometrial cells as well as between the endometrium and embryo. The most relevant cytokines involved in receptivity are:
  - LIF: regulates the proportions and amounts of immune cells in the endometrium at the time of implantation [25], mediates interactions between decidual leukocytes and invading trophoblast [26], controls the status of the endometrium through its receptor signaling, and forms pinopodes [25, 27].
  - IL-6: this cytokine may play a paracrine or autocrine role in the periimplantation period since its receptors exist in both endometrium and blastocysts. Furthermore, IL-6 may be a valid predictor of blastocyst quality [28, 29].
  - IL-11 contributes to the decidualization process and stimulates the production of LIF by the endometrium [25, 27, 30].

Besides, some biological processes as the immune response are involved in this phenomenon. Immune cells not only protect the organism against invaders but also allow invasion and maintenance of the fetal semi-allograft in the endometrium. In the human endometrium, several different leukocyte subpopulations exist: uterine natural killer (uNK) cells, macrophages, dendritic cells, and T cells [31]. Further, these cells can contribute to reproductive problems, such as recurrent miscarriage (RM), infertility, and implantation failure, but unfortunately it has been difficult to associate these cells with reproductive failure in women or to develop them as clinical targets, and hence the prognostic value of measuring immune cell parameters remains uncertain [32–34].

How to Evaluate the Receptive Phenotype: The ERA Development

From the moment when the WOI concept was established, many investigations have been focused on finding a receptivity marker that may identify a receptive endometrium. Passing from histological to molecular techniques, transcriptomics has proven its clinical applicability [35].

This transcriptomic approach has allowed identifying different mRNA expression patterns in the endometrium during the whole menstrual cycle, revealing a specific signature for each endometrial stage [35]. In fact, during the acquisition of the receptive transcriptomic profile, an up/downregulation of different genes has been observed. Some of the molecules regulated are implied in the immune response, such as CXCL14, which acts as a major recruitment stimulus for immune cells during the receptive period [36] and as chemotaxis of natural killer cells to cluster around epithelial glands [37]; glycodelin, which is implied in the decreasing of the maternal immune response during implantation [38]; and IL-15, involved in uNK cell proliferation and differentiation [39] from peripheral blood CD16 (−) NK cells [40]. Also, some other molecules are related to the protection of the embryo and endometrium, such as the metallothioneins and glutathione peroxidases (GPXs) (antioxidants), which protect against heavy metals, free radicals, and oxidative damage [41].

Based on endometrial transcriptomics, our group developed a molecular tool known as endometrial receptivity analysis (ERA) [42]. The goal was to translate more than 10 years of research in transcriptomics into the clinical practice in order to evaluate the endometrial receptivity objectively, highlighting the relevance of the maternal contribution in the implantation process.

Even though the ERA protocol was developed using microarray technologies, it is based nowadays in next-generation sequencing (NGS) techniques. RNA extracted from an endometrial biopsy is analyzed in order to obtain the expression pattern of 248 selected genes [43]. This information is analyzed by a computational predictor which classifies the endometrium in one of the different endometrial stages regardless of its histological appearance: proliferative, pre-receptive, receptive, or post-receptive, for the specific day in which the biopsy was taken.

The transition to NGS was accompanied by the development of a new ERA predictor based on machine learning algorithms improved by the acquired know-how after more than 20,000 endometrial biopsies analyzed. For this updating, several supervised machine learning methods were compared (random forest, classification tree, support vector machine, and K-nearest neighbor), resulting in the random forest as the one with the best performance in terms of accuracy (0.88), sensitivity (0.90), and specificity (0.97). This study also evaluated the success rate of the ERA prediction, according to the result obtained in a second endometrial biopsy. This technique has been refined and improved such that the predictor potency provides more detailed insights into the use of gene signature profiles for patient stratification, so the new ERA predictor defines a shorter, optimal WOI frame.

The ERA accuracy was evaluated by comparing its results with those obtained based on Noyes histological criteria, the classical method to date the endometrium [44]. To this aim, a dating set comprising 49 endometrial biopsies was analyzed using ERA by two independent pathologists. The concordance between each method was statistically analyzed by the quadratic weighted Kappa index, and results showed a high concordance for ERA (0.922) against the two pathologists (0.685 and 0.618).

The most important contribution of the ERA has been the objective diagnosis of the WOI, leading to the creation of the concept of personalized embryo transfer (pET). The basis of the test implies that pET must be performed after a receptive result in a subsequent cycle (or even several cycles later) under the same conditions (day and type of cycle) as the original endometrial biopsy.

Displaced Window of Implantation and its Assessment Under Different Clinical Conditions

For many years, it has been thought that the acquisition of endometrial receptivity was common for all women. In that way, the blastocyst transfer was performed routinely after 5 full days of progesterone administration in hormone replacement therapy (HRT) cycles or 7 days after the LH surge in natural cycles. However, the ERA test revealed that there is a proportion of women in whom, after the standard endometrial preparation protocols, their endometrium remains non-receptive [45].

For those patients with a pre-receptive profile, the endometrial receptivity will be reached later than when the biopsy was taken, needing more time of progesterone exposure. On the other hand, those cases with a post-receptive profile have already passed the receptive stage, requiring fewer days of progesterone exposure [45]. This mismatch implies a displacement of their WOI, revealing that it is not open at the same time for all the women.

A WOI displacement is highly relevant in ART since it results in an asynchrony between the embryo and the endometrium. Some patients could be more susceptible to suffer a WOI displacement, which leads to recurrent implantation failure (RIF). This term refers to a situation when repeatedly good-quality embryos are transferred without achieving pregnancy. Due to its dependence on multiple factors, such as general laboratory quality, embryonic and uterine/endometrial factors, etc. [46], there is no international consensus on the definition of RIF. One suggestion, found beyond others, defines RIF as the failure of pregnancy after a total of three cycles with reasonably good-quality embryos being transferred [46]. More strict criteria would speak of the failure of implantation in at least three consecutive IVF attempts, in which one to two embryos of high-grade quality are transferred in each cycle [47].

It is assumed that the endometrial factor contributes to 1/3 of RIF cases. According to a prospective multicenter trial published in 2013 [45], the WOI was delayed or advanced in one out of four RIF patients with more than three previous failed IVF cycles. In these cases, an embryo that is transferred on the “standard”

WOI will find a pre- or post-receptive endometrium, being too early or too late for successful implantation. This study showed that 84% of patients with displaced WOI were pre-receptive at the time when previous embryo transfer had failed, while the remaining 16% were post-receptive. These results were subsequently validated with a second ERA test performed at the time indicated by the first ERA.

Obese patients constitute another risk group for displaced WOI since this condition is associated with infertility. The poor outcome of patients with an elevated body mass index (BMI) could be provoked by the egg and/or embryo quality, the endometrium, or a combination of them. A retrospective study performed in 2014 evaluated the role of the endometrial receptivity in infertile obese women (ref). In this study, three study groups were established based on the BMI; women were classified as normal (BMI 19–24.9; $n = 163$), overweight (BMI 25–30; $n = 47$), or obese (BMI >30; $n = 11$), and the ERA test was performed. The analysis of endometrial receptivity showed that there were no statistically significant differences in overweight women compared to normal weight controls in terms of WOI timing. However, obese patients showed a slight increase in the non-receptive status during the expected WOI compared to normal or overweight patients [48]. In line with this, Comstock et al. published a prospective study in 2017 establishing the same categories of patients based on their BMI and found that the transcriptomic profile of the endometrium during the WOI was altered in obese patients. Interestingly, the more the BMI increased in the subjects, the more pronounced was the displacement of endometrial receptivity and the dysregulation of endometrial gene expression. This data evidence that altered endometrial gene expression in obese patients may contribute to their increased risk of infertility [49].

The influence of endometrial thickness to predict a positive result of IVF treatment and its possible association with receptivity has also been extensively studied. Most authors agree that an endometrium that reaches a thickness of at least 6 mm measured before the administration of exogenous progesterone in the HRT cycles indicates a receptive endometrium that can lead to pregnancy. To demonstrate this using a reliable molecular method, the ERA test was retrospectively analyzed in endometrial samples of HRT cycles classified into three groups according to their thickness: atrophic endometrium (<6 mm), normal endometrium (6–12 mm), and hypertrophic endometrium (>12 mm). The findings showed that samples from normal and increased endometrial thickness maintained a normal ratio of receptive versus non-receptive results. However, it was found that endometrial atrophic samples revealed a significantly higher percentage of non-receptive profiles, with more than 50% of the samples analyzed. These results suggest that the WOI of these patients could be displaced due to the insufficient growth of their endometrium [50].

On the other hand, there are other conditions which are not directly related to a WOI displacement, as is the case of endometriosis. This pathological condition is associated with infertility and refers to the growth of ectopic endometrial tissue outside the uterine cavity. Currently, there is a lack of consensus on the functional mechanisms of this disorder and how many levels does it affect IVF patients. In order to clarify its relationship with endometrial receptivity, a prospective study was designed to assess the endometrial receptivity gene signature in patients with

different stages of endometriosis using the ERA test. The authors observed that there were not differential regulation for the ERA transcriptomics profiles in any of the different stages of the disease compared to healthy women controls, concluding that endometriosis does not increase the risk of having a displaced WOI [51].

The ERA not only reveals if a given patient has a displaced WOI but also predicts when that patient will reach receptivity, indicating specifically how much time of progesterone exposure is needed for her endometrium to acquire this phenotype. So, a receptive profile is divided into three sub-signatures: (1) an optimal receptive profile indicating that the embryo has a high chance of implantation if the transfer is performed under the same conditions in which the biopsy was obtained, (2) an early receptive endometrium indicating that the endometrium needs 12 hours more of progesterone exposure to achieve an optimally receptive profile, and (3) a late receptive profile indicating that 12 hours less of progesterone exposure are needed. On the other hand, in case that the endometrium results pre-receptive at the standard WOI (120 hours progesterone), it would imply that the endometrium needs 1 or 2 more days of progesterone exposure to reach receptivity, while a post-receptive result will imply that it required 1 or 2 days less with progesterone exposure.

Clinical Results of Personalized Embryo Transfer

Implantation failure is critical in reproductive medicine since, beyond monetary considerations, it is an important cause of psychological stress and drop-out factor [52]. In order to minimize the negative impact of repeated implantation failure on infertility patients, it is crucial to optimize conditions before the next embryo transfers. As previously mentioned, it is assumed that the endometrial factor contributes to 1/3 of RIF cases. When it is related to a morphological anomaly or to pathology, it could be treated by a specific intervention or treatment. However, in those cases in which the underlying problem is a displacement of the WOI, it could be reverted by personalizing the embryo transfer according to the individual WOI of each woman.

pET has been widely used around the world from 2010 when ERA was used at clinical level for the first time [45, 53, 54]. First clinical data obtained following pET were published in Ruiz-Alonso et al. [45]. It was a prospective, interventional, multicenter clinical trial composed of 85 RIF patients (with at least 3 previous failed embryo transfer cycles) and 25 control patients (1 or no failed ET cycle) [45]. Results showed that a lower receptivity rate for RIF patients (75% vs. 88%) is one possible reason for their RIF condition. For all the cases with displaced WOI, pET was performed guided by ERA, leading to an embryo transfer at a different day than the standard. Even though the RIF group had several previous failed cycles, once pET was performed, pregnancy rate (PR) and implantation rate (IR) rose to 50% and 38.5%, respectively. This outcome was similar to that of patients who had a receptive result at their first biopsy (PR: 51.7% and IR: 33.9%). Thus, RIF patients

related to endometrial factor can normalize their reproductive outcome through pET guided by ERA after identifying their individual WOI, since otherwise the following embryo transfers would always be performed on the same day regardless of their endometrial receptivity status.

To check the difference between transfer at a non-receptive endometrium versus doing it during the personalized WOI (pWOI), several retrospective studies have been published. First of all, a pilot study showed the comparison within the same patient, comparing previous embryo transfer in a non-receptive endometrium prior to doing the ERA test, and then the outcome once her pWOI was detected. The 17 RIF patients had an ovum donation transfer for both prior and after the ERA and when the day of the embryo transfer was changed in a personalized manner. PR was increased from 19% to 60% and IR from 11% to 40% [54].

Clinical data after analyzing more than 55,000 patients worldwide from more than 1500 IVF clinics indicate that around 70% of RIF patients are receptive on the day in which the biopsy was performed. Hence, 30% are non-receptive, of which 0.5% are proliferative, 10.5% post-receptive, and 89% pre-receptive (non-published data).

Data achieved by different investigators in the last years show similar findings. In an Indian population study [55], a displaced WOI was found in 27.5% of RIF patients. Although not significant, after pET, an ongoing pregnancy rate (OPR) of 42.4% and an IR of 33% were reported, which were equal to their IVF results over 1 year [55]. Similar findings were reported by a retrospective analysis among 50 RIF patients in Japan. After personalizing the embryo transfer, clinical pregnancy rates were 35.3% (12/34) per first pET in the receptive group and 50.0% (5/10) per first pET in the non-receptive group (after correcting their WOI). All the pregnant cases in the non-receptive group achieved pregnancy in their first pET. The IR was 32.8% (20/61) in the receptive group and 31.6% (6/19) in the non-receptive group [56]. On the other hand, in a Canadian study with euploid blastocysts transferred, although not significant, IR and OPR were reported higher (73.7% vs. 54.2% and 63.2% vs. 41.7%, respectively) after pET when compared to patients without pET. The authors concluded that a significant proportion of patients with a history of implantation failure of a euploid embryo have a displaced WOI as detected by the ERA. For these patients, pET using a modified progesterone protocol may improve the outcomes of subsequent euploid frozen embryo transfers [57].

On the other hand, the ERA has also been evaluated for its ability to improve outcomes on patients without previous implantation failures. This was the first prospective, randomized controlled study where patients were randomized using a computational system to three arms: fresh embryo transfer (FET), deferred embryo transfer with cryopreserved embryos (DET), and pET guided by ERA with cryopreserved embryos. Preliminary outcome showed significant differences between PR for pET arm (85.7%) versus FET (61.7%) and DET (60.8%). Although not yet significant, there were also differences in IR (47.8% for pET, 35.3% for FET and 41.4% for DET) and in OPR per embryo transfer (55.1% for pET, 43.3% for FET and 44.6% for DET) [58]. This study included 28 clinics worldwide recruiting patients younger than 37 years, with body mass index (BMI) between 18.5 and 30

and with a normal ovarian reserve. Exclusion criteria were recurrent pregnancy loss and/or severe male factor. PGS was neither an inclusion nor an exclusion criterion.

Finally, the improvement of the new ERA predictor based on NGS has been evaluated by the clinical outcome obtained after pET. For that, the reproductive outcome of 512 patients (from 10 different clinics around the world) was evaluated. These results showed 57.2% IR, 72.9% PR, and 56.3% OPR. When compared with previous publications based on the ERA microarray diagnosis, a significant increase of 17% in IR (95% confidence interval (CI) [0.12, 0.22], p-value <0.0001), 14.9% in PR (95% CI [0.09, 0.21], p-value<0.0001), and 10.6% in OPR (95% CI [0.04, 0.17], p-value = 0.0013) was observed in favor of the new ERA predictor [43].

All these data show how the ERA has enabled clinical assessment of the endometrial factor, helping thousands of patients worldwide by identifying their pWOI. All the published results highlight the relevance of personalized medicine, also at the endometrial level, by synchronizing embryonic development and endometrial receptivity.

Conclusions: What the Future Will Bring?

The complexity of the implantation process has presented a challenge in reproductive medicine, being widely studied to improve the success rate of the IVF clinics. Now, the development of new technologies is allowing the researchers to make strides in understanding the cross talk between embryo and endometrium.

Currently, the ERA test is being applied widely to determine the endometrial receptivity based on transcriptomics. This test has introduced the concept of pET by personalizing the moment in which the transfer should be done, according to the specific timing of the endometrium. Future studies about the endometrial receptivity will have to clarify the role of epigenetics, microRNA, and genetic variants, among others, in those patients with failed cycles despite having a receptive endometrium.

On the other hand, the improvement of RNA and DNA sequencing techniques coupled with the evolution of bioinformatics analysis will provide more and better information about the molecular events taking place during the period of optimal receptivity. Also, it remains a challenge to the possibility of assessing the endometrial factor in a noninvasive way, even in the same transfer cycle, influencing the economic and emotional implications for the patient.

References

1. Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. Lancet. 1978;2(8085):366.
2. Hertig AT, Rock J, Adams EC. A description of 34 human ova within the first 17 days of development. Dev Dynam. 1956;98:435–93.

3. Navot D, Scott RT, Droesch K, Veeck LL, Liu HC, Rosenwaks Z. The window of embryo transfer and the efficiency of human conception in vitro. Fertil Steril. 1991 Jan;55(1):114–8.
4. Lessey BA. Endometrial receptivity and the window of implantation. Baillieres Best Pract Res Clin Obstet Gynaecol. 2000;14(5):775–88.
5. Murphy CR. Uterine receptivity and the plasma membrane transformation. Cell Res. 2004;14(4):259–67.
6. Cha J, Vilella F, Dey SK, Simón C. Molecular interplay in successful implantation. In: Sanders S, editor. Ten critical topics in reproductive medicine. Washington, DC: Science/AAAS; 2013. p. 44–8.
7. Song H, Lim H, Paria BC, Matsumoto H, Swift LL, Morrow J, et al. Cytosolic phospholipase A2alpha is crucial [correction of A2alpha deficiency is crucial] for "on-time" embryo implantation that directs subsequent development. Development. 2002;129(12):2879–89.
8. Ye X, Hama K, Contos JJ, Anliker B, Inoue A, Skinner MK, et al. LPA3-mediated lysophosphatidic acid signalling in embryo implantation and spacing. Nature. 2005;435(7038):104–8.
9. Achache H, Tsafrir A, Prus D, Reich R, Revel A. Defective endometrial prostaglandin synthesis identified in patients with repeated implantation failure undergoing in vitro fertilization. Fertil Steril. 2010;94(4):1271–8.
10. Vilella F, Ramirez L, Berlanga O, Martinez S, Alama P, Messeguer M, et al. PGE2 and PGF2 concentrations in human endometrial fluid as biomarkers for embryonic implantation. J Clin Endocrinol Metab. 2013;98(10):4123–32.
11. Genbacev OD, Prakobphol A, Foulk RA, Krtolica AR, Llic D, Singer MS, et al. Trophoblast L-selectin-mediated adhesion at the maternal-fetal interface. Science. 2003;299(5605):405–8.
12. Nejatbakhsh R, Kabir-Salmani M, Dimitriadis E, Hosseini A, Taheripanah R, Sadeghi Y, et al. Subcellular localization of L-selectin ligand in the endometrium implies a novel function for pinopodes in endometrial receptivity. Reprod Biol Endocrinol. 2012;10:46.
13. Lessey BA, Castelbaum AJ, Wolf L, Greene W, Paulson M, Meyer WR, et al. Use of integrins to date the endometrium. Fertil Steril. 2000;73(4):779–87.
14. Germeyer A, Savaris RF, Jauckus J, Lessey B. Endometrial beta3 integrin profile reflects endometrial receptivity defects in women with unexplained recurrent pregnancy loss. Reprod Biol Endocrinol. 2014;12:53.
15. Ordi J, Creus M, Quintó L, Casamitjana R, Cardesa A, Balasch J. Within-subject between-cycle variability of histological dating, alpha v beta 3 integrin expression, and pinopod formation in the human endometrium. J Clin Endocrinol Metab. 2003;88(5):2119–25.
16. Creus M, Balasch J, Ordi J, Fabregues F, Casamitjana R, Quinto L, et al. Integrin expression in normal and out-of-phase endometria. Hum Reprod. 1998;13(12):3460–8.
17. Hii LL, Rogers PA. Endometrial vascular and glandular expression of integrin alpha(v)beta3 in women with and without endometriosis. Hum Reprod. 1998;13(4):1030–5.
18. Surrey ES, Lietz AK, Gustofson RL, Minjarez DA, Schoolcraft WB. Does endometrial integrin expression in endometriosis patients predict enhanced in vitro fertilization cycle outcomes after prolonged GnRH agonist therapy? Fertil Steril. 2010;93(2):646–51.
19. Meseguer M, Aplin JD, Caballero-Campo P, O'Connor JE, Martin JC, Remohi J, et al. Human endometrial mucin MUC1 is up-regulated by progesterone and down-regulated in vitro by the human blastocyst. Biol Reprod. 2001;64(2):590–601.
20. Fujimoto J, Ichigo S, Hori M, Tamaya T. Expressions of E-cadherin and alpha- and beta-catenin mRNAs in uterine endometrial cancers. Eur J Gynaecol Oncol. 1998;19(1):78–81.
21. Van der Linden PJ, de Goeij AF, Dunselman GA, Erkens HW, Evers JL. Expression of cadherins and integrins in human endometrium throughout the menstrual cycle. Fertil Steril. 1995;63(6):1210–6.
22. Béliard A, Donnez J, Nisolle M, Foidart JM. Localization of laminin, fibronectin, E-cadherin, and integrins in endometrium and endometriosis. Fertil Steril. 1997;67(2):266–72.
23. Dawood MY, Lau M, Khan-Dawood FS. E-cadherin and its messenger ribonucleic acid in periimplantation phase human endometrium in normal and clomiphene-treated cycles. Am J Obstet Gynecol. 1998;178(5):996–1001.

24. Poncelet C, Leblanc M, Walker-Combrouze F, Soriano D, Feldmann G, Madelenat P, et al. Expression of cadherins and CD44 isoforms in human endometrium and peritoneal endometriosis. Acta Obstet Gynecol Scand. 2002;81(3):195–203.
25. Kimber SJ. Leukaemia inhibitory factor in implantation and uterine biology. Reproduction. 2005;130(2):131–45.
26. Dimitriadis E, White CA, Jones RL, Salamonsen LA. Cytokines, chemokines and growth factors in endometrium related to implantation. Hum Reprod Update. 2005;11(6):613–30.
27. González RR, Rueda BR, Ramos MP, Littell RD, Glasser S, Leavis PC. Leptin-induced increase in leukemia inhibitory factor and its receptor by human endometrium is partially mediated by interleukin 1 receptor signaling. Endocrinology. 2004;145(8):3850–7.
28. Kopf M, Baumann H, Freer G, Freudenberg M, Lamers M, Kishimoto T, et al. Impaired immune and acute-phase responses in interleukin-6-deficient mice. Nature. 1994;368(6469):339–42.
29. Salamonsen LA, Dimitriadis E, Robb L. Cytokines in implantation. Semin Reprod Med. 2000;18(3):299–310.
30. Krüssel J-S, Bielfeld P, Polan ML, Simón C. Regulation of embryonic implantation. Eur J Obstet Gynecol Reprod Biol. 2003;110 Suppl 1:S2–9.
31. Gnainsky Y, Dekel N, Granot I. Implantation: mutual activity of sex steroid hormones and the immune system guarantee the maternal-embryo interaction. Semin Reprod Med. 2014;32(5):337–45.
32. Vujisić S, Lepej SZ, Aksamija A, Jerkovic L, Sokolic B, Kupesic S, et al. B- and T-cells in the follicular fluid and peripheral blood of patients undergoing IVF/ET procedures. Am J Reprod Immunol. 2004;52(6):379–85.
33. Matteo M, Cicinelli E, Greco P, Massenzio F, Baldini D, Falagario T, et al. Abnormal pattern of lymphocyte subpopulations in the endometrium of infertile women with chronic endometritis. Am J Reprod Immunol. 2009;61(5):322–9.
34. Liu B, Mariee N, Laird S, Smith J, Li J, Li TC. The prognostic value of uNK cell count and histological dating in the mid-luteal phase of women with reproductive failure. Eur J Obstet Gynecol Reprod Biol. 2014;181:171–5.
35. Diaz-Gimeno P, Ruíz-Alonso M, Blesa D, Simón C. Transcriptomics of the human endometrium. Int J Dev Biol. 2014;58(2–4):127–37.
36. Talbi S, Hamilton AE, Vo KC, Tulac S, Overgaard MT, Dosiou C, et al. Molecular phenotyping of human endometrium distinguishes menstrual cycle phases and underlying biological processes in normo-ovulatory women. Endocrinology. 2006;147(3):1097–121.
37. Mokhtar NM, Cheng CW, Cook E, Bielby H, Smith SK, Charnock-Jones DS. Progestin regulates chemokine (C-X-C motif) ligand 14 transcript level in human endometrium. Mol Hum Reprod. 2010;16(3):170–7.
38. Aghajanova L, Hamilton AE, Giudice LC. Uterine receptivity to human embryonic implantation: histology, biomarkers, and transcriptomics. Semin Cell Dev Biol. 2008;19(2):204–11.
39. Okada H, Kuhn C, Feillet H, Bach J-F. The "hygiene hypothesis" for autoimmune and allergic diseases: an update. Clin Exp Immunol. 2010;160(1):1–9.
40. Kitaya K, Yamaguchi T, Honjo H. Central role of interleukin-15 in postovulatory recruitment of peripheral blood CD16(−) natural killer cells into human endometrium. J Clin Endocrinol Metab. 2005;90(5):2932–40.
41. Shankar K, Zhong Y, Kang P, Lau F, Blackbum ML, Chen JR, et al. Maternal obesity promotes a proinflammatory signature in rat uterus and blastocyst. Endocrinology. 2011;152(11):4158–70.
42. Díaz-Gimeno P, Horcajadas JA, Martínez-Conejero JA, Esteban FJ, Alamá P, Pellicer A, et al. A genomic diagnostic tool for human endometrial receptivity based on the transcriptomic signature. Fertil Steril. 2011;95(1):50–60.
43. Clemente-Ciscar M, Ruiz-Alonso M, Blesa D, Jimenez-Almazan J, Bahceci M, Banker M, et al. Endometrial receptivity analysis (ERA) using a next generation sequencing (NGS) predictor improves reproductive outcome in recurrent implantation failure (RIF) patients when compared to ERA arrays. Hum Reprod. 2018;33:8–8.

44. Díaz-Gimeno P, Ruiz-Alonso M, Blesa D, Bosch N, Martínez-Conejero JA, Alamá P, et al. The accuracy and reproducibility of the endometrial receptivity array is superior to histology as a diagnostic method for endometrial receptivity. Fertil Steril. 2013;99(2):508–17.
45. Ruiz-Alonso M, Blesa D, Díaz-Gimeno P, Gómez E, Fernández-Sánchez M, Carranza F, et al. The endometrial receptivity array for diagnosis and personalized embryo transfer as a treatment for patients with repeated implantation failure. Fertil Steril. 2013;100(3):818–24.
46. Margalioth EJ, Ben-Chetrit A, Gal A, Eldar-Geva T. Investigation and treatment of repeated implantation failure following IVF-ET. Hum Reprod. 2006;21(12):3036–43.
47. Simon A, Laufer N. Repeated implantation failure: clinical approach. Fertil Steril. 2012;97(5):1039–43.
48. Lathi RB, Ruiz M, Gómez E, Rincon-Bertolin A, Blesa D, Simón C. Obesity and endometrial receptivity as assessed by the endometrial receptivity array (ERA). Fertil Steril. 2014;101(2):e30–1.
49. Comstock IA, Diaz-Gimeno P, Cabanillas S, Bellver J, Sebastian-Leon P, Shah M, et al. Does an increased body mass index affect endometrial gene expression patterns in infertile patients? A functional genomics analysis. Fertil Steril. 2017;107(3):740–748.e2.
50. Valbuena D, Ruiz-Alonso M, Marin C, Soria J, Simon C, Garcia-Velasco JA. Endometrial thickness does not predict endometrial receptivity. Hum Reprod. 2016;31:255–6.
51. Garcia-Velasco JA, Fassbender A, Ruiz-Alonso M, Blesa D, Hooghe TD, Simón C. Is endometrial receptivity transcriptomics affected in women with endometriosis? A pilot study. Reprod Biomed Online. 2015;31(5):647–54.
52. Boivin J, Griffiths E, Venetis CA. Emotional distress in infertile women and failure of assisted reproductive technologies: meta-analysis of prospective psychosocial studies. Br Med J. 2011;342:d223.
53. Garrido-Gómez T, Ruiz-Alonso M, Blesa D, Díaz-Gimeno P, Vilella F, Simón C. Profiling the gene signature of endometrial receptivity: clinical results. Fertil Steril. 2013;99(4):1078–85.
54. Ruiz-Alonso M, Galindo N, Pellicer A, Simón C. What a difference two days make: "personalized" embryo transfer (pET) paradigm: a case report and pilot study. Hum Reprod. 2014;29(6):1244–7.
55. Mahajan N. Endometrial receptivity array: clinical application. J Hum Reprod Sci. 2015;8(3):121.
56. Hashimoto T, Koizumi M, Doshida M, Toya M, Sagara E, Oka N, et al. Efficacy of the endometrial receptivity array for repeated implantation failure in Japan: a retrospective, two-centers study. Reprod Med Biol. 2017;16(3):290–6.
57. Tan J, Kan A, Hitkari J, Taylor B, Tallon N, Warraich G, et al. The role of the endometrial receptivity array (ERA) in patients who have failed euploid embryo transfers. J Assist Reprod Genet. 2018;35(4):683–92.
58. Simón C, Vladimirov G, Castillon Cortes G. Prospective randomized study of the endometrial receptivity analysis (ERA) test in the infertility work-up to guide personalized embryo transfer versus fresh transfer or deferred embryo transfer. Fertil Steril. 2016;106(3):e46–7.

Chapter 7
Endometrium Gene Expression and Epigenetic Regulation in Reproductive Failure

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Understanding Endometrium and Reproductive Disorder

The primary function of the endometrium is to support embryo implantation, a process whereby the embryo attaches itself to the endometrium, followed by migration across the luminal epithelium and invasion into the endometrial stroma layer to become embedded. The process involves a complex sequence of cellular and molecular changes [1]. The deranged endometrial function may result in a spectrum of reproductive disorders, ranging from implantation failure, miscarriage, abnormal placentation, fetal growth restriction, and possibly preeclampsia.

Many methods have been used to study the endometrium and understand if the observed reproductive disorder in a particular individual is due to an underlying endometrial problem. Histological examination of the endometrial biopsy is often utilized to assess if the histological transformation of the endometrium, especially secretory transformation, is adequate. Immunohistochemical techniques offer to examine the expression of a specific protein or putative marker of implantation. Cell counting techniques are used to determine the density of certain immune cell types

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such as uterine natural killer cell. Imaging techniques such as ultrasonography or hysteroscopy are utilized to examine the uterine cavity, and the sampling of fluid in the endometrial cavity provides an opportunity to measure proteins or cytokine concentrations in the endometrial fluid.

Depending on the outcome of the initial investigation, one of the frequently asked questions is why and how the observed abnormality is brought up and if there is an effective treatment available to correct the underlying abnormality. In this regard, the genomic study of the endometrium is gaining increasing popularity as a modern approach to the study of endometrial receptivity and its regulation. A well-known earlier example is microarray analysis of endometrial biopsy using endometrial samples obtained at the putative time of embryo transfer to determine if the endometrial development at the time of implantation is in-phase, ahead, or behind schedule.

Prerequisites for the Genomic Study of the Endometrium

An important prerequisite for conducting a genomic study of the endometrium is to collect the specimen precisely at a well-defined time point in the menstrual cycle, because the endometrium undergoes profound, rapid changes in the secretory phase, especially around the time of implantation. Endometrial genes, including LIF, HOXA10, MUC1, EMX2, IGFBP-1, CSF-1, and IL-1, have been shown to fluctuate during the menstrual cycle [2–4] profoundly. The examination of RNA transcripts in precisely timed endometrial specimens and the comparison of results between women with recurrent miscarriage and recurrent implantation failure have provided new insights into how these two types of reproductive failure differ from each other [2].

A second prerequisite of genomic study of the endometrium is to collect an endometrial sample from a homogenous, well-defined population. The specific type of reproductive failure to be studied must be clearly defined. For example, it is necessary to understand that subjects with “infertility” of “recurrent implantation failure” or “recurrent miscarriage” are all rather heterogeneous with several underlying etiologies. Every effort must be made to define the inclusion criteria and exclusion criteria carefully to rule out known contributory or confounding factors such as the presence of any congenital or acquired uterine anomalies such as fibroid, congenital uterine anomaly, or intrauterine adhesions.

A third prerequisite is the need to recruit a suitable group of fertile, control subjects with whom the result of the studied population can be compared. Without a proper control group, it is difficult to draw meaningful conclusions. The control groups ought to have proven fertility in the recent past and similarly have undergone investigations to verify the absence of uterine anomalies.

Transcriptomic Study

Transcriptomic studies by using microarray analysis or RNA sequencing have been applied to examine the endometrium during the peri-implantation period (before or during the window of implantation) [3, 4] using various patient

populations such as PCOS [5], RIF [6–8], or RM [9, 10], with different hormonal treatments [11, 12], and data were compared between unexplained RM and RIF [2]. It is of interest that the endometrial gene expression in unexplained RM and recurrent RIF shared something in common. For example, IL-15 has been reported to be increased in both conditions [13, 14]. On the other hand, certain markers are deranged in one condition but not the other. A notable example is beta3 integrin, which is downregulated in RM [15] but not in RIF [16], whereas leukemia inhibitory factor (LIF) is downregulated in RIF [17], but not in RM [18, 19]. Recently, Brosens et al. [20–22] hypothesized that RM is associated with an over-receptive endometrium which would allow defective or abnormal embryos to implant and in turn leads to super-fertility, but followed by an increased risk of miscarriage of an abnormal embryo. In contrast, in women with RIF, implantation often fails to take place despite the replacement of many good-quality embryos, implying an underlying etiology of a different nature, possibly a presence of endometrial pathology.

Genes in complement and coagulation cascade pathways of women with RIF are upregulated in the endometrium when compared to those of women with RM. The complement system, represented by complement component 3 (C3), is a proteolytic cascade in plasma, which also mediates innate immunity. One of the major immune functions of this pathway is to form a membrane attack complex, which leads to cell lysis [23]. Genetic association study also found that the loss of functional mutation of some genes in this pathway was associated with RM [24] and other adverse pregnancy outcomes, such as preeclampsia [25]. It seems like that the upregulation of the complement pathway is associated with impairment of endometrial receptivity leading to implantation failure. The downregulation of this pathway observed in the endometrium of women with RM may explain the apparent increase in endometrial receptivity observed in this group of women.

On the contrary, the calcium signaling pathway in the endometrium of women with RIF is downregulated when compared with RM. It has long been known that Ca^{2+} channels are involved in a variety of implantation processes, and increased Ca^{2+} mobilization can assist blastocyst-endometrium adhesion [20, 26, 27]. It is suggested that in women with RM, the endometrium is more favorable for implantation when compared with RIF, consistent with the in vitro study carried out by Brosens and co-workers [20].

Single-Cell Gene Expression Study

One of the limitations of endometrial gene expression study is a lack of information about gene expression patterns in various cell types. Given the fact that the endometrium consists of various cell populations including luminal epithelium, glandular epithelium, stroma, blood vessels, fibroblasts, and immune cells, it would be more informative if gene expression changes are investigated in various cell types. For example, C3 gene expression is low in both epithelial and stromal cells in the proliferative phase endometrium. However, it gradually increases during the cycle, reaching a significantly higher level in the mid-secretory phase [2].

When stimulated by human chorionic gonadotropin (hCG), C3 gene expression is further increased in stromal cells but not in glandular cells. Similarly, after endometrial scratch, *CCL19* and *ITGB1* gene expressions were altered in the stromal but not in glandular cells.

On the other hand, single-cell sequencing (Sc-seq) may be performed in a single cell and amplified by whole genome or transcriptome for high-throughput sequencing, which reveals the genetic structure and gene expression status of the particular individual cell [28, 29]. Unlike a conventional sequencing technique, Sc-seq is the sequencing of the genome of a single cell, which avoids the heterogeneity of multiple cells by ordinary sequencing [29]. As a result, it helps to better understand the difference in genomic expression between different cell types [30]. The single-cell sequencing technology includes single-cell separation, cytolysis and genomic DNA acquisition, whole genome amplification, sequencing, and data analysis. The difficulty often lies in the isolation of single-cell samples and whole genome amplification [31].

Earlier, the single-cell isolation was made by the manual selection under the microscope and serial dilution method, which were problematic but did not require special instruments [32]. Later on, laser capture microdissection (LCM) and flow cytometry emerged and utilized for the single-cell isolation [33]. However, these techniques have a certain impact on cell viability, and throughput is not high. Recently, updated single-cell sorting methods, including fluorescence activated cell sorting (FACS), magnetic activated cell sorting (MACS), microdroplet, microfluidics, and nanopore chip technology, are available [34]. These new technologies cannot only achieve high throughput but have the advantage of sorting thousands of the same cells at a time [35, 36].

A special challenge of single-cell genomic study relates to whole genome amplification (WGA). High-throughput sequencing requires at least 200 ng of DNA sample, but the total DNA in a single cell is only a few picograms, so even with the use of third-generation sequencing technology, it may not be able to meet the minimal amount of DNA required for the analysis [29]. Therefore, DNA needs to be amplified for the single-cell sequencing [37]. Early whole genome amplification technology was performed by PCR, including ligation-mediated PCR (LM-PCR), primer extension pre-amplification PCR (PEP-PCR), and degenerate oligonucleotide-primed PCR (DOP-PCR). At present, multi-displacement amplification (MDA) and primase-based whole genome amplification (pWGA) are the most commonly used whole genome amplification methods based on constant temperature conditions [38]. Recently, a new method called multiple annealing and looping-based amplification cycles (MALBAC), which combines the former two amplification methods, has been introduced [39]. These genome amplification methods can be selected according to different experimental requirements (Table 7.1).

In the endometrium, Sc-seq has the potential to detect cell-to-cell variability, map possible subpopulations, discover possible rare cell types, and study clinically relevant but rare endometrial adult stem cells [40]. The first and so far the only study on endometrial Sc-seq was performed in 2016. Frozen-thawed endometrial biopsy samples were used for ScRNA-seq. Endometrial stromal cells were cultured from two patients obtained in the mid-secretory (Day 21, LH + 8) and late-secretory phase (Day 25). However, due to a small number of the endometrial epithelial cell sorted by

Table 7.1 A comparison of various WGA techniques used in the genomic study of the endometrium

Technique	Principle	Advantages	Limitations
DOP-PCR	Partial random primer method	Simple operation, the minimum starting template amount is 50 pg, and the product fragment size is 0.5–10 kb	Large deviation of amplification when the amount of starting template is low
LM-PCR	Connection-mediated PCR reaction	High yield, long fragment, low requirement for template DNA quality and quantity	Operation is cumbersome; the multi-step operation is easy to lose templates DNA
PEP-PCR	Complete random primer method	The template DNA quality and quantity requirements are low, the operation is simple, and the improvement is easy. The 50 ng starting template can produce 0.2–0.5 μg of the product, and the minimum starting template amount can reach 5 pg	Low yield and poor fidelity
MDA	Multiple displacement amplification	High yield, 50 ng of starting template can produce 10–20 μg of product, the minimum starting template amount can reach 10 pg, and the faithfulness is good	Large deviation of amplification when the amount of starting template is low
pWGA	Reconstitution of T7 phage DNA replication in vitro	High output, low requirements on template quality and quantity, simple operation, minimum starting template amount up to 100 fg	Less fidelity
MALBAC	Multiple annealing cyclic circulation amplification	Simple operation, high yield, a minimum of a few picograms of the starting template, reliable, and repeatable results	Increased difficulty in amplification when the amount of starting template is extremely low

FACS and low reads mapping, this research only focused on endometrial stromal cells. The results showed that cultured cells had more high-quality mRNA than frozen-thawed biopsy sample. Besides, of the 8622 detected genes, 2661 were more active in cultured stromal cells than those in the biopsy sample [41]. It suggests that ScRNA-seq technique may be utilized to study the function of different compartments and cell types of human endometrium. The single-cell preparation method for endometrial epithelial cells needs to be optimized due to the minimal amount of transcriptome data per individual epithelial cell, which compromised further analysis.

Epigenetics

Classical genetics refers to genes as the molecular basis of inheritance in terms of nucleic acids as the structural and functional unit of an organism. A gene determines a protein required for activities of life [42]. However, it is now known that many

genetic phenomena cannot be entirely explained by classical genetics. Change of the genome without affecting the nucleic acids not only can affect the function of genes in an individual but also can be inherited to the offspring. This phenomenon is known as epigenetics, which is first introduced by Waddington in 1942 [43]. The current definition of epigenetics is the science studying the inheritance of gene function that cannot be explained by nucleic acids changes during mitosis and meiosis [44]. In general, epigenetics has three characteristics, including being heritable, reversible, and regulatory, without DNA sequences change [45]. They are influenced by genetic variability and other factors, such as environmental and nutritional status. Currently, epigenetics includes DNA methylation, histone modification, non-coding RNA, genomic imprinting, and chromosomal inactivation, and each affects gene transcription in various ways. DNA methylation adds methyl to cytosine in a gene, particularly promotor regions, resulting in gene silencing. Histone modification alters coiling of genes for replication and functioning. Non-coding RNAs target complementary sequence interrupting transcription or post-transcription of genes. Genomic imprinting activates allele, while chromosomal inactivation changes chromatin location and structure.

Several studies have shown that epigenetics plays an important role in embryo implantation, placental formation, organ formation, and fetal growth [46]. Epigenetic modification may result in aberrant endometrial receptivity, leading to a reproductive failure [47].

DNA Methylation and Endometrium Receptivity

DNA methylation is the epigenetic regulatory mechanism which was discovered earliest and still one of the hotspots in current epigenetic research. It refers to the selective addition of methyl to cytosine in CpG dinucleotides to form 5-methylcytosine (5-mc) catalyzed by DNA methyltransferases (DNMTs), including DNMT1, DNMT3a, DNMT3b, and DNMT3l. DNA methylation is generally associated with gene silencing, and DNA demethylation is associated with gene activation [48].

DNA methylation in the endometrium is involved in the regulation of endometrial receptivity-related cytokines, gene expression, cell adhesion mechanism, and sex hormones. The endometrium undergoes periodic morphological and functional changes during the menstrual cycle to prepare for the implantation of the embryo. These changes include many gene modifications under the regulation of estrogen and progesterone, and DNA methylation is also involved in the regulation [49].

On the one hand, DNA methylation regulates the cyclical changes of the endometrium during the menstrual cycle. The expression of DNMTs in the endometrium was significantly changed during the menstrual cycle. At the mRNA expression level, the expression of DNMT1, DNMT3a, and DNMT3b in the secretory phase was significantly lower than that in the proliferative phase, with the lowest expression in the mid-secretory phase [50]. However, another research used whole

genome-wide methylation sequencing to investigate the changes in DNA methylation during the menstrual cycle and found methylome remained relatively stable during the menstrual cycle with small changes affecting only 5% of the CpG sites [51]. These findings suggest that DNA methylation probably plays a significant but a specific role in the endometrium.

Several studies examined how DNA methylation regulates the expression of genes involved in endometrial receptivity. E-cadherin and homeobox genes 10 (HOXA10) are important for endometrial receptivity and implantation. Rahnama et al. used an in vitro embryo implantation model and demonstrated that inhibition of DNA methylation promoted E-cadherin expression which was regulated by DNMT-1, 3a, and 3b. Also, hypermethylation of HOXA10 promoter led to reduced expression of HOXA10 and then resulted in poor endometrial receptivity [52]. 5-Aza-2′-deoxycytidine (AZA) inhibits DNA methyltransferase and in turn increases implantation rate with increased HOXA10 gene expression in Jeg-3 spheroid-endometrial cells [53]. It is now known that high progesterone (P) on the day of hCG trigger in fresh ART cycle was associated with poor endometrium receptivity. The expression of 5-mC in the high P group was significantly higher than that in the normal P group in human endometrial glandular epithelium. It indicated that DNA methylation modification associated with high P level might lead to poor endometrium receptivity [54]. However, the effects of high progesterone on the whole genome methylation changes in the endometrium are still lacking. While DNA methylation is considered an important factor in the regulation of endometrial receptivity, the specific role of DNA methylation in this important process requires further research.

Histone Modification and Endometrial Receptivity

Histone modifications refer to the basal amino-terminal tail of histones undergoing posttranscriptional modifications (PTMs), such as methylation, acetylation, phosphorylation, and ubiquitination [55]. Modifications constitute a rich "histone code" that affects the degree of compression tightness of chromatin and therefore plays an important regulatory role in gene expression [56].

The most studied histone modifications in endometrial receptivity and embryo implantation are histone acetylation and deacetylation. Histone acetylation is mainly regulated by two opposite enzymes: histone acetylase (HATs) and histone deacetylases (HDACs) [57, 58]. HATs and HDACs are expressed in the endometrium in the different menstrual cycles, which regulate the cyclical changes of the endometrium. The histone deacetylase inhibitors (HDACI) induced the expression, morphology, and function of endometrial stromal and epithelial cell differentiation markers and cell differentiation [59]. During the menstrual cycle, HDAC-1, HDAC-2, and HDAC-3 mRNAs were expressed periodically in the endometrium. HDAC-1 expression was significantly different among individuals, while HDAC-2 expression was significantly increased in the secretory phase, and HDAC-3 expression constituted throughout the menstrual cycle [60]. Estella et al. showed that histone

acetylation affected the balance of extracellular matrix regulators, which in turn inhibited trophoblast invasion into the endometrium [61].

Histone modification and DNA methylation can also be regulated by each other. For example, DNMTs can recruit complexes containing HDACs [50]. Inhibition of HDAC can lead to DNA methylation modification [62]. Further study on the interaction of histone modification and DNA methylation in endometrial receptivity may help better understand the embryo implantation process in synchronized endometrium.

ncRNAs and Endometrial Receptivity

Non-coding RNAs (ncRNAs) are RNA molecules that do not translate into a protein. ncRNAs contain microRNAs (miRNAs), transcribed ultraconserved region (T-UCR), small nucleolar RNAs (snoRNAs), PIWI-interacting RNAs (piRNAs), long non-coding RNAs (lncRNAs), and many other RNA molecules, accounting for more than 97% of the human genome [63]. Although ncRNAs rarely encode or do not have the function to encode proteins, they are involved in many biological processes such as stem cell maintenance, embryo development, cell proliferation, apoptosis, and differentiation [64]. Among them, miRNAs are the most studied ncRNAs in human endometrium receptivity. The research on other ncRNAs in human endometrium receptivity is still in its infancy, with only limited reports on related functions and mechanisms.

MicroRNA is a small ncRNA molecule of about 21–23 nucleotides widely distributed in eukaryotes [65]. It specifically inhibits posttranscriptional gene expression by binding to target messenger RNA (mRNA). It plays an important role in regulating gene expression and involves in cells cycles, developmental timing, and many other biological aspects [66]. More than 700 miRNAs have been discovered in humans, many of which are mutated or downregulated during disease development, leading to abnormal gene expression and related diseases [67].

Similar to DNA methylation and histone modifications, the expression of miRNAs is also cycle-dependent. Lam showed that expressions of 12 kinds of miRNAs (miR-29b, miR-29c, miR-30b, miR-30d, miR-31, miR-193a-3p, miR-203, miR-204, miR-200c, miR-210, miR-582-5p, and miR-345) in the secretory phase were significantly higher than those in the proliferative phase [68]. Some miRNAs (miR-15b, miR-20a, and miR-21) have higher expression in the proliferative phase, while others (e.g., miR-135a) have lower expression in the proliferative phase [69].

Revel et al. compared the expression of different miRNAs in the secretory phase between women with recurrent implantation failures (RIF) and normal fertile women. The result showed that 13 miRNAs were abnormally expressed, and these 13 miRNAs potentially regulated the expression of 3800 genes. Through miRNA-mRNA parallel analysis, molecular signaling pathways regulated by miRNAs were observed, including cell adhesion, Wnt, P53, and cell cycle. These molecular pathways are closely related to endometrial receptivity [70]. Petracco et al. observed that miRNA-135a and miRNA-135b inhibited the expression of

HOXA10, and overexpression of the miRNA may inhibit the expression of genes involved in embryo implantation, such as HOXA10 and cyclooxygenase-2 (COX-2) [71]. It showed miRNAs might affect endometrial receptivity by regulating genes and signaling pathways involved in embryo implantation and implantation window.

At present, miRNAs are one of the hotspots in the field of epigenetics research, and its role in endometrial receptivity is far from clear. Further work will be required to better understand how miRNA regulates endometrial receptivity.

Genomic Imprinting and Endometrial Receptivity

In 1984, scientists proposed that mammalian parental genomes have asymmetric functions. In these genes, one of the alleles is expressed in a family-dependent way, and the other is not expressed or expressed very weakly. This is called genomic imprinting [72]. The expression of these imprinted genes depends on whether they are from the father or the mother. The paternal and maternal genes have the opposite effect. The paternal genes direct the development of extraembryonic tissues, while the maternal genes direct the embryonic development. Therefore, genomic imprinting is a self-regulation and monitoring mechanism, which can effectively prevent parthenogenesis, ensure the simultaneous existence of both embryonic parental genomes, and maintain genetic diversity. However, it increases the disease risk caused by the mutation as well [73, 74]. At present, there are 73 imprinted genes in mice, and 243 are already discovered in humans [75].

It has been shown that the expression of imprinted genes may be involved in the establishment of endometrial receptivity [8]. Specifically, the expression of imprinted gene H19 in the endometrium appears to be related to the development of endometrial cancer [76]. Expression of H19 gene in sheep embryos can only be detected in the embryonic tissue after implantation, and no expression of H19 is observed in the embryonic ectoderm at any time before the implantation [77]. It seems, therefore, that normal expression of imprinted genes may play an important role in endometrial receptivity. Abnormal expression of imprinted genes may lead to structural and functional changes of endometrial microenvironment and thus reduce the implantation rate.

Chromosomal Inactivation and Endometrium Receptivity

X-chromosomal inactivation (XCI) refers to the silencing of one of the X chromosome pairs in the mammalian female embryo at the transcriptional level. Up to 1000 silencing genes on the X chromosome cause a balanced expression of sex-linked genes between male and female. The balance of gene expression is a mechanism to maintain dose-compensation effects in mammalian individuals and an important part of epigenetic research [78]. However, there is so far no reported study on the association between XCI and endometrium receptivity.

Sequencing Techniques for Epigenetic Study

Given that there are various types of epigenetic study, the laboratory methods should be chosen depending on the specific question to be addressed. First, there are two categories of methods to study DNA methylation: the one category is the methylation analysis at the whole genome level, including high-performance liquid chromatography, methylation-sensitive amplification polymorphism (MSAP) detection, DNA methylation immunoprecipitation (MeDIP), and DNA methylation chip technology [79–82]. The other category is a DNA methylation analysis for target sites, including bisulfite sequencing, sodium bisulfite treatment combined with enzymatic analysis, methylation-sensitive single-nucleotide amplification, methylation-sensitive high-resolution amplification, and methylation fluorescence PCR [83, 84]. These methods are generally based on the principle that cytosine becomes uracil under the treatment of bisulfite, while methylated cytosine does not change. This principle is the gold standard for DNA methylation analysis.

As for histone modifications, there are many types, including methylation, acetylation, phosphorylation, ubiquitination, and ADP ribosylation. To study histone modification, the most commonly used method is chromatin immunoprecipitation (ChIP) technique, including CHIP-chip and ChIP-seq [85, 86].

For non-coding RNA detection, the methods used include Northern blot, real-time PCR and expression library cloning, as well as ChIP technology, surface-enhanced Raman spectroscopy, and next-generation sequencing. ChIP technology and next-generation sequencing are the most commonly used technology for miRNA [87, 88].

Conclusions

Genomic study of the endometrium holds a great promise to increase our knowledge of the biology of implantation and its regulation, especially in women suffering from various forms of reproductive failure. In planning a study to examine genomic regulation of implantation, it is necessary to obtain precisely timed endometrial biopsy specimens and to recruit not only subjects with a well-defined category of reproductive failure but also a separate population of fertile control subjects.

References

1. Cha J, Sun X, Dey SK. Mechanisms of implantation: strategies for successful pregnancy. Nat Med. 2012;18(12):1754–67.
2. Huang J, Qin H, Yang Y, Chen X, Zhang J, Laird S, et al. A comparison of transcriptomic profiles in endometrium during window of implantation between women with unexplained recurrent implantation failure and recurrent miscarriage. Reproduction. 2017;153(6):749–58.

3. Diaz-Gimeno P, Horcajadas JA, Martinez-Conejero JA, Esteban FJ, Alama P, Pellicer A, et al. A genomic diagnostic tool for human endometrial receptivity based on the transcriptomic signature. Fertil Steril. 2011;95(1):50–60, e1–15.
4. Hu S, Yao G, Wang Y, Xu H, Ji X, He Y, et al. Transcriptomic changes during the pre-receptive to receptive transition in human endometrium detected by RNA-Seq. J Clin Endocrinol Metab. 2014;99(12):E2744–53.
5. Qiao J, Wang L, Li R, Zhang X. Microarray evaluation of endometrial receptivity in Chinese women with polycystic ovary syndrome. Reprod Biomed Online. 2008;17(3):425–35.
6. Koot YE, van Hooff SR, Boomsma CM, van Leenen D, Groot Koerkamp MJ, Goddijn M, et al. An endometrial gene expression signature accurately predicts recurrent implantation failure after IVF. Sci Rep. 2016;6:19411.
7. Ruiz-Alonso M, Blesa D, Diaz-Gimeno P, Gomez E, Fernandez-Sanchez M, Carranza F, et al. The endometrial receptivity array for diagnosis and personalized embryo transfer as a treatment for patients with repeated implantation failure. Fertil Steril. 2013;100(3):818–24.
8. Ledee N, Munaut C, Aubert J, Serazin V, Rahmati M, Chaouat G, et al. Specific and extensive endometrial deregulation is present before conception in IVF/ICSI repeated implantation failures (IF) or recurrent miscarriages. J Pathol. 2011;225(4):554–64.
9. Kosova G, Stephenson MD, Lynch VJ, Ober C. Evolutionary forward genomics reveals novel insights into the genes and pathways dysregulated in recurrent early pregnancy loss. Hum Reprod. 2015;30(3):519–29.
10. Othman R, Omar MH, Shan LP, Shafiee MN, Jamal R, Mokhtar NM. Microarray profiling of secretory-phase endometrium from patients with recurrent miscarriage. Reprod Biol. 2012;12(2):183–99.
11. Mirkin S, Nikas G, Hsiu JG, Diaz J, Oehninger S. Gene expression profiles and structural/functional features of the peri-implantation endometrium in natural and gonadotropin-stimulated cycles. J Clin Endocrinol Metab. 2004;89(11):5742–52.
12. Haouzi D, Assou S, Mahmoud K, Tondeur S, Reme T, Hedon B, et al. Gene expression profile of human endometrial receptivity: comparison between natural and stimulated cycles for the same patients. Hum Reprod. 2009;24(6):1436–45.
13. Tuckerman E, Mariee N, Prakash A, Li TC, Laird S. Uterine natural killer cells in peri-implantation endometrium from women with repeated implantation failure after IVF. J Reprod Immunol. 2010;87(1–2):60–6.
14. Tuckerman E, Laird SM, Prakash A, Li TC. Prognostic value of the measurement of uterine natural killer cells in the endometrium of women with recurrent miscarriage. Hum Reprod. 2007;22(8):2208–13.
15. Germeyer A, Savaris RF, Jauckus J, Lessey B. Endometrial beta3 integrin profile reflects endometrial receptivity defects in women with unexplained recurrent pregnancy loss. Reprod Biol Endocrinol. 2014;12:53.
16. Coughlan C, Sinagra M, Ledger W, Li TC, Laird S. Endometrial integrin expression in women with recurrent implantation failure after in vitro fertilization and its relationship to pregnancy outcome. Fertil Steril. 2013;100(3):825–30.
17. Mariee N, Li TC, Laird SM. Expression of leukaemia inhibitory factor and interleukin 15 in endometrium of women with recurrent implantation failure after IVF; correlation with the number of endometrial natural killer cells. Hum Reprod. 2012;27(7):1946–54.
18. Xu B, Sun X, Li L, Wu L, Zhang A, Feng Y. Pinopodes, leukemia inhibitory factor, integrin-beta3, and mucin-1 expression in the peri-implantation endometrium of women with unexplained recurrent pregnancy loss. Fertil Steril. 2012;98(2):389–95.
19. Karaer A, Cigremis Y, Celik E, Urhan GR. Prokineticin 1 and leukemia inhibitory factor mRNA expression in the endometrium of women with idiopathic recurrent pregnancy loss. Fertil Steril. 2014;102(4):1091–5 e1.
20. Brosens JJ, Salker MS, Teklenburg G, Nautiyal J, Salter S, Lucas ES, et al. Uterine selection of human embryos at implantation. Sci Rep. 2014;4:3894.
21. Teklenburg G, Salker M, Heijnen C, Macklon NS, Brosens JJ. The molecular basis of recurrent pregnancy loss: impaired natural embryo selection. Mol Hum Reprod. 2010;16(12):886–95.

22. Macklon NS, Brosens JJ. The human endometrium as a sensor of embryo quality. Biol Reprod. 2014;91(4):98.
23. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. Nat Immunol. 2010;11(9):785–97.
24. Mohlin FC, Mercier E, Fremeaux-Bacchi V, Liszewski MK, Atkinson JP, Gris JC, et al. Analysis of genes coding for CD46, CD55, and C4b-binding protein in patients with idiopathic, recurrent, spontaneous pregnancy loss. Eur J Immunol. 2013;43(6):1617–29.
25. Salmon JE, Heuser C, Triebwasser M, Liszewski MK, Kavanagh D, Roumenina L, et al. Mutations in complement regulatory proteins predispose to preeclampsia: a genetic analysis of the PROMISSE cohort. PLoS Med. 2011;8(3):e1001013.
26. Ruan YC, Chen H, Chan HC. Ion channels in the endometrium: regulation of endometrial receptivity and embryo implantation. Hum Reprod Update. 2014;20(4):517–29.
27. Thie M, Denker H-W. In vitro studies on endometrial adhesiveness for trophoblast: cellular dynamics in uterine epithelial cells. Cells Tissues Organs. 2002;172(3):237–52.
28. Nawy T. Single-cell sequencing. Nat Methods. 2014;11(1):18.
29. Lasken RS. Single-cell genomic sequencing using multiple displacement amplification. Curr Opin Microbiol. 2007;10(5):510–6.
30. Tang F, Barbacioru C, Wang Y, Nordman E, Lee C, Xu N, et al. mRNA-Seq whole-transcriptome analysis of a single cell. Nat Methods. 2009;6(5):377–82.
31. Shintaku H, Nishikii H, Marshall LA, Kotera H, Santiago JG. On-chip separation and analysis of RNA and DNA from single cells. Anal Chem. 2014;86(4):1953–7.
32. Zhang K, Martiny AC, Reppas NB, Barry KW, Malek J, Chisholm SW, et al. Sequencing genomes from single cells by polymerase cloning. Nat Biotechnol. 2006;24(6):680–6.
33. Chiou PY, Ohta AT, Wu MC. Massively parallel manipulation of single cells and microparticles using optical images. Nature. 2005;436(7049):370–2.
34. Hosic S, Murthy SK, Koppes AN. Microfluidic sample preparation for single cell analysis. Anal Chem. 2016;88(1):354–80.
35. Papalexi E, Satija R. Single-cell RNA sequencing to explore immune cell heterogeneity. Nat Rev Immunol. 2018;18(1):35–45.
36. Baslan T, Hicks J. Unravelling biology and shifting paradigms in cancer with single-cell sequencing. Nat Rev Cancer. 2017;17(9):557–69.
37. Telenius H, Carter NP, Bebb CE, Nordenskjold M, Ponder BA, Tunnacliffe A. Degenerate oligonucleotide-primed PCR: general amplification of target DNA by a single degenerate primer. Genomics. 1992;13(3):718–25.
38. Nakamura T, Yabuta Y, Okamoto I, Aramaki S, Yokobayashi S, Kurimoto K, et al. SC3-seq: a method for highly parallel and quantitative measurement of single-cell gene expression. Nucleic Acids Res. 2015;43(9):e60.
39. Zong C, Lu S, Chapman AR, Xie XS. Genome-wide detection of single-nucleotide and copy-number variations of a single human cell. Science. 2012;338(6114):1622–6.
40. Vassena R, Eguizabal C, Heindryckx B, Sermon K, Simon C, van Pelt AM, et al. Stem cells in reproductive medicine: ready for the patient? Hum Reprod. 2015;30(9):2014–21.
41. Krjutskov K, Katayama S, Saare M, Vera-Rodriguez M, Lubenets D, Samuel K, et al. Single-cell transcriptome analysis of endometrial tissue. Hum Reprod. 2016;31(4):844–53.
42. Weiling F. Historical study: Johann Gregor Mendel 1822-1884. Am J Med Genet. 1991;40(1):1–25; discussion 6.
43. Waddington CH. The epigenotype. 1942. Int J Epidemiol. 2012;41(1):10–3.
44. Berger SL, Kouzarides T, Shiekhattar R, Shilatifard A. An operational definition of epigenetics. Genes Dev. 2009;23(7):781–3.
45. Kanherkar RR, Bhatia-Dey N, Csoka AB. Epigenetics across the human lifespan. Front Cell Dev Biol. 2014;2:49.
46. Horsthemke B, Ludwig M. Assisted reproduction: the epigenetic perspective. Hum Reprod Update. 2005;11(5):473–82.
47. Das L, Parbin S, Pradhan N, Kausar C, Patra SK. Epigenetics of reproductive infertility. Front Biosci (Schol Ed). 2017;9:509–35.

48. Koukoura O, Sifakis S, Spandidos DA. DNA methylation in the human placenta and fetal growth (review). Mol Med Rep. 2012;5(4):883–9.
49. Houshdaran S, Zelenko Z, Irwin JC, Giudice LC. Human endometrial DNA methylome is cycle-dependent and is associated with gene expression regulation. Mol Endocrinol. 2014;28(7):1118–35.
50. Yamagata Y, Asada H, Tamura I, Lee L, Maekawa R, Taniguchi K, et al. DNA methyltransferase expression in the human endometrium: down-regulation by progesterone and estrogen. Hum Reprod. 2009;24(5):1126–32.
51. Kukushkina V, Modhukur V, Suhorutsenko M, Peters M, Magi R, Rahmioglu N, et al. DNA methylation changes in endometrium and correlation with gene expression during the transition from pre-receptive to receptive phase. Sci Rep. 2017;7(1):3916.
52. Rahnama F, Thompson B, Steiner M, Shafiei F, Lobie PE, Mitchell MD. Epigenetic regulation of E-cadherin controls endometrial receptivity. Endocrinology. 2009;150(3):1466–72.
53. Wang L, Tan YJ, Wang M, Chen YF, Li XY. DNA methylation inhibitor 5-Aza-2'-deoxycytidine modulates endometrial receptivity through upregulating HOXA10 expression. Reprod Sci. 2019;26(6):839–46.
54. Xiong Y, Wang J, Liu L, Chen X, Xu H, Li TC, et al. Effects of high progesterone level on the day of human chorionic gonadotrophin administration in in vitro fertilization cycles on epigenetic modification of endometrium in the peri-implantation period. Fertil Steril. 2017;108(2):269–76 e1.
55. Strahl BD, Allis CD. The language of covalent histone modifications. Nature. 2000;403(6765):41–5.
56. Jenuwein T, Allis CD. Translating the histone code. Science. 2001;293(5532):1074–80.
57. Thiagalingam S, Cheng KH, Lee HJ, Mineva N, Thiagalingam A, Ponte JF. Histone deacetylases: unique players in shaping the epigenetic histone code. Ann N Y Acad Sci. 2003;983:84–100.
58. Uchida H, Maruyama T, Arase T, Ono M, Nagashima T, Masuda H, et al. Histone acetylation in reproductive organs: significance of histone deacetylase inhibitors in gene transcription. Reprod Med Biol. 2005;4(2):115–22.
59. Uchida H, Maruyama T, Nagashima T, Ono M, Masuda H, Arase T, et al. Human endometrial cytodifferentiation by histone deacetylase inhibitors. Hum Cell. 2006;19(1):38–42.
60. Krusche CA, Vloet AJ, Classen-Linke I, von Rango U, Beier HM, Alfer J. Class I histone deacetylase expression in the human cyclic endometrium and endometrial adenocarcinomas. Hum Reprod. 2007;22(11):2956–66.
61. Estella C, Herrer I, Atkinson SP, Quinonero A, Martinez S, Pellicer A, et al. Inhibition of histone deacetylase activity in human endometrial stromal cells promotes extracellular matrix remodelling and limits embryo invasion. PLoS One. 2012;7(1):e30508.
62. Maass N, Biallek M, Rosel F, Schem C, Ohike N, Zhang M, et al. Hypermethylation and histone deacetylation lead to silencing of the maspin gene in human breast cancer. Biochem Biophys Res Commun. 2002;297(1):125–8.
63. Esteller M. Non-coding RNAs in human disease. Nat Rev Genet. 2011;12(12):861–74.
64. Alexander RP, Fang G, Rozowsky J, Snyder M, Gerstein MB. Annotating non-coding regions of the genome. Nat Rev Genet. 2010;11(8):559–71.
65. Ambros V. The functions of animal microRNAs. Nature. 2004;431(7006):350–5.
66. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116(2):281–97.
67. Creighton CJ, Benham AL, Zhu H, Khan MF, Reid JG, Nagaraja AK, et al. Discovery of novel microRNAs in female reproductive tract using next generation sequencing. PLoS One. 2010;5(3):e9637.
68. Lam EW, Shah K, Brosens JJ. The diversity of sex steroid action: the role of microRNAs and FOXO transcription factors in cycling endometrium and cancer. J Endocrinol. 2012;212(1):13–25.

69. Ramon LA, Braza-Boils A, Gilabert-Estelles J, Gilabert J, Espana F, Chirivella M, et al. microRNAs expression in endometriosis and their relation to angiogenic factors. Hum Reprod. 2011;26(5):1082–90.
70. Revel A, Achache H, Stevens J, Smith Y, Reich R. MicroRNAs are associated with human embryo implantation defects. Hum Reprod. 2011;26(10):2830–40.
71. Petracco R, Grechukhina O, Popkhadze S, Massasa E, Zhou Y, Taylor HS. MicroRNA 135 regulates HOXA10 expression in endometriosis. J Clin Endocrinol Metab. 2011;96(12):E1925–33.
72. McGrath J, Solter D. Completion of mouse embryogenesis requires both the maternal and paternal genomes. Cell. 1984;37(1):179–83.
73. Haig D, Graham C. Genomic imprinting and the strange case of the insulin-like growth factor II receptor. Cell. 1991;64(6):1045–6.
74. Moore T, Haig D. Genomic imprinting in mammalian development: a parental tug-of-war. Trends Genet. 1991;7(2):45–9.
75. Regha K, Latos PA, Spahn L. The imprinted mouse Igf2r/Air cluster--a model maternal imprinting system. Cytogenet Genome Res. 2006;113(1–4):165–77.
76. Tanos V, Ariel I, Prus D, De-Groot N, Hochberg A. H19 and IGF2 gene expression in human normal, hyperplastic, and malignant endometrium. Int J Gynecol Cancer. 2004;14(3):521–5.
77. Lee RS, Depree KM, Davey HW. The sheep (Ovis aries) H19 gene: genomic structure and expression patterns, from the preimplantation embryo to adulthood. Gene. 2002;301(1–2):67–77.
78. Lyon MF. Gene action in the X-chromosome of the mouse (Mus musculus L.). Nature. 1961;190:372–3.
79. Yan PS, Chen CM, Shi H, Rahmatpanah F, Wei SH, Huang TH. Applications of CpG island microarrays for high-throughput analysis of DNA methylation. J Nutr. 2002;132(8 Suppl):2430S–4S.
80. Schilling E, Rehli M. Global, comparative analysis of tissue-specific promoter CpG methylation. Genomics. 2007;90(3):314–23.
81. Down TA, Rakyan VK, Turner DJ, Flicek P, Li H, Kulesha E, et al. A Bayesian deconvolution strategy for immunoprecipitation-based DNA methylome analysis. Nat Biotechnol. 2008;26(7):779–85.
82. Butcher LM, Beck S. AutoMeDIP-seq: a high-throughput, whole genome, DNA methylation assay. Methods. 2010;52(3):223–31.
83. Begemann M, Leisten I, Soellner L, Zerres K, Eggermann T, Spengler S. Use of multilocus methylation-specific single nucleotide primer extension (MS-SNuPE) technology in diagnostic testing for human imprinted loci. Epigenetics. 2012;7(5):473–81.
84. Konishi Y, Hayashi H, Suzuki H, Yamamoto E, Sugisaki H, Higashimoto H. Comparative analysis of methods to determine DNA methylation levels of a tumor-related microRNA gene. Anal Biochem. 2015;484:66–71.
85. Sandmann T, Jakobsen JS, Furlong EE. ChIP-on-chip protocol for genome-wide analysis of transcription factor binding in Drosophila melanogaster embryos. Nat Protoc. 2006;1(6):2839–55.
86. O'Neill LP, Turner BM. Immunoprecipitation of chromatin. Methods Enzymol. 1996;274:189–97.
87. Zhu J, Fu H, Wu Y, Zheng X. Function of lncRNAs and approaches to lncRNA-protein interactions. Sci China Life Sci. 2013;56(10):876–85.
88. Liu CG, Spizzo R, Calin GA, Croce CM. Expression profiling of microRNA using oligo DNA arrays. Methods. 2008;44(1):22–30.

Chapter 8
Endometrial Gene Expression for HHV-6

Carolyn Coulam

Introduction

The human endometrium is comprised of glandular epithelium, stroma, and leukocytes and is regenerated nearly 450 times in a woman's lifetime [1, 2]. The primary function of the endometrium is to provide an attachment site and a source of nourishment to an early embryo. Genome-wide analyses have yielded insight into mRNA expression changes during the natural endometrial cycle [3], and growing evidence supports the concept of a receptive gene expression profile which may be disrupted in patients experiencing recurrent implantation failure [4]. The causes of implantation failure are diverse and include both embryonic and uterine factors such as uterine abnormalities, hormonal or metabolic disorders, thrombophilias, immunological factors, and infections [5]. Subclinical endometrial infection or inflammation has been suggested to play a role in implantation failure after in vitro fertilization (IVF), spontaneous abortion, and preterm birth [6]. Diagnosis of such infections has been limited by the use of culture-based technologies [7]. New investigative technologies including DNA fingerprinting, microarrays and genome sequencing have empowered the study of metagenomics by analyzing organisms contained in samples based on their genetic information. While the most commonly reported example a pathology caused by an altered endometrial microbiota has been chronic endometritis [8], more recent publications have implicated human herpesvirus 6 (HHV-6) uterine infection as an important factor for the development of unexplained female infertility [9, 10] including recurrent implantation failure after IVF [10].

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Human Herpesvirus 6 (HHV-6)

Human herpesvirus 6 (HHV-6) was the sixth herpesvirus discovered. Its structure is shown in Fig. 8.1. HHV-6 is a set of two closely related herpesviruses known as HHV-6A and HHV-6B. Since herpesviruses rely on their hosts for replication, it is not in their interest to kill or seriously injure their host. Like the other herpesviruses—Epstein-Barr virus, chickenpox, and herpes simplex—HHV-6 establishes lifelong latency and can become reactivated later in life (Fig. 8.2). Primary infection with HHV-6 occurs within the first 2 years of life and is usually associated with a febrile illness and classic skin manifestations of roseola infantum [11]. During acute infection, replication occurs in lymphocytes, macrophages, histiocytes, endothelial cells, and epithelial cells. After primary infection with HHV-6, the viral genome persists in peripheral blood mononuclear cells and salivary glands, and viral DNA can be routinely detected in saliva. The exact mode by which HHV-6 is transmitted has yet to be elucidated fully. Children likely acquire infection through contact with adult caretakers' saliva or from older siblings. DNA restriction enzyme profile studies have shown mothers' isolates to be genetically similar to their infants'. Vertical transmission of

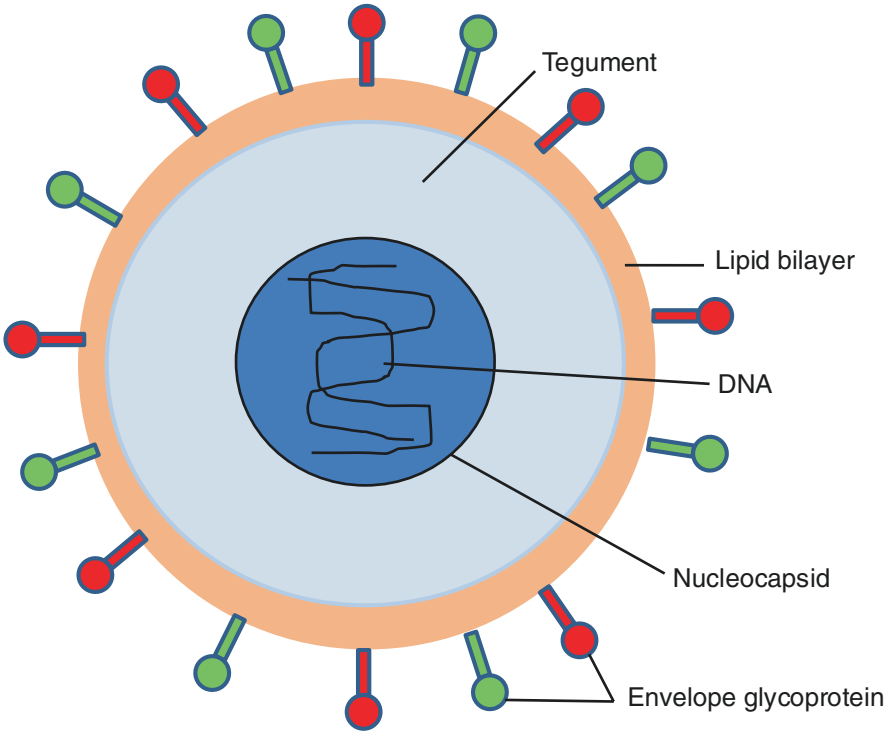

Fig. 8.1 Structure of human herpesvirus 6 (HHV-6) depicted by HHV-6 foundation

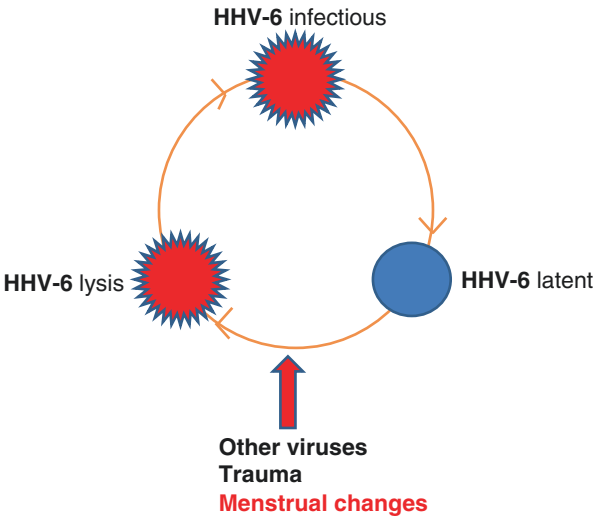

Fig. 8.2 Replication phases of HHV-6

HHV-6 has been documented; however, this mode of transmission represents only 1–2% of all births [12]. The cellular receptor for HHV-6 is CD46, a 52- to 57-kD type 1 transmembrane glycoprotein expressed on the surface of all cells [13]. The cell attachment protein of HHV-6 has not been identified. Entry occurs through receptor-mediated endocytosis. Subsequent stages of viral replication include latent and lytic phases (Fig. 8.2).

A replication phase that yields progeny virions is referred to as the lytic cycle of infection. This genome is packaged into a viral coat and released from the cell. This process of viral release from the cells results in lysis of cells, and hence, it is termed the "lytic phase." In another phase of replication, referred to as the "latent phase," the virus lays dormant. Latent infections have the ability to be reactivated into a lytic form. The ability to move back and forth from latent to lytic infections helps the virus spread from infected individuals to uninfected individuals. Reactivation is the mechanism whereby a latent virus that has infected a host cell switches to a lytic stage, undergoing productive viral replication and allowing the virus to spread. HHV-6 remains latent unless the immune system is stressed or compromised, at which time the virus may reactivate. Viral reactivation is associated with several stress factors including viral infection (with other viruses), nerve trauma, and physiologic and physical changes (e.g., fever, exposure to sunlight, and menstruation) [14].

HHV-6 and Infertility

Viral infections have been considered as possible environmental factors in human infertility [15]. In particular, herpesviruses have been implicated in male infertility [16]. Data exist suggesting that HHV-6 can also infect and replicate in the female genital tract [17–22]. HHV-6 DNA has been detected in genital tract secretions from

pregnant and nonpregnant women with several studies reporting low-level HHV-6 shedding from the genital tract in up to 25% of women [18–20]. These data suggest that the female genital tract may be a secondary site for HHV-6 infection, although the impact of herpes infection on fertility has only recently been reported [9] and confirmed [10].

Since the discovery of HHV-6 over 30 years ago, the viral variants, HHV-6A and HHV-6B, have been recognized as different viral species [23] with important differences in cell tropism between them. HHV-6A but not HHV-6B has been found in endometrial epithelial cells from women with unexplained infertility [9] suggesting that HHV-6 variants use different cell receptors. HHV-6A uses CD46, a ubiquitous molecule present on all cell types [13], as a receptor, while HHV-6B uses CD134, expressed mainly on activated Treg lymphocytes [24].

Two studies have confirmed an association between HHV-6 expression in endometrial biopsies and infertility [9, 10] (Fig. 8.3). Marci et al. [6] first reported HHV-6 DNA present in 43% of endometrial cells from women with unexplained infertility, whereas no fertile control women harbored the virus. Subsequently, Coulam et al. [10] demonstrated a similar prevalence (37%) of positive HHV-6 in their endometrial biopsies of another cohort of infertile women (those experiencing recurrent implantation failure after IVF/ET) and 0% in the fertile controls. Both studies also found variations in uterine immune markers between HHV-6+ and HHV-6- samples. The Coulam study [10] also measured expression levels of CD3e, CD19, CD16a, CD16b, and CD57 and found significantly higher levels of neutrophil-specific CD16b mRNA in HHV-6+ endometrial samples compared to HHV-6- samples [10].

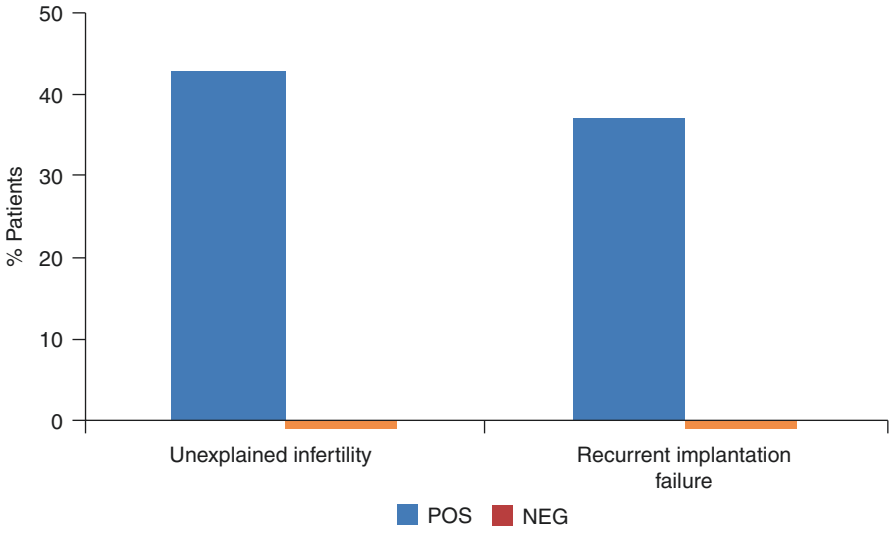

Fig. 8.3 Prevalence of endometrial biopsies positive for activated HHV-6 among women experiencing unexplained infertility and recurrent implantation failure after in vitro fertilization procedures. (Marci et al. [9] and Coulam et al. [10])

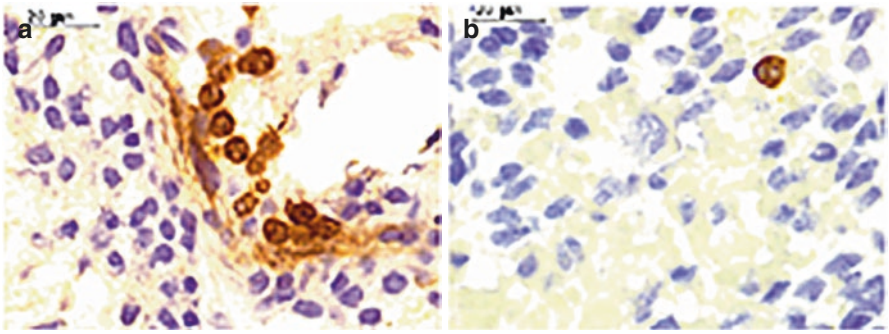

Fig. 8.4 Expression of neutrophil-specific CD16b mRNA in HHV-6 positive endometrial biopsy samples obtained from women with recurrent implantation failure (**a**) in comparison to HIV-6 negative samples from controls (**b**). (Adapted from Ref. [10])

In addition, immunostaining of CD16b revealed an increased presence of neutrophils in the HHV-6 positive tissue (Fig. 8.4). Rizzo and DiLuca (2018) demonstrated HLA G and HLA E molecules, both necessary for local immunomodulation of maternal immune system rejection of invading trophoblast, were not expressed on the surface of endometrial epithelial cells from HHV-6-positive women [25]. Taken together, these data support the concept that HHV-6 infection in the uterus of some women can contribute to infertility. Of interest, a correlation between the level of serum estradiol and presence of an HHV-6A infection ($p = 0.02$) was found, and the virus was active only during the secretory phase of the menstrual cycle when estradiol levels were highest [9]. Estradiol has also been shown to cause HSV1 reactivation [26].

Methods of Detection of Uterine HHV-6

The aim of virological diagnosis is first to provide proof of HHV-6 infection, i.e., the presence of the virus(es) in an investigated endometrial biopsy. Second, it is necessary to define the status of this infection as latent or active. Technologies used to identify HHV-6 in endometrial biopsies have included immunofluorescence assays and mRNA analyses.

Immunofluorescence Assay

HHV-6 DNA presence and the load are analyzed by PCR and real-time quantitative (qPCR) specific for the U94 and U42 genes [27]. HHV-6 variant A or B identification is obtained by restriction enzyme digestion with HindIII enzyme of the U31 nested PCR amplification product [27]. Digestion products are then visualized on

ethidium bromide-stained agarose gel after migration. Immunofluorescence for HHV-6 antigen expression is performed with a mouse monoclonal antibodies (mAb) directed against p41 and IE2 (early antigen) and glycoprotein gp116 (late antigen) of HHV-6 A and B (ABI, Columbia, MD, USA) [27].

mRNA Expression Analysis

Total RNA transcribed into cDNA is used in qRT-PCR with TaqMan assay designed with HHV-6A/B U94 primers [28]. B2M TaqMan endogenous control is included in qRT-PCR to obtain relative abundance values for viral U94 expression for samples where U94 amplification was recorded (CT values $\leq$28) using the comparative quantification method (ΔCT) CT.

Concluding Remarks

Human herpesvirus 6 (HHV-6) is a widespread herpesvirus that has effectively colonized the vast majority of the human population. It is spread with great efficiency during early childhood and establishes a persistent lifelong relationship with its host. Primary virus infection is frequently manifested as classical roseola (exanthem subitum). HHV-6 exhibits a wide cell tropism. In adults, like other herpesviruses, viral reactivation can occur. While the majority of outcomes of such reactivations involve skin and neurons, recent reports provide evidence of HHV-6 involvement in the reproductive system. The independent findings of Marci et al. [9] and Coulam et al. [10] describing a similar prevalence of HHV-6 in women who are unable to conceive (43% vs. 37%), as well as an absence of HHV-6 in the endometrium of fertile controls (both 0%), are compelling suggestions of a role of HHV-6 in the etiology of infertility [9, 10]. Clinical trials using an anti-HHV-6 agent to treat women demonstrating activated HHV-6 in their endometrial biopsies and experiencing unexplained infertility or recurrent implantation failure will be very useful in determining whether the virus is a causative factor behind female infertility. If HHV-6 is found to cause or contribute to RIF and/or unexplained infertility, treating the virus could improve the chance of successful pregnancy outcomes, and reduce the emotional and financial toll of infertility, in up to 40% of women with these conditions.

References

1. Milne SA, Critchley HO, Drudy TA, Kelly RW, Baird DT. Perivascular interleukin-8 messenger ribonucleic acid expression in human endometrium varies across the menstrual cycle and in early pregnancy decidua. J Clin Endocrinol Metab. 1999;84:2563–7.

2. Seli MD, Senturk LM, Bahtiyar OM, Kayisli UA, Arici A. Expression of aminopeptidase N in human endometrium and regulation of its activity by estrogen. Fertil Steril. 2001;75:1172–6.
3. Díaz-Gimeno P, Horcajadas JA, Martínez-Conejero JA, Esteban FJ, Alamá P, Pellicer A, et al. A genomic diagnostic tool for human endometrial receptivity based on the transcriptomic signature. Fertil Steril. 2011;95:50–60.
4. Talbi S, Hamilton AE, Vo KC, Tulac S, Overgaard MT, Dosiou C, et al. Molecular phenotyping of human endometrium distinguishes menstrual cycle phases and underlying biological processes in normo-ovulatory women. Endocrinology. 2006;147:1097–121.
5. Timeva T, Shterev A, Kyurkchiev S. Recurrent implantation failure: the role of the endometrium. J Repro Infertil. 2014;15:173–83.
6. Romero R, Espinoza J, Mazor M. Can endometrial infection/inflammation explain implantation failure, spontaneous abortion, and preterm birth after in vitro fertilization? Fertil Steril. 2004;82:799–804.
7. Rhoads DD, Cox SB, Rees EJ, Sun Y, Wolcott RD. clinical identification of bacteria in human chronic wound infections: culturing vs. 16S ribosomal DNA sequencing. BMC Infect Dis. 2012;12:321–6.
8. Cicinelli E, Matteo M, Tinelli R, Lepera A, Alfonso R, Indraccolo U, et al. Prevalence of chronic endometritis in repeated unexplained implantation failure and the IVF success rate after antibiotic therapy. Hum Reprod. 2015;30:323–30.
9. Marci R, Gentili V, Bortolotti D, Lo Monte G, Caselli E, Bolzan S, et al. Presence of HHV-6A in endometrial epithelial cells from women with primary unexplained infertility. PLoS One. 2016;11:e0158304.
10. Coulam CB, Bilal M, Salazar Garcia MD, Katukurundage D, Elazzamy H, Fernandez EF, et al. Prevalence of HHV-6 in endometrium from women with recurrent implantation failure. Am J Reprod Immunol. 2018;80:e12862.
11. Yamanishi K, Okuno T, Shiraki K, Takahashi M, Kondo T, Asano Y, et al. Identification of human herpesvirus-6 as a causal agent for exanthem subitum. Lancet. 1988;1:1065–7.
12. De Bolle L, Naesens L, De Clercq E. Update on human herpesvirus 6 biology, clinical features, and therapy. Clin Microbiol Rev. 2005;18:217–45.
13. Santoro F, Kennedy PE, Locatelli G, Malnati MS, Berger EA, Lusso P. CD46 is a cellular receptor for human herpesvirus 6. Cell. 1999;99:817–27.
14. Cook SD, Paveloff MJ, Doucet JJ, Cottingham AJ, Sedarati F, Hill JM. Ocular herpes simplex virus reactivation in mice latently infected with latency-associated transcript mutants. Investig Ophthalmol. 1991;32:1558–61.
15. Baecher-Lind LE, Miller W, Wilcox AJ. Infectious disease and reproductive health: a review. Obstet Gynecol Surv. 2010;65:53–65.
16. Naumenko V, Tyulenev Y, Kurilo L, Shileiko L, Sorokina T, Evdokimov V, et al. Detection and quantification of human herpes viruses types 4–6 in sperm samples of patients with fertility disorders and chronic inflammatory urogenital tract diseases. Androl. 2014;2:687–94.
17. Wang H, Chen M, Berneman ZN, Delgado G, DiPaolo JA. Detection of human herpesvirus-6 in paraffin-embedded tissue of cervical cancer by polymerase chain reaction. J Virol Methods. 1994;47:297–305.
18. Ohashi M, Yoshikawa T, Ihira M, Suzuki K, Suga S, Tada S, et al. Reactivation of human herpesvirus 6 and 7 in pregnant women. J Med Virol. 2002;67:354–8.
19. Okuno T, Oishi H, Hayashi K, Nonogaki M, Tanaka K, Yamanishi K. Human herpesviruses 6 and 7 in cervixes of pregnant women. J Clin Microbiol. 1995;33:1968–70.
20. Baillargeon J, Piper J, Leach CT. Epidemiology of human herpesvirus 6 (HHV-6) infection in pregnant and nonpregnant women. J Clin Virol. 2002;16:149–57.
21. Maeda T, Okuno T, Hayashi K, Nagata M, Ueda M, Terashima K, et al. Outcomes of infants whose mothers are positive for human herpesvirus-6 DNA within the genital tract in early gestation. Acta Paediatr Jpn. 1997;39:653–7.
22. Leach CT, Newton ER, McParlin S, Jenson HB. Human herpesvirus 6 infection of the female genital tract. J Infect Dis. 1994;169:1281–3.

23. Aubin JT, Collandre H, Candotti D, Ingrand D, Rouzioux C, Burgard M, et al. Several groups among human herpesvirus 6 strains can be distinguished by Southern blotting and polymerase chain reaction. J Clin Microbiol. 1991;29:367–72.
24. Tang H, Serada S, Kawabata A, Ota M, Hayashi E, Naka T, et al. CD134 is a cellular receptor specific for human herpesvirus-6B entry. Proc Natl Acad Sci U S A. 2013;110:9096–9.
25. Rizzo R, DiLuca D. Endometrium infection by human herpes virus 6A: implication in female idiopathic infertility. Fertil Steril. 2018;110:e128.
26. Miguel RDV, Sheridan BS, Harvey SAK, Schreiner RS, Hendricks RL, Cherpes TL. 17-β estradiol promotion of herpes simplex virus type 1 reactivation is estrogen receptor dependent. J Virol. 2010;84:565–72.
27. Caselli E, Zatelli M, Rizzo R, Benedetti S, Martorelli D, Trasforini G, et al. Virologic and immunologic evidence supporting an association between HHV-6 and Hashimoto's thyroiditis. PLoS Pathog. 2012;8:e1002951.
28. Davies ML, Dambaeva SV, Katukurundage D, Repak M, Gilman-Sachs A, Kwak-Kim J, Beaman KD. Predicting NK cell subsets using gene expression levels in peripheral blood and endometrial biopsy specimens. Am J Reprod Immunol. 2017;78:e12730.

Chapter 9
Infection and Endometrial Gene Expression: HHV-6 and Infertility

Roberta Rizzo

Endometrial Microbiome

In contrast to the conventional wisdom about human endometrium as a sterile tissue, several recent studies suggest the endometrial presence of resident populations of microorganisms, which reaches only a 30% of concordance with those of the cervical-vaginal flora [1]. The most frequent infectious agents detected at the endometrial level are common bacteria, accounting for 69% of all cases. In particular, streptococci were found in 27% of cases and bacteria from intestinal flora (*Enterococcus faecalis* and *Escherichia coli*) in 31% of cases; *Ureaplasma urealyticum* was detected in 10% of cases and *Mycoplasma* in 0.2% of cases [2]. Early prospective studies evaluating the role of the endometrial microbial contamination suggested that positive microbiological endometrial culture, obtained from the tip of the transfer catheter in patients undergoing in vitro fertilization (IVF), had negative effects on implantation and pregnancy rates. In particular, *Enterobacteriaceae* spp., *Streptococcus* spp., *Staphylococcus* spp., *Escherichia coli*, and Gram-negative bacteria have been associated with decreased implantation rates and poor pregnancy outcomes [3, 4]. No consensus has been reached regarding the origin and genus of these pathogens and the mechanisms by which they might interfere with embryonic implantation.

Endometrial Virome

Although few virome studies have been performed on the female reproductive tract, a series of researches has clearly shown that viral infection might alter the normal

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mechanism mediating the interaction between the placenta, maternal tissues, and the normal microbiota present at the implantation site. These changes most likely would induce damage to the tolerance status leading to fertility-associated problems. It has been proposed that endogenous retroviruses mediate regulatory evolution and that this is an important mechanism underlying the placental development [5, 6]. The suggestion is that retroviruses might integrate into the host germ cell genome and that viral activity regulates developmental genes responsible for establishing the ancestral trophoblast cell type. It is proposed that epigenetic aberrations could cause global endogenous retroviral repression and placental defects, such as pre-eclampsia, suggesting a central role of viruses in reproduction.

Endometrium and Herpes Virus Infection

Clinical maternal viral infection is not uncommon in pregnancy, and when it occurs, either as a primary infection or as reactivation with viremia, the fetus is placed at risk of infection through the transplacental transmission. Subclinical maternal viral infection must be common as there is nucleic acid evidence of viral exposure in 40–44% of healthy newborns [7]. It has been postulated that fetal viral infection in utero may increase the risk of adverse pregnancy outcomes (APOs), such as pregnancy-induced hypertensive disorders (PIHD), birth weight <tenth percentile (small for gestational age, SGA) and preterm birth (PTB) [8].

Herpesviruses (including human cytomegalovirus (HCMV), herpes simplex viruses (HSV) 1 and 2, varicella-zoster virus (VZV), Epstein-Barr virus (EBV), and human herpesviruses (HHV) 6, 7, and 8) and enteroviruses are capable of crossing the placenta and causing in utero infection [9, 10] that could potentially contribute directly or indirectly to APOs. It has been shown that HCMV infection impairs critical aspects of cytotrophoblast function, which may explain some of the deleterious effects of this virus on pregnancy outcome [11]. The likelihood of maternal infection resulting in fetal infection varies according to the specific virus, whether the infection is primary or recurrent, and the gestational age of the fetus at the time of infection. Once the infection has crossed the placenta into the fetal circulation, there is the potential for adverse fetal outcomes. These can be caused by the infectious agent directly or indirectly through the fetal and/or placental inflammatory response to infection, where proinflammatory cytokines may adversely affect the developing brain and perhaps also placental function [12].

HHV-6

Human herpesvirus 6 (HHV-6) is a betaherpesvirus that exists as two closely related variants, HHV-6A and HHV-6B. HHV-6A has not been etiologically linked to any disease; HHV-6B is the causative agent of exanthema subitum (ES), a childhood

disease characterized by high fever and a mild skin rash, occasionally complicated by seizures or encephalitis. The term HHV-6 remains in usage and collectively refers to the two species.

HHV-6 exhibits wide cell tropism in vivo and, as with other herpesviruses, induces a lifelong latent infection in humans. HHV-6 preferentially replicates in activated CD4+ T lymphocytes [13, 14] and uses specific cell receptors permitting virus anchorage to the cell surface: HHV-6A uses CD46, a regulator of complement activation expressed on all nucleated cells, while CD134 (also called OX40), a member of the tumor necrosis factor (TNF) receptor superfamily present only on activated T lymphocytes, functions as a specific entry receptor for HHV-6B [15, 16]. HHV-6 can infect in vitro, in addition to CD4+ T lymphocytes, CD8+ T lymphocytes (only with HHV-6A), fibroblasts, natural killer (NK) cells, liver cells, epithelial cells, endothelial cells, microglial cells, astrocytes, and oligodendrocytes [13, 17–24]. The in vivo host tissue range appears to be broader than expected from in vitro studies and includes the brain, salivary glands, tonsils, kidneys, lymph nodes, liver, heart, gastrointestinal tract, lungs, and monocytes/macrophages [13, 25–27]. The preferential sites for latency are suspected to be monocytes/macrophages, bone marrow progenitors and central nervous system cells [28–30]. Fecal-oral spread, a common transmission route among young children, has not been documented for HHV-6, although stool specimens were found positive for HHV-6 DNA [31]. Thus, the most probable route for HHV-6 transmission (at least for the B variant) is through saliva [32].

Interestingly, HHV-6 DNA can be integrated into the subtelomeric region of host chromosomes as an inherited chromosomally integrated HHV-6 (iciHHV-6), present in about 1% of the general population passed through generations via vertical transmission [33].

HHV-6 and Pregnancy

HHV-6 can be found in the female genital tract [34] with low rates of HHV-6 shedding in the genital tract in both pregnant and nonpregnant women [35], making unlikely a perinatal transmission [36] (Table 9.1).

The detection of HHV-6 DNA in cord blood specimens of healthy newborns in the absence of serum immunoglobulin M (IgM) and fetuses following spontaneous abortion supports the possibility of intrauterine transmission [37]. The incidence of vertical HHV-6 transmission is about 1–2% of all births and is not related to intrauterine HCMV transmission [38, 39]. Severe neurological complications were observed after intrauterine HHV-6 infection [40]. During months 3–8 of pregnancy, HHV-6 has been found in the blood of 41–44% of women, and it has likewise been found in 25% of samples during delivery and 24% of samples from nonpregnant women [39]. HHV-6 has been mainly detected in the cervixes of pregnant women (7.5–19.4%) and placental samples [41, 42], with HHV-6B detection in nonpregnant women, whereas HHV-6A accounted for 17% of positive cases among pregnant

Table 9.1 HHV-6 infection in pregnancy

Author	Sample	HHV-6 results	Conclusions	References
Leach et al.	345 pregnant women 224 nonpregnant women	2% of pregnant women had HHV-6 DNA positive 3.7% of nonpregnant women had HHV-6 DNA positive	HHV-6 is present in the female genital tract	[34]
Maeda et al.	110 pregnant women	25.5% of pregnant women had HHV-6 DNA positive	HHV-6 is present in the female genital tract	[36]
Adams et al.	305 newborns	1.6% of newborns had HHV-6 DNA positive	Congenital infection	[37]
Boutolleau et al.	54 amniotic fluids	0% of amniotic fluid samples had HHV-6 DNA positive	No HHV-6 in amniotic fluids	[38]
Dahl et al.	104 pregnant women 107 women at delivery 211 cord blood samples	41–44% pregnant women had HHV-6 DNA positive 25% of women at delivery had HHV-6 DNA positive 1% of cord blood samples had HHV-6 DNA positive	HHV-6 reactivation during pregnancy	[39]
Lanari et al.	One newborn	HHV-6 DNA was detected in cerebrospinal fluid	Association with neurological sequelae in vertical transmission	[40]
Okuno et al.	72 cervical swabs from pregnant women	19.4% of the samples were HHV-6 DNA positive	HHV-6 in the cervix during late pregnancy that may cause perinatal infection	[41]
Caserta et al.	104 pregnant women and 31 controls	7.5% of pregnant women had HHV-6 DNA positive One placental sample had active HHV-6 replication	Active placental infection HHV-6A in pregnant women.	[42]
Baillargeon et al.	345 pregnant women 224 nonpregnant women	2% of pregnant women had HHV-6 DNA positive 3% of nonpregnant women had HHV-6 DNA positive. Mainly HHV-6A	Both groups shed virus at low rates in the genital tract.	[35]
D'Agaro et al.	187 dried cord blood spots from HIV-positive	3.2% of babies born from HIV-positive mothers had HHV-6 DNA positive	HIV-infected mothers transmit HHV-6 to their babies more frequently than uninfected women	[43]

(continued)

Table 9.1 (continued)

Author	Sample	HHV-6 results	Conclusions	References
Hall et al.	5638 cord blood samples	1% of cord blood samples were HHV-6 DNA positive	Congenital HHV-6 infections occurred in 1% of births	[44]
Hall et al.	85 infants	43 infants had congenital HHV-6 infections 42 infants had postnatal infections 86% of congenital infections from ciHHV-6 14% of infants had transplacental infections	HHV-6 congenital infection results primarily from chromosomally integrated virus	[45]

women [42]. HHV-6 vaginal infection has been reported in the 2–18% of pregnant women [35, 36], during the first (3.7%) and the third trimester (12.2%), the 3.7% of nonpregnant women [35], and the 10% of women attending a sexually transmitted disease clinic [34]. The positivity for HHV-6 in the cord blood samples from healthy women was 1.0% [37, 39] [43], and in the placental tissues was about 9.1% (3.0% HHV-6A) [42].

Interestingly, the 1% of normal neonates present congenital HHV-6 infections, 10% of which were reactivated, and one-third of which were HHV-6A [44]. 86% of HHV-6 congenital infections were the result of ciHHV-6 [45].

HHV-6 and Pregnancy-Related Disorders

The presence of HHV-6 vaginal infections is associated with a twofold increased risk of idiopathic fetal loss [36], even if the newborns from women positive for HHV-6 vaginal infection (25.5%) were unaffected by the presence of the virus in both the blood and saliva [36] (Table 9.2). Interestingly, the presence of HHV-6 DNA was reported in hydrops fetalis [46, 47], where HHV-6A sequences were found in the heart, lung, liver, kidney, and placental tissue, and in Down's syndrome [46] and intrauterine fetal death, where HHV-6B was detected in the heart, kidney, liver, lung, and placental tissue [46]. Repeated miscarriages [48] and spontaneous abortions [49] have also been correlated with the presence of HHV-6 infection. The appearance of pityriasis rosea, a common, acute, self-limiting papulosquamous skin disorder linked to HHV-6 reactivation [50–53], during the first 15 weeks of pregnancy, increased the risk of spontaneous abortion [49].

The presence of HHV-6 infection impacts also on the health of the mother, increasing the risk of peripartum cardiomyopathy [54], pregnancy-induced

Table 9.2 HHV-6 infection in pregnancy-related disorders

Author	Sample	HHV-6 results	Conclusions	References
Maeda et al.	110 pregnant women	25.5% HHV-6 DNA-positive pregnant women	A twofold increase in the risk of idiopathic fetal loss	[36]
Al-Buhtori et al.	73 cases of fetal death 27 cases of elective termination of pregnancy	2 fetal deaths were HHV-6 DNA positive	The risk factor in cases of fetal loss	[46]
Ashshi et al.	8 fetuses with hydrops 10 non-hydropic dead fetuses	Two fetuses with hydrops and none of the non-hydropic dead fetuses were HHV-6A DNA positive	The risk factor in fetal hydrops	[47]
Revest et al.	Spontaneous abortion at 24 weeks of pregnancy	HHV-6 maternal-fetal infection	Risk of abortion	[48]
Drago et al.	38 women who developed pityriasis rosea (PR)	62% of the women who developed PR within 15 weeks gestation aborted and were HHV-6 DNA positive	PR may be associated with an active HHV-6 infection. In pregnancy, PR may foreshadow premature delivery with neonatal hypotonia and even fetal demise especially if it develops within 15 weeks gestation	[52]
Bültmann et al.	26 patients with peripartum cardiomyopathy	30.7% HHV-6 DNA positive	Prevalence of virus-associated inflammatory changes in peripartum cardiomyopathy	[54]
Gibson et al.	717 adverse pregnancy cases and 609 controls	Increased risk of developing PIHD	Fetal exposure to HHV-6 infection was associated with PIHD	[55]
Gervasi et al.	729 mid-trimester amniocentesis samples	1% HHV-6 DNA positive	Gestational hypertension at term Preterm premature rupture of membranes	[56]
Wiersbitzky et al.	Two cases of pre- and perinatal HHV-6 infection	Dramatic clinical symptoms of illness with death or cerebral defects	Pre- and perinatal HHV-6 infections related to lethal outcome or severe residual encephalopathy	[57]

(continued)

Table 9.2 (continued)

Author	Sample	HHV-6 results	Conclusions	References
Yoshikawa et al.	One neonatal HHV-6B infection	HHV-6 B was isolated from peripheral blood at the onset of the illness. A significant increase in viral antibody titers was not observed	Atypical clinical feature	[58]
Rentz et al.	156 infants (55% without pathologies and 45% with congenital anomaly)	No clinical association	HHV-6 DNA is present in 8% of infants	[59]
Marci et al.	30 unexplained primary infertile women 36 fertile women	HHV-6A DNA in 43% of endometrial biopsies from primary unexplained infertile women	Factor in female unexplained infertility development	[60]
Coulam et al.	30 RIF 10 fertile women	37% of RIF were HHV-6 DNA positive in endometrial biopsies 0% fertile women was HHV-6 DNA positive in endometrial biopsies	HHV-6 infection is an important factor in RIF	[62]

PIHD pregnancy-induced hypertensive disorders, *RIF* recurrent implantation failure

hypertensive disorders and preterm birth [55], antepartum hemorrhage and preterm premature rupture of membranes [56]. Prenatal and perinatal HHV-6 infections might result in cerebral deficits and death [57], the development of seizures and consequent severe neurological sequelae [40] and increased atypical lymphocytes, fever, and maculopapular rash [58]. In HHV-6-positive newborns (8%), there was an increased incidence of idiopathic respiratory failure (25%), congenital anomalies (17%), and signs of infection (50%) [59].

HHV-6A and Female Idiopathic Infertility

HHV-6, and especially HHV-6A, has recently been involved in female infertility. In the first study testing for HHV-6 in the endometrial tissue of women with idiopathic primary infertility and fertile women, results conveyed a striking difference in

infection patterns between the two groups. HHV-6 DNA was detected by quantitative PCR in the endometrial epithelial cells of 43% of infertile women but in none of the fertile controls [60]. Moreover, decreased levels of uterine CD56brightCD16- natural killer (NK) cells were found in HHV-6-positive samples compared to those collected from infertile, HHV-6-negative women and fertile women. Th2 cytokine (IL-10) was higher, and Th1 cytokine (IFN-gamma) was lower in the uterine flushing samples from HHV-6-positive women, resulting in a higher Th1/Th2 ratio. The authors suggested that endometrial NK cells experience a shift toward greater cytotoxicity in the presence of HHV-6A-infected endometrial epithelial cells [61]. These results suggest HHV-6A as a cofactor in determining female idiopathic infertility with the abnormal activation of endometrial-resident NK cells toward the subclinical HHV-6A infection undermining the permissiveness of the endometrium to the embryo implantation and fetal development.

These data have been confirmed in a cohort of women who were unable to conceive after undergoing several cycles of in vitro fertilization [62]. HHV-6 mRNA was isolated from the endometrial biopsies of 37% of women with recurrent implantation failure (RIF), while transcripts were not detected in endometrial samples collected during the luteal/secretory phase of the menstrual cycle from fertile women. Notably, HHV-6 immediate early protein 2 (IE2) was previously detected only during this phase of the menstrual cycle, while viral DNA was present during all phases. Abnormal immune profiles were seen in 10 of 11 HHV-6-positive RIF samples, 14 of 19 HHV-6-negative RIF samples, and only 1 of 10 control samples. Increased numbers of neutrophils and higher expression of CD16b were present in HHV-6-positive tissues, while NK cell phenotypes were not analyzed.

HHV-6A and Endometrial Gene Expression

When the effect of HHV-6A infection on endometrial gene expression was evaluated, it was clear that the virus perturbates the molecular environment that might disadvantage embryo implantation and placentation, causing an incorrect engagement of eNK cells [61] (Fig. 9.1). HHV-6A, as previously reported for HHV-6B [63], decreased the expression of activating NKG2D receptor ligand (NKG2DL) on endometrial epithelial cells, maintaining in vivo *endometrial natural killer cells* (eNK) with a low killer profile, allowing the persistence of subclinical HHV-6A infection.

Conversely, human leukocyte antigen (HLA)-E molecules, the ligand of the inhibitory NKG2A receptor, are down-modulated during HHV-6A infection and slightly reduced in endometrial epithelial cells from HHV-6A-positive infertile women, thus promoting the activatory profile observed in eNK cells from idiopathic infertile women [17, 64]. On the contrary, classical HLA class I molecules maintain their level of expression during HHV-6A infection. The difference between HLA-E and classical HLA-I molecules might reside in their intrinsic functional differences, where HLA-E has an immune-regulatory function while classical HLA-I antigens are totally involved in antigen presentation [17]. HHV-6A might interfere with

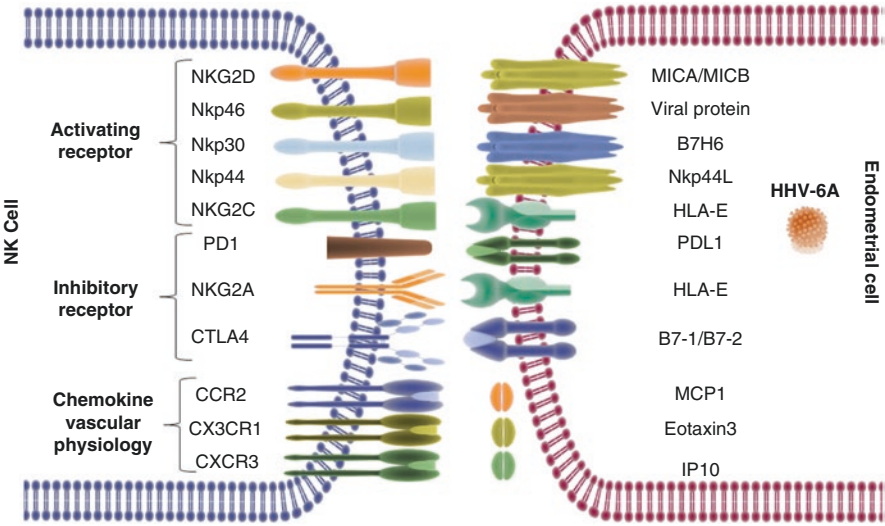

Fig. 9.1 Immune checkpoint at the endometrial and endometrial natural killer (eNK) cell interface. eNK cells express a repertoire of activating and inhibitory receptors that are controlled by the specific ligands expressed on the surface of endometrial cells. Receptors for chemokines secreted by endometrial cells are also expressed by eNK cells, and control vascular physiology. HHV-6A infection modifies the expression of NKG2D activating receptor and chemokine receptors (CCR2, CXCR3, CX3CR1) on the surface of endometrial cells and the expression of MCP1, eotaxin-3, and IP10 on the surface of endometrial cells

HLA-E surface expression impairing protein translocation from the endoplasmic reticulum. Endometrial epithelial cells acquired a de novo expression of HLA-II DR molecules, as previously reported for thyrocytes [65]. This "APC-like" phenotype during HHV-6A infection might be involved in viral clearance by immune cells. The expression of HLA-II antigens and chemokine receptor ligands could facilitate eNK cells homing to HHV-6A-infected endometrial cells, as demonstrated by in vitro culture model of endometrial epithelial cells. The persistence of activated eNK and subclinical HHV-6A infection seems to alter the endometrial environment, as demonstrated by the increase in chemokines, mainly IP10, and FasL in uterine flushing samples from HHV-6A-positive infertile women [61]. This perturbation of the molecular environment might disadvantage embryo implantation and placentation, which require a correct engagement of eNK cells. The presence of activated eNK cells can potentially have serious adverse side effects, as incorrect or insufficient remodeling of the spiral arteries leading to complications of pregnancy such as preeclampsia, fetal growth restriction, and stillbirth [66].

Endometrium and Innate Immune Response

The endometrium, with a resident immune cell population, is the first line of defense against pathogens that ascend the female genital tract [67] (Fig. 9.2).

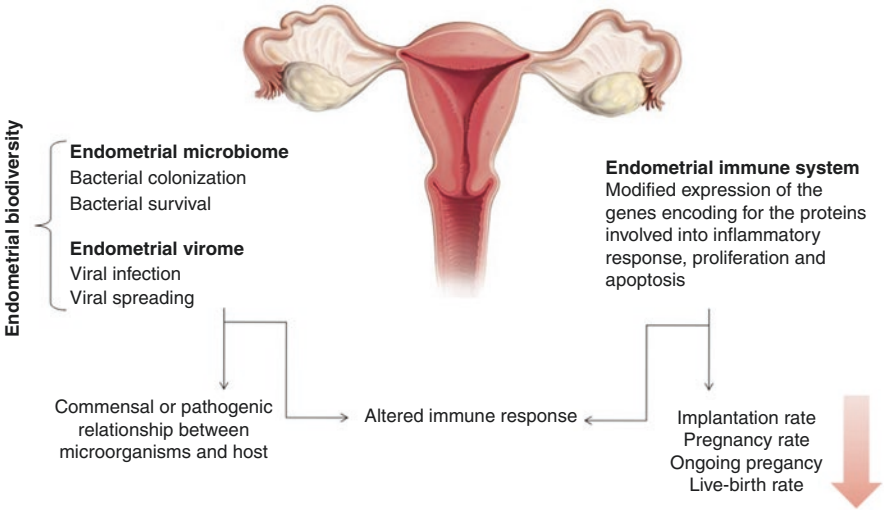

Fig. 9.2 Microbiome/virome interaction with the endometrial immune system. The modification of the endometrial environment might cause pregnancy-related diseases, affecting embryo implantation and live-birth rate

eNK cells are a heterogeneous population that seems to arise from in situ progenitors and/or from homing peripheral blood NK (pNK) cells [68]. The absolute numbers of eNK cells in the endometrium increase after ovulation, although the proportion remains constant (30%) across the menstrual cycle in relation to other leukocyte subtypes. When pregnancy occurs, this proportion increases to around 70% of the total leukocyte population. Increasing evidence indicates that eNK cells are involved in the success of implantation and maintenance of pregnancy [68, 69]. In comparison with pNK counterpart, eNK shows lesser cytotoxic activity, possesses fewer receptors of activation, and increased inhibitory receptors. They selectively express galectin-1 and glycodelin, and both of them are associated with the immunomodulatory activity. Galectin inhibits T-cell proliferation and survival as well as reduces T-cell production of tumor necrosis factor (TNF)-α, IL-2, and interferon (IFN)-γ, and the macrophage secretion of IL-12. Glycodelin downregulates T-cell activation [70]. The major mechanism preventing the eNK-mediated attack of trophoblast cells is now considered their ability to interact through inhibitory receptors with human leucocyte antigen (HLA)-G, HLA-E, and HLA-C expressed on trophoblast cells. Following this interaction, lytic activity is inhibited, and a T helper (Th)-2 type response is activated [70]. Human in vitro studies provided evidence for the eNK cells production of angiogenic factors such as vascular endothelial growth factor (VEGF), placental growth factor (PlGF), and angiopoietin 2 in both mice and humans. Similarly, murine studies have indicated that eNK cells are essential for the induction of spiral arteries, mediated via their production of IFN-γ [71, 72]. The role of eNK cells in the control of decidual angiogenesis and placental development has been supported by the observation that eNK-deficient mice exhibit compromised placentation and fetal growth [73].

HHV-6A and Endometrial NK Cell Gene Expression

During HHV-6A infection, eNK cells seem to become cytotoxic to limit viral infection [61]. Functional studies showed that NKG2D activating receptor and FasL are involved in the acquired cytotoxic function of eNK cells during HHV-6A infection of endometrial epithelial cells.

The engagement of NKG2D might trigger cytokine production (i.e., IFN-type 1, IFN-gamma, TNF-alpha) that control the cytotoxicity of eNK cells by Fas/FasL pathway [74]. The upregulation of FasL on the surface of eNK cells and in the uterine flushing samples of HHV-6A-positive infertile women suggest that eNK cell killing of HHV-6A-infected endometrial epithelial cells proceeds through NKG2D engagement and subsequent mechanisms which are dependent on the death receptor-ligand Fas/FasL pathway [61]. Interestingly, both eNK cells from fertile and infertile HHV-6-negative women present an increase in specific lysis when cocultured with HHV-6A-infected endometrial cells. On the contrary, eNK cells from HHV-6A-positive infertile women showed significantly higher lysis of HHV-6A-infected endometrial cells that overcomes the natural cytotoxicity of eNK cells. Moreover, in the presence of HHV-6A infection, eNK cells increased the expression of CCR2, CXCR3, and CX3CR1 chemokine receptors.

Meanwhile, HHV-6 infection of NK cells induces significant modifications in the expression of miRNAs known for their role in NK cell development, maturation and effector functions (miR-146, miR-155, miR-181, miR-223), and other 13 miRNAs with a role in inflammation and autoimmunity [17]. Also, the expression of transcription factors is significantly modified by HHV-6 infection, with an early increase of ATF3, JUN, and FOXA2, with HHV-6A specifically inducing a 15-fold decrease of POU2AF1, and HHV-6B an increase of FOXO1 and a decrease of ESR1. The remarkable effect of HHV-6 infections on the expression of miRNAs and transcription factors in NK cells might be important in the induction of eNK cell function impairment, virus escape strategies, and pregnancy-related disorders.

Conclusions

Pathogens represent a tremendous burden on the reproductive fitness of humans and are a major selective force in the evolution of our species [75]. The endometrial immune cell variations are tailored to microorganisms present in local environments [76]. As such, the immune phenotypes can have a profound effect on how an infectious pathogen might reduce the reproductive fitness of individuals in a population.

As HHV-6 is considered a ubiquitous virus that can undergo low-level reactivation in healthy individuals, it is often difficult to determine whether HHV-6 infection is a cause or effect of several pathological conditions. In addition, HHV-6 may reactivate in tissues without a corresponding rise in blood viremia, so it is necessary to analyze the site of infection to recognize viral replication. Moreover, the presence of two variants, HHV-6A and HHV-6B, known to be two

separate species with differing functionality, complicate the definition of disease associations. As HHV-6A was the only variant detected in HHV-6-positive endometrial samples when the virus was typed, it is probable that the two species of HHV-6 do not have the same impact on reproductive potential. The effect of HHV-6A endometrial infection on genetic expression and molecular environment might contribute to an aberrant distribution and function in the physiological endometrial immune molecules, with a possible association with poor obstetric conditions including miscarriage, preterm birth, preeclampsia, and intrauterine growth retardation [77–79].

Even if there is an increased awareness of diseases associated with HHV-6 infection and reactivation, no pharmaceutical compound is yet approved for the clinical management of HHV-6 infection. The drugs utilized are anti-HCMV agents as ganciclovir, cidofovir, and foscarnet. For this, an anti-HHV-6 therapy in infertile women is still far from realization. A possibility could be the use of treatments for the control of NK cells activation, as prednisolone, intravenous IgG (IVIG), intralipid, and TNF-α-blocking agent. Interesting results have been obtained with IVIG treatment in antiphospholipid syndrome with persistent presence of autoantibodies against beta2 glycoprotein 1, where NK cell expansion [80] and Th1 shift [81] seem to be implicated in recurrent miscarriage. However, it is necessary to underline that during viral infections, it is not inhibition of eNK cells that is needed, but rather the right degree of activation that is of importance. The goal of future studies will be both to maintain the correct activation of eNK cells toward HHV-6A infection without altering their functional role during implantation and to avoid the onset of implantation disorders, which is currently often diagnosed as idiopathic infertility.

References

1. Baker JM, Chase DM, Herbst-Kralovetz MM. Uterine microbiota: residents, tourists, or invaders? Front Immunol. 2018;9:208.
2. Cicinelli E, Ballini A, Marinaccio M, Poliseno A, Coscia MF, Monno R, De Vito D. Microbiological findings in endometrial specimen: our experience. Arch Gynecol Obstet. 2012;285:1325–9.
3. Egbase PE, al-Sharhan M, al-Othman S, al-Mutawa M, Udo EE, Grudzinskas JG. Incidence of microbial growth from the tip of the embryo transfer catheter after embryo transfer in relation to clinical pregnancy rate following in-vitro fertilization and embryo transfer. Hum Reprod. 1996;11:1687–9.
4. Moore DE, Soules MR, Klein NA, Fujimoto VY, Agnew KJ, Eschenbach DA. Bacteria in the transfer catheter tip influence the live-birth rate after in vitro fertilization. Fertil Steril. 2000;74:1118–24.
5. Chuong EB. Retroviruses facilitate the rapid evolution of the mammalian placenta. BioEssays. 2013;35:853–61.
6. Chuong EB, Rumi MA, Soares MJ, Baker JC. Endogenous retroviruses function as species-specific enhancer elements in the placenta. Nat Genet. 2013;45:325–9.
7. Gibson CS, MacLennan AH, Goldwater PN, Haan EA, Priest K, Dekker GA, South Australian Cerebral Palsy Research. Neurotropic viruses and cerebral palsy: population based case-control study. BMJ. 2006;332:76–80.

8. Sun Y, Pei W, Wu Y, Jing Z, Zhang J, Wang G. Herpes simplex virus type 2 infection is a risk factor for hypertension. Hypertens Res. 2004;27:541–4.
9. McLean LK, Chehab FF, Goldberg JD. Detection of viral deoxyribonucleic acid in the amniotic fluid of low-risk pregnancies by polymerase chain reaction. Am J Obstet Gynecol. 1995;173:1282–6.
10. Wenstrom KD, Andrews WW, Bowles NE, Towbin JA, Hauth JC, Goldenberg RL. Intrauterine viral infection at the time of second trimester genetic amniocentesis. Obstet Gynecol. 1998;92:420–4.
11. Fisher S, Genbacev O, Maidji E, Pereira L. Human cytomegalovirus infection of placental cytotrophoblasts in vitro and in utero: implications for transmission and pathogenesis. J Virol. 2000;74:6808–20.
12. Adams Waldorf KM, McAdams RM. Influence of infection during pregnancy on fetal development. Reproduction. 2013;146:R151–62.
13. De Bolle L, Naesens L, De Clercq E. Update on human herpesvirus 6 biology, clinical features, and therapy. Clin Microbiol Rev. 2005;18:217–45.
14. Braun DK, Dominguez G, Pellett PE. Human herpesvirus 6. Clin Microbiol Rev. 1997;10:521–67.
15. Santoro F, Kennedy PE, Locatelli G, Malnati MS, Berger EA, Lusso P. CD46 is a cellular receptor for human herpesvirus 6. Cell. 1999;99:817–27.
16. Tang H, Serada S, Kawabata A, Ota M, Hayashi E, Naka T, Yamanishi K, Mori Y. CD134 is a cellular receptor specific for human herpesvirus-6B entry. Proc Natl Acad Sci U S A. 2013;110:9096–9.
17. Rizzo R, Soffritti I, D'Accolti M, Bortolotti D, Di Luca D, Caselli E. HHV-6A/6B infection of NK cells modulates the expression of miRNAs and transcription factors potentially associated to impaired NK activity. Front Microbiol. 2017;8:2143.
18. Caruso A, Caselli E, Fiorentini S, Rotola A, Prandini A, Garrafa E, Saba E, Alessandri G, Cassai E, Di Luca D. U94 of human herpesvirus 6 inhibits in vitro angiogenesis and lymphangiogenesis. Proc Natl Acad Sci U S A. 2009;106:20446–51.
19. Caruso A, Favilli F, Rotola A, Comar M, Horejsh D, Alessandri G, Grassi M, Di Luca D, Fiorentini S. Human herpesvirus-6 modulates RANTES production in primary human endothelial cell cultures. J Med Virol. 2003;70:451–8.
20. Caselli E, Campioni D, Cavazzini F, Gentili V, Bortolotti D, Cuneo A, Di Luca D, Rizzo R. Acute human herpesvirus-6A infection of human mesothelial cells modulates HLA molecules. Arch Virol. 2015;160:2141–9.
21. Gu B, Zhang GF, Li LY, Zhou F, Feng DJ, Ding CL, Chi J, Zhang C, Guo DD, Wang JF, Zhou H, Yao K, Hu WX. Human herpesvirus 6A induces apoptosis of primary human fetal astrocytes via both caspase-dependent and -independent pathways. Virol J. 2011;8:530.
22. Li C, Goodrich JM, Yang X. Interferon-gamma (IFN-gamma) regulates production of IL-10 and IL-12 in human herpesvirus-6 (HHV-6)-infected monocyte/macrophage lineage. Clin Exp Immunol. 1997;109:421–5.
23. Robert C, Aubin JT, Visse B, Fillet AM, Huraux JM, Agut H. Difference in permissiveness of human fibroblast cells to variants A and B of human herpesvirus-6. Res Virol. 1996;147:219–25.
24. Harberts E, Yao K, Wohler JE, Maric D, Ohayon J, Henkin R, Jacobson S. Human herpesvirus-6 entry into the central nervous system through the olfactory pathway. Proc Natl Acad Sci U S A. 2011;108:13734–9.
25. Donati D, Akhyani N, Fogdell-Hahn A, Cermelli C, Cassiani-Ingoni R, Vortmeyer A, Heiss JD, Cogen P, Gaillard WD, Sato S, Theodore WH, Jacobson S. Detection of human herpesvirus-6 in mesial temporal lobe epilepsy surgical brain resections. Neurology. 2003;61:1405–11.
26. Roush KS, Domiati-Saad RK, Margraf LR, Krisher K, Scheuermann RH, Rogers BB, Dawson DB. Prevalence and cellular reservoir of latent human herpesvirus 6 in tonsillar lymphoid tissue. Am J Clin Pathol. 2001;116:648–54.
27. Fox JD, Briggs M, Ward PA, Tedder RS. Human herpesvirus 6 in salivary glands. Lancet. 1990;336:590–3.

28. Kondo K, Kondo T, Okuno T, Takahashi M, Yamanishi K. Latent human herpesvirus 6 infection of human monocytes/macrophages. J Gen Virol. 1991;72(Pt 6):1401–8.
29. Luppi M, Barozzi P, Maiorana A, Marasca R, Torelli G. Human herpesvirus 6 infection in normal human brain tissue. J Infect Dis. 1994;169:943–4.
30. Luppi M, Barozzi P, Morris C, Maiorana A, Garber R, Bonacorsi G, Donelli A, Marasca R, Tabilio A, Torelli G. Human herpesvirus 6 latently infects early bone marrow progenitors in vivo. J Virol. 1999;73:754–9.
31. Suga S, Yazaki T, Kajita Y, Ozaki T, Asano Y. Detection of human herpesvirus 6 DNAs in samples from several body sites of patients with exanthem subitum and their mothers by polymerase chain reaction assay. J Med Virol. 1995;46:52–5.
32. Mukai T, Yamamoto T, Kondo T, Kondo K, Okuno T, Kosuge H, Yamanishi K. Molecular epidemiological studies of human herpesvirus 6 in families. J Med Virol. 1994;42:224–7.
33. Arbuckle JH, Medveczky PG. The molecular biology of human herpesvirus-6 latency and telomere integration. Microbes Infect. 2011;13:731–41.
34. Leach CT, Newton ER, McParlin S, Jenson HB. Human herpesvirus 6 infection of the female genital tract. J Infect Dis. 1994;169:1281–3.
35. Baillargeon J, Piper J, Leach CT. Epidemiology of human herpesvirus 6 (HHV-6) infection in pregnant and nonpregnant women. J Clin Virol. 2000;16:149–57.
36. Maeda T, Okuno T, Hayashi K, Nagata M, Ueda M, Terashima K, Kawashima T, Miyamoto H, Mori T, Yamada Y. Outcomes of infants whose mothers are positive for human herpesvirus-6 DNA within the genital tract in early gestation. Acta Paediatr Jpn. 2000;39:653–7.
37. Adams O, Krempe C, Kogler G, Wernet P, Scheid A. Congenital infections with human herpesvirus 6. J Infect Dis. 1998;178:544–6.
38. Boutolleau D, Cointe D, Gautheret-Dejean A, Mace M, Agut H, Grangeot-Keros L, Ingrand D. No evidence for a major risk of roseolovirus vertical transmission during pregnancy. Clin Infect Dis. 2003;36:1634–5.
39. Dahl H, Fjaertoft G, Norsted T, Wang FZ, Mousavi-Jazi M, Linde A. Reactivation of human herpesvirus 6 during pregnancy. J Infect Dis. 1999;180:2035–8.
40. Lanari M, Papa I, Venturi V, Lazzarotto T, Faldella G, Gabrielli L, Guerra B, Landini MP, Salvioli GP. Congenital infection with human herpesvirus 6 variant B associated with neonatal seizures and poor neurological outcome. J Med Virol. 2003;70:628–32.
41. Okuno T, Oishi H, Hayashi K, Nonogaki M, Tanaka K, Yamanishi K. Human herpesviruses 6 and 7 in cervixes of pregnant women. J Clin Microbiol. 1995;33:1968–70.
42. Caserta MT, Hall CB, Schnabel K, Lofthus G, McDermott MP. Human herpesvirus (HHV)-6 and HHV-7 infections in pregnant women. J Infect Dis. 2007;196:1296–303.
43. D'Agaro P, Burgnich P, Comar M, Dal Molin G, Bernardon M, Busetti M, Alberico S, Poli A, Campello C, Group SI. HHV-6 is frequently detected in dried cord blood spots from babies born to HIV-positive mothers. Curr HIV Res. 2008;6:441–6.
44. Hall CB, Caserta MT, Schnabel KC, Boettrich C, McDermott MP, Lofthus GK, Carnahan JA, Dewhurst S. Congenital infections with human herpesvirus 6 (HHV6) and human herpesvirus 7 (HHV7). J Pediatr. 2004;145:472–7.
45. Hall CB, Caserta MT, Schnabel K, Shelley LM, Marino AS, Carnahan JA, Yoo C, Lofthus GK, McDermott MP. Chromosomal integration of human herpesvirus 6 is the major mode of congenital human herpesvirus 6 infection. Pediatrics. 2008;122:513–20.
46. Al-Buhtori M, Moore L, Benbow EW, Cooper RJ. Viral detection in hydrops fetalis, spontaneous abortion, and unexplained fetal death in utero. J Med Virol. 2011;83:679–84.
47. Ashshi AM, Cooper RJ, Klapper PE, Al-Jiffri O, Moore L. Detection of human herpes virus 6 DNA in fetal hydrops. Lancet. 2000;355:1519–20.
48. Revest M, Minjolle S, Veyer D, Lagathu G, Michelet C, Colimon R. Detection of HHV-6 in over a thousand samples: new types of infection revealed by an analysis of positive results. J Clin Virol. 2011;51:20–4.
49. Drago F, Broccolo F, Javor S, Drago F, Rebora A, Parodi A. Evidence of human herpesvirus-6 and -7 reactivation in miscarrying women with pityriasis rosea. J Am Acad Dermatol. 2014;71:198–9.

50. Yasukawa M, Sada E, MacHino H, Fujita S. Reactivation of human herpesvirus 6 in pityriasis rosea. Br J Dermatol. 1999;140:169–70.
51. Tanaka-Taya K, Kondo T, Nakagawa N, Inagi R, Miyoshi H, Sunagawa T, Okada S, Yamanishi K. Reactivation of human herpesvirus 6 by infection of human herpesvirus 7. J Med Virol. 2000;60:284–9.
52. Drago F, Malaguti F, Ranieri E, Losi E, Rebora A. Human herpes virus-like particles in pityriasis rosea lesions: an electron microscopy study. J Cutan Pathol. 2002;29:359–61.
53. Watanabe T, Kawamura T, Jacob SE, Aquilino EA, Orenstein JM, Black JB, Blauvelt A. Pityriasis rosea is associated with systemic active infection with both human herpesvirus-7 and human herpesvirus-6. J Invest Dermatol. 2002;119:793–7.
54. Bultmann BD, Klingel K, Nabauer M, Wallwiener D, Kandolf R. High prevalence of viral genomes and inflammation in peripartum cardiomyopathy. Am J Obstet Gynecol. 2005;193:363–5.
55. Gibson CS, Goldwater PN, MacLennan AH, Haan EA, Priest K, Dekker GA, South Australian Cerebral Palsy Research G. Fetal exposure to herpesviruses may be associated with pregnancy-induced hypertensive disorders and preterm birth in a Caucasian population. BJOG. 2008;115:492–500.
56. Gervasi MT, Romero R, Bracalente G, Chaiworapongsa T, Erez O, Dong Z, Hassan SS, Yeo L, Yoon BH, Mor G, Barzon L, Franchin E, Militello V, Palu G. Viral invasion of the amniotic cavity (VIAC) in the midtrimester of pregnancy. J Matern Fetal Neonatal Med. 2012;25:2002–13.
57. Wiersbitzky S, Ratzmann GW, Bruns R, Wiersbitzky H. Reactivation in children of juvenile chronic arthritis and chronic iridocyclitis associated with human herpesvirus-6 infection. Padiatr Grenzgeb. 1993;31:203–5.
58. Yoshikawa T, Ohashi M, Miyake F, Fujita A, Usui C, Sugata K, Suga S, Hashimoto S, Asano Y. Exanthem subitum-associated encephalitis: nationwide survey in Japan. Pediatr Neurol. 2009;41:353–8.
59. Rentz AC, Stevenson J, Hymas W, Hillyard D, Stoddard GJ, Taggart EW, Byington CL. Human herpesvirus 6 in the newborn intensive care unit. Eur J Clin Microbiol Infect Dis. 2009;26:297–9.
60. Marci R, Gentili V, Bortolotti D, Lo Monte G, Caselli E, Bolzani S, Rotola A, Di Luca D, Rizzo R. Presence of HHV-6A in endometrial epithelial cells from women with primary unexplained infertility. PLoS One. 2016;11:e0158304.
61. Caselli E, Bortolotti D, Marci R, Rotola A, Gentili V, Soffritti I, D'Accolti M, Lo Monte G, Sicolo M, Barao I, Di Luca D, Rizzo R. HHV-6A infection of endometrial epithelial cells induces increased endometrial NK cell-mediated cytotoxicity. Front Microbiol. 2017;8:2525.
62. Coulam CB, Bilal M, Salazar Garcia MD, Katukurundage D, Elazzamy H, Fernandez EF, Kwak-Kim J, Beaman K, Dambaeva SV. Prevalence of HHV-6 in endometrium from women with recurrent implantation failure. Am J Reprod Immunol. 2018;80:e12862.
63. Schmiedel D, Tai J, Levi-Schaffer F, Dovrat S, Mandelboim O. Human herpesvirus 6B downregulates expression of activating ligands during lytic infection to escape elimination by natural killer cells. J Virol. 2016;90:9608–17.
64. Rizzo R, Lo Monte G, Bortolotti D, Graziano A, Gentili V, Di Luca D, Marci R. Impact of soluble HLA-G levels and endometrial NK cells in uterine flushing samples from primary and secondary unexplained infertile women. Int J Mol Sci. 2015;16:5510–6.
65. Caselli E, Zatelli MC, Rizzo R, Benedetti S, Martorelli D, Trasforini G, Cassai E, degli Uberti EC, Di Luca D, Dolcetti R. Virologic and immunologic evidence supporting an association between HHV-6 and Hashimoto's thyroiditis. PLoS Pathog. 2012;8:e1002951.
66. Yougbare I, Tai WS, Zdravic D, Oswald BE, Lang S, Zhu G, Leong-Poi H, Qu D, Yu L, Dunk C, Zhang J, Sled JG, Lye SJ, Brkic J, Peng C, Hoglund P, Croy BA, Adamson SL, Wen XY, Stewart DJ, Freedman J, Ni H. Activated NK cells cause placental dysfunction and miscarriages in fetal alloimmune thrombocytopenia. Nat Commun. 2017;8:224.
67. Herath S, Fischer DP, Werling D, Williams EJ, Lilly ST, Dobson H, Bryant CE, Sheldon IM. Expression and function of toll-like receptor 4 in the endometrial cells of the uterus. Endocrinology. 2006;147:562–70.

68. Moffett A, Colucci F. Uterine NK cells: active regulators at the maternal-fetal interface. J Clin Invest. 2014;124:1872–9.
69. Gaynor LM, Colucci F. Uterine natural killer cells: functional distinctions and influence on pregnancy in humans and mice. Front Immunol. 2017;8:467.
70. Koopman LA, Kopcow HD, Rybalov B, Boyson JE, Orange JS, Schatz F, Masch R, Lockwood CJ, Schachter AD, Park PJ, Strominger JL. Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential. J Exp Med. 2003;198:1201–12.
71. Chen SJ, Liu YL, Sytwu HK. Immunologic regulation in pregnancy: from mechanism to therapeutic strategy for immunomodulation. Clin Dev Immunol. 2012;2012:258391.
72. Torry DS, Leavenworth J, Chang M, Maheshwari V, Groesch K, Ball ER, Torry RJ. Angiogenesis in implantation. J Assist Reprod Genet. 2007;24:303–15.
73. Faas MM, de Vos P. Uterine NK cells and macrophages in pregnancy. Placenta. 2017;56:44–52.
74. Park DS, Lee H, Frank PG, Razani B, Nguyen AV, Parlow AF, Russell RG, Hulit J, Pestell RG, Lisanti MP. Caveolin-1-deficient mice show accelerated mammary gland development during pregnancy, premature lactation, and hyperactivation of the Jak-2/STAT5a signaling cascade. Mol Biol Cell. 2002;13:3416–30.
75. Fumagalli M, Sironi M, Pozzoli U, Ferrer-Admetlla A, Pattini L, Nielsen R. Signatures of environmental genetic adaptation pinpoint pathogens as the main selective pressure through human evolution. PLoS Genet. 2011;7:e1002355.
76. Grindstaff JL, Hasselquist D, Nilsson JK, Sandell M, Smith HG, Stjernman M. Transgenerational priming of immunity: maternal exposure to a bacterial antigen enhances offspring humoral immunity. Proc Biol Sci. 2006;273:2551–7.
77. Vinketova K, Mourdjeva M, Oreshkova T. Human decidual stromal cells as a component of the implantation niche and a modulator of maternal immunity. J Pregnancy. 2016;2016:8689436.
78. Liu S, Diao L, Huang C, Li Y, Zeng Y, Kwak-Kim JYH. The role of decidual immune cells on human pregnancy. J Reprod Immunol. 2017;124:44–53.
79. Wilczynski JR. Immunological analogy between allograft rejection, recurrent abortion and pre-eclampsia - the same basic mechanism? Hum Immunol. 2006;67:492–511.
80. Oliver-Minarro D, Gil J, Aguaron A, Rodriguez-Mahou M, Fernandez-Cruz E, Sanchez-Ramon S. NK cell expansion in obstetrical antiphospholipid syndrome: guilty by association? Eur J Obstet Gynecol Reprod Biol. 2009;145:227.
81. Benagiano M, Gerosa M, Romagnoli J, Mahler M, Borghi MO, Grassi A, Della Bella C, Emmi G, Amedei A, Silvestri E, Emmi L, Prisco D, Meroni PL, D'Elios MM. beta2 glycoprotein I recognition drives Th1 inflammation in atherosclerotic plaques of patients with primary antiphospholipid syndrome. J Immunol. 2017;198:2640–8.

Chapter 10
Endometrial Immune-Inflammatory Gene Signatures in Endometriosis

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Introduction

Endometriosis is a debilitating gynecological disorder that affects 176 million reproductive-aged women worldwide and presents clinically as chronic pelvic pain and infertility. Endometriosis places a substantial burden on healthcare systems and national economies ($1.8 billion in Canada [1] and $22 billion in the USA [2]) and can have devastating effects on the personal and family lives of patients. Despite its prevalence and impact, the cause of endometriosis remains unknown. The disease is characterized by the benign growth of endometrial-like tissue lesions in ectopic sites such as the ovary, peritoneum, and broad ligament and more rarely in the lung, brain, pericardium, and sites of surgical incision. These endometriotic lesions are composed largely of endometrial epithelial, glandular, and stromal compartments, but are also highly vascularized and can be highly fibrotic, innervated and infiltrated with immune cell populations [3–5]. Other hallmark features of endometriosis include estrogen dependence, progesterone resistance, and chronic inflammation [6, 7]. While it is known that ovarian hormones and inflammation drive the pathology of the disease, the pathogenesis of endometriosis remains unknown. The most common theory to explain the etiology of the disease is Sampson's Theory of Retrograde Menstruation, which suggests that shed menstrual tissue is refluxed during menstrual contractions and once in the peritoneum, this tissue adheres to peritoneal structures [8]. To explain the presence of endometriosis in rare locations and surrounding lymph nodes, theories such as the metaplasia theory and lymphatic spread theory have been suggested [9–12]. Other theories including coelomic metaplasia

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and stem cell theories have also been proposed, suggesting endometriotic lesions originate from stem cells [7, 13].

Additionally, due to the lack of knowledge, merely procuring a diagnosis can be difficult for endometriosis patients. Symptoms are often confused for other abdominal or gynecological diseases, which lead to misdiagnosis [14]. Further, because a noninvasive diagnostic biomarker does not exist, diagnosis is confirmed only through an invasive surgical procedure and physician visualization of endometriotic lesions. These factors and others contribute to an average diagnostic delay of approximately 6.7 years [15, 16]. Therefore, there is an urgent need for noninvasive biomarkers, which has been extensively reviewed [17–19]. Diagnosis of endometriosis relies heavily on the American Society of Reproductive Medicine's (ASRM) weighted staging system (ranging from stage I (minimal), stage II (mild), stage III (moderate) and stage IV (severe) endometriosis) and when appropriate, the Enzian Scoring System of deep infiltrating endometriosis and Endometriosis Fertility Index [20, 21]. However, due to the heterogeneity even within these classifications, optimal ways of classifying and sub-classifying endometriosis are often discussed. Endometriotic lesions in different anatomical locations have been proposed to represent distinct entities of endometriosis and have been shown to have differing symptomology [22–25]. Deep infiltrating endometriosis, commonly located in the rectouterine area and rectovaginal pouch or on the surface of the bowel, is typically regarded as the most severe due to pelvic pain association [24, 25] along with a more invasive, inflammatory, and fibrotic phenotype (as reviewed [26]). The most commonly reported type of endometriosis is ovarian endometrioma, which despite being regarded as less severe symptomatically, has recently garnered significant concern due to its strong association with epithelial clear cell ovarian carcinoma [27, 28]. Similarly, superficial peritoneal endometriosis is also viewed as less severe and is often found on the abdominal wall [22]. However, despite significant efforts, patients within the ASRM stages, as well as anatomical classifications, still exhibit vast heterogeneity, associated symptoms, and response to treatment. Therefore, a superior classification system or sub-classification system needs to be developed to help us accurately understand the biology of the disease.

In addition to difficulties with diagnosis and classification of endometriosis, treating this disease effectively is an evolving challenge. Current treatments typically focus on decreasing levels of estrogen using hormonal therapeutics and/or surgical excision or ablation of the endometriotic lesions, which can temporarily relieve symptoms. However, disease recurrence is common. Indeed, approximately 30–60% of patients who undergo surgical excision of the endometriotic lesion will experience a recurrence within 12 months [14, 29]. Interestingly, after surgical ablation, lesions typically form in similar locations [30], which suggests that the microenvironment surrounding that particular anatomical area is likely facilitating the regrowth of the lesion. To treat endometriosis-associated infertility, endometriotic lesions are surgically ablated, and patients then undergo assisted reproductive technologies. Randomized control studies show that surgical ablation of the endometriotic lesion prior to assisted reproductive technology use significantly improves fertility in these patients [14, 31, 32]. Because it is well established that the systemic

and local inflammatory environment alters following surgical removal of the lesion [33], it is possible that the inflammatory environment stimulated by the endometriotic lesion contributes to the alteration in embryo-endometrial cross talk leading to infertility-related complications. Pain management in endometriosis consists largely of surgical intervention and medical therapeutics including nonsteroidal anti-inflammatory drugs, oral contraceptives, progestogens, GnRH agonists, GnRH antagonists, and aromatase inhibitors [29, 34]. Again, surgically and chemically, these therapies transiently dampen inflammation and create a hypoestrogenic state in endometriosis patients. While these therapeutics often provide pain relief, many of them also impact fertility, forcing patients to choose between treating their pain and infertility. Therefore, non-hormonal therapeutics, which do not impact fertility, are urgently needed.

In the context of understanding the disease pathophysiology, it is well accepted that endometriosis patients exhibit chronic inflammation. Numerous studies have shown that endometriosis patients present with anti-endometrial autoantibodies, elevated cytokines and chemokines, and aberrant immune cell populations and activation (recently reviewed [35]). In fact, 90% of normal cycling women with patent fallopian tubes have been shown to also experience the reflux and retrograde transport of menstrual debris during menstrual contractions [36]; however, it is only in 10% of women who go on to develop endometriosis. Therefore, endometriosis patients likely have trouble clearing menstrual debris or experience immune evasion. To further support an immune dysfunction theory, endometriosis patients commonly present with other chronic inflammatory comorbidities including type 1 hypersensitivities and allergies, autoimmune diseases (such as systemic lupus erythematosus and rheumatoid arthritis), cardiovascular disease as well as a number of malignancies [37, 38].

Due to this, endometriosis is categorized as a chronic inflammatory condition. It has been postulated that the excessive production of ovarian hormones, heme accumulation, oxidative stress, the endometrial tissue itself, or a combination of all of these initiates inflammation in endometriosis [39, 40]. Ultimately though, it remains unclear how chronic inflammation is sustained in the peritoneum while simultaneously exhibiting the ability for endometriotic lesions to evade immune clearance. However, it is well understood that inflammation plays a central, pathogenic role in the progression of the disease. This can be partly explained based on the knowledge that cytokines, chemokines, and growth factors that are upregulated systemically and in the local microenvironment of endometriosis patients can act as mediators in the progression and survival of endometriotic lesions. Additionally, it has been shown that this inflammatory microenvironment contributes to the pain symptoms and infertility in these patients [3, 41, 42].

With the rise in popularity of large-scale molecular profiling, many studies sought to understand alterations in genetic variants, epigenetics, gene expression, and protein levels to provide answers to the multitude of questions and challenges faced by endometriosis patients, healthcare providers, and researchers alike. From this data, efforts have been made to distinguish a unique gene expression profile, specific to endometriosis and inflammation. Further understanding of an immune-

inflammatory gene signature could support previous claims regarding immune dysfunction in endometriosis but could also identify molecular pathways that have not been previously identified. This could then lead to further understanding of the pathophysiology of the disease. It is important to note that transcript expression does not always correlate with equivalent changes in protein levels. Therefore, alterations in gene transcripts should also be validated proteomically. Additionally, an immune-inflammatory gene signature could improve the diagnostic procedure. More specifically, if a gene signature from endometrial biopsies could better predict infertility or if gene signatures from an endometriotic lesion could better predict susceptibility to pain medication or their overall ovarian cancer risk, diagnostics in the field of endometriosis could be potentially improved. Finally, because extensive heterogeneity between patients, even within the same disease stage, understanding altered immune pathways in specific patients could create subclassifications of the disease. Keeping this knowledge and potential gaps in view, here we will provide a comprehensive overview of the alterations in immune-inflammatory gene signatures and their functional significance in the context of endometriosis.

Transcriptional Alterations in the Endometrium

Early transcriptomic reports in endometriosis patients were focused on identifying gene candidates in the endometrium that could signify or predict implantation failure and infertility [43]. Experimentally, it has been shown that the presence of an endometriotic lesion has the ability to transcriptionally alter the endometrium in a baboon model of endometriosis [44, 45]. This suggests that the presence of an endometriotic lesion likely stimulates a peritoneal inflammatory microenvironment that contributes to the alterations in the endometrium. Unsupervised clustering of the gene transcripts revealed that the endometrium of endometriosis patients had a distinct gene profile [43]. Genes among the most strongly elevated were involved with apoptosis, secretory proteins, signal transduction (including mitogen-activated protein kinases), cell surface proteins (including major histocompatibility complex-related genes), transcription factors, and inflammatory pathways (including a number of cytokines and their receptors), while most strongly downregulated included immune function in natural killer (NK) cell receptors and T-cell receptor [43]. It is clear from early transcriptional analysis reports that candidate genes for identifying endometriosis and endometriosis-associated infertility include immune and inflammatory genes. More recently, Ahn et al., demonstrated 91 differentially expressed immune-inflammatory genes in the endometrium from endometriosis patients compared to the endometrium from healthy fertile controls [46]. The highest elevated gene transcripts in the endometrium of infertile endometriosis patients included genes encoding for chemokines and their receptors (including *CXCL10, CXCR2*), immune cell markers (including *CD19, LILRA5, CD40, CD3EAP*), cytokines (including *IL-32, IL-17A*), adhesion molecules (including *ICAM3, SELL*) and immune modulating transcription factors (*NOTCH1, STAT6*). Based on the

transcriptional analysis of the endometrium, another study showed that diagnostic classifiers could accurately distinguish endometriosis from other uterine/pelvic pathologies [47]. These diagnostic classifiers included many immune-inflammatory genes such as genes involved in immune activation (including antigen presentation, cytokine activity, and leukocyte activation) [47]. Additionally, transcriptional analysis of endocervical tissue from women with deep infiltrating endometriosis compared to healthy fertile controls show differentially expressed immune and inflammatory gene signatures, particularly chemokines and their receptors (including *CCL21, CXCL14, CCR5, CCL13)* and genes modifying dendritic cell activation (*ICAM2, CXCL12)* [48]. Interestingly, both menstrual effluent-derived stromal stem cells and endometrial biopsy-derived stem cells from women with endometriosis had differential expression of genes encoding for immune activation and activity compared to cells derived from menstrual effluent and endometrial biopsy of healthy fertile women [49, 50]. Contrary to this significant data, others found similar transcriptomic profiles between the endometrium of endometriosis patients and healthy fertile women [51, 52]. This suggests that the classification of endometriosis perhaps needs to be re-evaluated keeping in view what types of endometriotic lesions stimulate a specific inflammatory profile in the patient's matched endometrium before being able to better predict endometrial alterations and associated infertility.

Nonetheless, there is sufficient literature evidence to conclude that immune and inflammatory transcripts are altered in the endometrium of women with endometriosis [46, 47]. Because implantation and pregnancy require a delicate balance of inflammation and immune regulation, it is well established that pelvic inflammation, whether from pelvic infection or chronic inflammatory gynecological diseases, can lead to the development of recurrent implantation failure and/or infertility [53]. However, the molecular mechanisms that cause infertility in endometriosis are not known. Therefore, this immune-inflammatory gene signature could offer considerable insight into the molecular mechanisms underlying endometriosis-associated infertility.

Transcriptional Alterations in Mononuclear Cells

Because endometriosis is classified as a chronic inflammatory disease, it is of interest to understand whether peripheral blood immune cells will capture the alterations reflective of the disease and if that can elucidate associated molecular mechanisms. If so, the knowledge gained could contribute toward developing noninvasive diagnostic markers or aid in the development of a new sub-classification system or non-hormonal therapeutic. Indeed, the transcriptome of peripheral blood mononuclear cells (PBMCs) isolated from endometriosis patients was determined through the use of a microarray and compared to PBMCs from healthy fertile controls [54]. After analysis by gene ontology (GO), several gene pathways were altered including innate immune response, cell-cell signaling, response to steroid hormone stimulus, response to external stimulus, and others [54]. Interestingly, the PBMC transcriptome of endometriosis patients showed remarkable similarities to alterations in

PBMCs from psoriasis patients [54]. Specifically, GO analysis revealed similar alterations in pathways including but not limited to responses to steroid hormone stimulus and external stimulus [54]. Because of these commonalities, strategies for specific targeting of immune effector pathways that have been shown to be successful in psoriasis or other chronic inflammatory diseases such as inflammatory bowel disease and rheumatoid arthritis could be utilized in endometriosis. Additionally, peritoneal fluid mononuclear cells from endometriosis patients showed differential expression of inflammatory genes encoding for TNF-α compared to healthy fertile controls [55]. Analysis of peripheral and peritoneal immune cells and their transcriptomic alterations in the endometriosis setting offers a unique perspective on the systemic and localized microenvironment and supports our current theories surrounding immune dysfunction and chronic inflammation in endometriosis. Further research is required to understand specific molecular pathways that are functionally aberrant in order to understand the pathology and improve diagnostics and clinical treatments.

Transcriptional Alterations in the Endometriotic Lesion

In two separate studies from two independent research groups, the nCounter® GX Human Immunology Kit (from Nanostring) was utilized to understand the inflammatory gene profile of endometriotic lesions from women with endometriosis. In both studies, unsupervised hierarchical clustering successfully distinguished endometriotic lesions from matched endometrium from the same patient [46] and endometriosis-associated ovarian cancer [56] in addition to comparing to the endometrium from healthy fertile controls [46, 56]. Using DAVID bioinformatics, genes were grouped into GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, which identifies altered pathways [46]. From both studies, the endometriotic lesion transcriptome had altered immune and inflammatory gene pathways including innate and adaptive inflammatory responses, defensive immune response, leukocyte activation, cell proliferation and regulation, cytokine/chemokine response, chemotaxis, complement activation, apoptosis, wound-healing mechanisms, antiviral, antibacterial, cell death, cell adhesion, and transcription factor activity [46, 56]. These studies show that endometriotic lesions have a distinct immune-inflammatory gene profile, which could be explored further to develop new diagnostic and symptomology predictors.

Cytokine and Cytokine-Cytokine Receptor Expression

Several studies have attempted to delineate the role of cytokines in endometriosis. Collectively, it has been shown that cytokines and chemokines are dysregulated in their expression (both at mRNA and protein) levels in endometriosis. While these studies provide correlative evidence that cytokines (such as IL-1, IL-6, TNF-α, IFN-γ, TGF-β) are involved in endometriosis (as previously reviewed [57, 58]),

specific studies are required to establish how and whether these cytokines play a functional role in the initiation of lesion establishment and subsequent progression of the disease. Due to the elevated levels of cytokines associated with a T-helper type 2 immune response, which is the immune response that contributes to chronic inflammation in asthma and allergy, it has long been suggested that endometriosis could be skewed toward a type 2 immune response [59]. Indeed, gene profiles indicated elevated expression of *IL4* and *IL13*, which are type 2 immune cytokines, in endometriotic lesions [46, 60]. However, IL-12 associated cytokines (*IL12A, IL12RB1,* IL27), which produce a type 1 response, have also been shown to have elevated expression [46]. Therefore, based on the transcriptome, it appears that both a type 1 and type 2 immune responses are involved in the pathophysiology of endometriosis. Additionally, elevated protein levels of chemokines (responsible for immune cell recruitment), such as IL-8, CCL4, CCL5, and CCL7 were found to be elevated in peritoneal fluid from endometriosis patients compared to controls [57]. Further, mRNA transcripts indicate elevated expression of chemokines, and their receptors (*IL8, CCL5, CCR2*, *CCL11, CCL2, CCL3, CCL5, CCL7, CXCL1, CXCL12, CXCR1, CXCR7,* and *CXCR2*) were significantly elevated in endometriotic lesions compared to the endometrium of healthy fertile controls [46]. These chemokines recruit immune cells to the lesion and peritoneal environment and could be contributing to vascularization and the overall pathology of the disease.

Taken together, it is clear that the transcriptomic profile of endometriosis patients has elevated expression of many different types of cytokines [46, 61, 62]. Due to many functional roles of individual cytokines and the combination of cytokines during chronic inflammation, utilizing cytokine transcript expression and/or protein levels has not been proven to be useful as a single biomarker or a panel for improving diagnosis [63]. Indeed, in a systematic review published in 2010, authors curated the literature for 25 years and found 100 putative biomarkers that met the criteria in serum, plasma, and urine. Unfortunately, they could not find a clinical utility of any of the biomarkers [18]. Nevertheless, this knowledge has contributed and could continue to contribute to a better understanding of the dynamic nature of microenvironment around the endometriotic lesion.

Immune Cell Surface Markers and Immune Cell Activation

While it is established that immune cell numbers are aberrant in the microenvironment of endometriosis, mechanistic evidence surrounding why they are aberrant and how that affects the disease requires further study. Transcripts of immune cell surface markers including *CD14* (monocyte marker); *CD19, BCL10,* and *LILRB3* (B cell markers); *CD3D, CD4, CD5, CD8A, CD8B, CD45RO,* and *FoxP3* (T-cell markers); *FCER1A* (mast cell marker); *MARCO* (macrophage receptor); and *CD48* (marker expressed on a variety of myeloid and lymphatic immune cells) were found to be elevated in the endometriotic lesions compared to the endometrium of healthy

fertile controls [64]. The expression profiles of these immune cell markers support current findings showing elevated monocytes and macrophages [39, 61], elevated and activated B and T cells, specifically regulatory T cells [65–67], and elevated and activated mast cells [68, 69] in endometriosis. Interestingly, NK cells have been repeatedly shown to be decreased in numbers and have reduced cytotoxicity in endometriosis patients [70]. Therefore, decreased expression of NK cell markers (*KLRC1, KLRC2, KLRC3, KLRD1, KLRF1, KLRF2,* and *KIR*), NK cell-related development genes (*Nfil3, IL15, GNLY*), as well as T cell and NK cell cytotoxicity markers (*GZMA* and *GZMB*) provides mechanistic evidence and contributes to the knowledge of aberrant NK cell function in endometriosis [46]. Future studies are required to understand the cross talk between and among NK cells, dendritic cells, macrophages, and effector lymphocyte populations that are found in the microenvironment of the lesion.

Human Leukocyte Antigens and Antigen Presentation

A common problem in other chronic inflammatory and autoimmune conditions is the inability to properly identify self-antigens from non-self-antigens. Typically, when non-self-antigens enter the body, antigen-presenting cells (APC) such as dendritic cells and macrophages are able to process these antigens through the use of a major histocompatibility complex (MHC) class II and present them to a CD4+ T cell. This CD4+ T cell can, in turn, mount an adaptive immune response to the antigen. When T-cell development and maturation is functioning properly, T cells that are able to mount an immune response to self-antigens are selectively identified and undergo apoptosis through a process called negative selection. However, in autoimmune and other chronic inflammatory states, T cells that are able to mount an immune response to self-tissue enter the bloodstream and mount this abnormal immune response. The genes that encode for MHC class II are human leukocyte antigen (HLA)-DR, HLA-DQ, and HLA-DP. HLA-DM is responsible for aiding in the loading of antigens onto the MHC complex. Elevated transcript expressions of *HLA-DPA1, HLA-DPB1, HLA-DQA1* [56]*, HLA-DRA,* and *HLA-DMA* were found in endometriotic lesions [46]. These findings point toward potentially elevated numbers of antigen presenting cells in the endometriotic lesions or that the antigen presenting cells could be expressing increased MHC class II. Indeed, other reports show elevated protein levels of MHC class II and HLA-DR protein in endometriotic lesions [71]. Additionally, single nucleotide polymorphisms (SNPs) that influence HLA-DRA have been shown to be a risk factor for endometriosis [72]. Interestingly, increases in expression of MHC class II and HLAs are associated with chronic inflammation and autoimmune diseases such as vitiligo [73]. Endometriosis has long been considered an autoimmune disease due to the presence of self-antibodies, specifically anti-endometrial antibodies [74, 75]. Therefore, perhaps alterations in antigen presentation could explain this autoimmune-like inflammatory response.

In contrast, the expression of MHC class II in peritoneal fluid immune cells has been shown to be variable in endometriosis with some reports indicating decreased expression of MHC class II [76] or no differences in MHC class II expression [77]. Interestingly, in advanced tumors, the loss of HLA contributes to the ability of tumors to evade the immune system [78]. While immune evasion has been an interesting speculation that has been long considered in endometriosis, we still do not have mechanistic evidence to prove whether the immune system is unable to mount an immune response or if there are alterations in distinguishing between self and non-self. Nevertheless, the findings of altered profiles of antigen presenting gene machinery provide insight into the likely scenario of immune evasion theory. Ultimately, further studies are required to understand how and whether endometriotic lesions can evade the immune system and why chronic inflammation results instead, and if this represents two distinct molecular mechanisms that arrive at the same pathology.

Defensive Immune Responses

Several studies have shown elevated expression of antiviral and antibacterial genes such as Toll-like receptors (TLR2, TLR4, TLR9) [46, 79–81], inflammasome activation [40, 82], and complement activation [46, 56, 61, 83] in endometriosis patients. Because endometriosis is thought to be a sterile condition, the source stimulating these antiviral and antibacterial responses has been studied. It has been postulated that oxidative stress, hypoxia, apoptosis, and necrosis, which are present in endometriosis, are thought to stimulate endogenous ligands and danger signals such as inflammasome activation, heat shock proteins, neutrophil elastases, and fibronectin, which ultimately stimulate these antiviral and antibacterial genes [84]. Indeed, using a mouse model of endometriosis and human cell lines, it was shown that ERβ regulates TNF-α-induced apoptosis, which in turn led to inflammasome activation and proliferation in endometriotic lesions [40]. However, recent reports challenge the claim of sterile inflammation in endometriosis. Peritoneal fluid and menstrual effluent, collected from 20 women with endometriosis and 15 women without endometriosis showed elevated levels of endotoxin and an increased number of colony forming units of *Escherichia coli* [85]. In another study, endocervical swabs, peritoneal fluid, and endometrial biopsies from 73 endometriosis patients and 31 healthy fertile controls investigated the presence of bacteria using PCR including *Mycoplasma genitalium, Mycoplasma hominis, Ureaplasma urealyticum,* and *Ureaplasma parvum* [79]. This study shows no statistical difference between the presence of mollicutes in women with endometriosis compared to women without endometriosis, but they do show correlations between the presence of *U. parvum* with dyspareunia in endometriosis patients [79]. Perhaps women with endometriosis who also had or currently have *U. parvum* infection could have alterations in pelvic pain-related symptomology. Additionally, there is a well-established role of bacterial, viral,

and mycoplasmic infections in the progression of pelvic inflammatory disease and infertility. These findings add a new dimension to the already complex pathology of endometriosis. However, to date, comprehensive evidence suggesting the involvement of microorganisms in the pathogenesis of endometriosis is still lacking.

Complement

The complement system has been traditionally regarded as a component of innate immunity that plays a critical role in immune surveillance and the defense against microbial pathogens [86]. In recent years, however, emerging evidence has revealed that aberrant complement activation may create an immune imbalance that promotes tumorigenesis and the perpetuation of inflammation [87, 88]. In the context of endometriosis, Ahn et al. demonstrated increased expression of genes encoding numerous proteins of both the classical and alternative complement pathways in ectopic endometriotic lesions compared to control endometrium, including *C1QA, C1QB, C1R, C1S, C2, C3, C4A/B, C5, C6, C7, C8A, CFB, CFH,* and *CFI* [46]. In contrast, the gene encoding for C4BPA, an inhibitor of the classical complement pathway, was the only complement gene that exhibited reduced expression in endometriotic lesions compared to healthy endometrium [46]. In concordance with these findings, Suryawanshi et al. (2014) reported that the complement pathway was the most significantly dysregulated immune pathway in endometriosis and endometriosis-associated ovarian cancer [56]. Elevated gene expression of six complement pathway genes, including *C3, C4A, C7, CFH, CFD, CFB*, as well as decreased expression of *MASP1*, a component of the lectin pathway, was shown in endometriotic lesions compared to control endometrium [56]. Other reports have also highlighted the increased expression of complement components *C3* and *C5* in endometriotic lesions compared to the patient's matched endometrium and healthy control endometrium [83, 89, 90]. In particular, RNA sequencing revealed that *C3* was the most significantly upregulated gene within cultured endometrial stromal cells derived from endometriomas compared to paired eutopic endometrium [89]. Additionally, because the aberrant complement activation is predominantly found in the epithelial compartment of endometriotic lesions and that elevated complement proteins such as C7 are shown to contribute to ovarian cell proliferation [56], it is possible that complement is involved in the transition of endometriosis to epithelial clear cell carcinoma of the ovary. As such, excessive or aberrant complement activation within the microenvironment of the endometriotic lesion could possibly act to enhance the inflammatory response within the peritoneal cavity, thereby dysregulating immune cell activation and function. However, further studies are required to understand the initiation of complement activation and the downstream effects of activation on the disease progression.

Wound-Healing Mechanisms: Fibrosis, Adhesion, and Invasion

A characteristic feature of endometriotic lesions is their ability to adhere to and invade various surfaces within the peritoneal microenvironment, a process likely facilitated by intercellular adhesion molecules and their interaction with extracellular matrix components. One marker of severity in the ASRM-weighted staging of endometriosis is the presence and density of adhesions that encase the peritoneal organs and a reduction in organ mobility [20]. In addition to causing a seemingly extensive pathological reaction in the form of enhanced fibrosis and adhesion, fibrotic nodules in endometriotic lesions have been shown to have elevated innervation, which has been associated with elevated pain, specifically in deep infiltrating endometriosis [3, 4, 91]. Therefore, the processes of collagen deposition, extracellular matrix production, wound healing and fibrosis have been extensively studied in endometriosis and have been identified as a potential therapeutic target [92]. Indeed, Ahn et al. reported that eutopic endometrium from endometriosis patients displayed increased expression of genes encoding the cell adhesion molecules, *ICAM-1,-2,-3,-4,-5* and *SELL*, compared to control endometrium [46]. Additionally, various studies have demonstrated the aberrant expression of genes relating to cellular adhesion within endometriotic lesions [46, 89, 93]. Specifically, Wu et al. (2006) compared the gene expression of epithelial cells isolated by laser capture microdissection from the matched endometriotic lesion and endometrium of endometriosis patients [93]. Thirteen differentially expressed genes relating to focal adhesion were identified by KEGG pathway analysis, including *ERBB2, AKT1, IGF1R, ITGA7, PDGFB, PDGFRA, MAPK6, BAD, PXN, RAC1, RAF1, CAV2,* and *ACTN3* [93]. Furthermore, stromal cells derived from endometriomas have also demonstrated differential expression of various genes encoding integrins (*ITGA4*, *ITGA7*, *ITGA10*, *ITGA11*, and *ITGB4*) as well as other cell adhesion pathway components (*ESAM*, *CD6*, *CDH3*, *MAG*, *LRRC4B*, *NFASC*, *NLGN1*, and *NRXN1*) compared to eutopic stromal cells [89]. Taken together, these findings highlight that both eutopic endometrium and endometriotic lesions from endometriosis patients possess an altered, and possibly enhanced adhesive capacity, which could facilitate the binding of refluxed endometrial cells to ectopic sites within the peritoneal cavity. Like many other similar processes, inflammation and immune regulation tightly regulate fibrosis and collagen deposition, and numerous studies have shown dysregulated immune profiles in highly fibrotic diseases [94–96]. Therefore, it is not surprising that endometriotic lesions from endometriosis patients have differentially expressed gene signatures from wound-healing pathways [46] and that somatic mutations in endometriosis patients have been connected to fibrogenesis [97].

Genetic Variants Implicated in Immune Mechanisms of Endometriosis

In addition to understanding transcriptional alterations in endometriosis, identifying genetic variants to one's genome, which are directly inherited from one's parents, could lead to key understandings in the pathogenesis of the disease. The heritability

of endometriosis is approximately 47–52% [98, 99]. Therefore, there are some women with a predisposed risk of developing endometriosis. To understand specifically how these patients differ from other women, genetic variants have been assessed, typically by genome-wide association studies (GWAS), to understand SNPs in patients with endometriosis compared to healthy women. To date, large-scale studies have identified a number of SNPs that are associated with endometriosis in Japanese and Caucasian populations [100–106]. These SNPs have revealed candidate genes that feed into functional pathways associated with endometriosis including estrogen responsivity, Wnt signaling, inflammation, cellular adhesion, and kinases [100–106]. While many gene candidates have been extracted from this data, inflammation and immune modulating genes are clearly involved. Implicating functional phenotypic pathways from the genome-wide association studies in endometriosis is adequately reviewed [107]. In particular, a number of cytokines and chemokines have been shown to be gene candidates [102], of particular interest is the IL1A locus [105, 106, 108]. However, the accurate functional role of these SNPs in the context of the disease may not be accurately depicted, as some gene candidates may have many different roles within the body. For example, the Wnt signaling pathway and mitogen-activated protein kinase signaling pathway, which have both been repeatedly selected as a gene candidate through GWAS studies [100, 102–104], have significant contributions for immune cell signaling and therefore immune responses [109] but also function in many other processes. Therefore, from genetic variants, SNPs can cause alterations in immune modulation and could be contributing to the immune evasion that we observe in endometriosis patients.

Summary

Overall, a staggering number of immune-inflammatory gene transcripts and gene variants are differentially expressed in endometriosis patients compared to healthy fertile controls. At this time, transcriptomic and genomic analyses have provided new avenues for research to identify molecular mechanisms of immune dysfunction. In turn, this has created many non-hormonal therapeutic targets; however, further research is required to understand the functional role of these targets and evaluate their potential use. It is also possible that immune-inflammatory-related gene signatures could be used to sub-classify patients or to provide risk-assessment for patients; however, subsequent studies with large patient cohorts are required.

References

1. Levy AR, Osenenko KM, Lozano-Ortega G, Sambrook R, Jeddi M, Bélisle S, Reid RL. Economic burden of surgically confirmed endometriosis in Canada. J Obstet Gynaecol Can. 2011;33:830–7.

2. Fuldeore M, Yang H, Du EX, Soliman AM, Wu EQ, Winkel C. Healthcare utilization and costs in women diagnosed with endometriosis before and after diagnosis: a longitudinal analysis of claims databases. Fertil Steril. 2015;103:163–71.
3. McKinnon BD, Bertschi D, Bersinger NA, Mueller MD. Inflammation and nerve fiber interaction in endometriotic pain. Trends Endocrinol Metab. 2015;26:1–10.
4. Anaf V, Simon P, El Nakadi I, Fayt I, Buxant F, Simonart T, Peny MO, Noel JC. Relationship between endometriotic foci and nerves in rectovaginal endometriotic nodules. Hum Reprod. 2000;15:1744–50.
5. Khan KN, Kitajima M, Hiraki K, Fujishita A, Nakashima M, Masuzaki H. Visible and occult microscopic lesions of endometriosis. Gynecol Minim Invasive Ther. 2014;3:109–14.
6. Aghajanova L, Tatsumi K, Horcajadas JA, Zamah AM, Esteban FJ, Herndon CN, Conti M, Giudice LC. Unique transcriptome, pathways, and networks in the human endometrial fibroblast response to progesterone in endometriosis. Biol Reprod. 2011;84:801–15.
7. Klemmt PAB, Starzinski-Powitz A. Molecular and cellular pathogenesis of endometriosis. Curr Womens Health Rev. 2018;14:106–16.
8. Sampson JA. Peritoneal endometriosis due to the menstrual dissemination of endometrial tissue into the peritoneal cavity. Am J Obstet Gynecol. 1927;14:422–69.
9. Giudice LC, Kao LC. Endometriosis. Lancet. 2004;364:1789–99.
10. Jerman LF, Hey-Cunningham AJ. The role of the lymphatic system in endometriosis: a comprehensive review of the Literature. Biol Reprod. 2015;92:64. https://doi.org/10.1095/biolreprod.114.124313.
11. Beavis AL, Matsuo K, Grubbs BH, Srivastava SA, Truong CM, Moffitt MN, Maliglig AM, Lin YG. Endometriosis in para-aortic lymph nodes during pregnancy: case report and review of literature. Fertil Steril. 2011;95:2429.e9–2429.e13.
12. Sampson JA. Metastatic or embolic endometriosis, due to the menstrual dissemination of endometrial tissue into the venous circulation. Am J Pathol. 1927;3:93–110.43.
13. Matsuura K, Ohtake H, Katabuchi H, Okamura H. Coelomic metaplasia theory of endometriosis: evidence from in vivo studies and an in vitro experimental model. Gynecol Obstet Investig. 1999;47:18–22.
14. Giudice LC. Clinical practice. Endometriosis. N Engl J Med. 2010;362:2389–98.
15. BALLARD K, LOWTON K, WRIGHT J. What's the delay? A qualitative study of women's experiences of reaching a diagnosis of endometriosis. Fertil Steril. 2006;86:1296–301.
16. Nnoaham KE, Hummelshoj L, Webster P, d'Hooghe T, de Cicco NF, de Cicco NC, Jenkinson C, Kennedy SH, Zondervan KT, World Endometriosis Research Foundation Global Study of Women's Health consortium. Impact of endometriosis on quality of life and work productivity: a multicenter study across ten countries. Fertil Steril. 2011;96:366–373.e8.
17. Nisenblat V, Bossuyt PM, Shaikh R, Farquhar C, Jordan V, Scheffers CS, Mol BWJ, Johnson N, Hull ML. Blood biomarkers for the non-invasive diagnosis of endometriosis. Cochrane Database Syst Rev. 2016; https://doi.org/10.1002/14651858.CD012179.
18. May KE, Conduit-Hulbert SA, Villar J, Kirtley S, Kennedy SH, Becker CM. Peripheral biomarkers of endometriosis: a systematic review. Hum Reprod Update. 2010;16:651–74.
19. Fassbender A, Burney RO, O DF, D'Hooghe T, Giudice L. Update on biomarkers for the detection of endometriosis. Biomed Res Int. 2015;2015:1–14.
20. Schenken RS, Guzick DS. Endometriosis classification and infertility. Fertil Steril. 1997;67(5):815–6.
21. Johnson NP, Hummelshoj L, Adamson GD, et al. World Endometriosis Society consensus on the classification of endometriosis. Hum Reprod. 2017;32:315–24.
22. Nisolle M, Donnez J. Peritoneal endometriosis, ovarian endometriosis, and adenomyotic nodules of the rectovaginal septum are three different entities. Fertil Steril. 1997;68:585–96.
23. Bertschi D, McKinnon BD, Evers J, Bersinger NA, Mueller MD. Enhanced inflammatory activity of endometriotic lesions from the rectovaginal septum. Mediat Inflamm. 2013;2013:450950.

24. Chapron C, Fauconnier A, Dubuisson J-B, Barakat H, Vieira M, Bréart G. Deep infiltrating endometriosis: relation between severity of dysmenorrhoea and extent of disease. Hum Reprod. 2003;18:760–6.
25. Koninckx PR, Meuleman C, Demeyere S, Lesaffre E, Cornillie FJ. Suggestive evidence that pelvic endometriosis is a progressive disease, whereas deeply infiltrating endometriosis is associated with pelvic pain. Fertil Steril. 1991;55:759–65.
26. Tosti C, Pinzauti S, Santulli P, Chapron C, Petraglia F. Pathogenetic mechanisms of deep infiltrating endometriosis. Reprod Sci. 2015;22:1053–9.
27. Kvaskoff M, Horne AW, Missmer SA. Informing women with endometriosis about ovarian cancer risk. Lancet. 2017;390:2433–4.
28. Wendel JRH, Wang X, Hawkins SM. The Endometriotic tumor microenvironment in ovarian cancer. Cancers (Basel). 2018;10 https://doi.org/10.3390/cancers10080261.
29. Practice Committee of the American Society for Reproductive Medicine. Treatment of pelvic pain associated with endometriosis: a committee opinion. Fertil Steril. 2014;101:927–35.
30. Vignali M, Bianchi S, Candiani M, Spadaccini G, Oggioni G, Busacca M. Surgical treatment of deep endometriosis and risk of recurrence. J Minim Invasive Gynecol. 2005;12:508–13.
31. Marcoux S, Maheux R, Bérubé S, Endometriosis the CCG on. Laparoscopic surgery in infertile women with minimal or mild endometriosis. N Engl J Med. 1997;337:217–22.
32. Jacobson TZ, Duffy JM, Barlow DH, Farquhar C, Koninckx PR, Olive D. Laparoscopic surgery for subfertility associated with endometriosis. Cochrane Database Syst Rev. 2010; https://doi.org/10.1002/14651858.CD001398.pub2.
33. Monsanto SP, Edwards AK, Zhou J, Nagarkatti P, Nagarkatti M, Young SL, Lessey BA, Tayade C. Surgical removal of endometriotic lesions alters local and systemic proinflammatory cytokines in endometriosis patients. Fertil Steril. 2016;105:968–977.e5.
34. Dun EC, Taylor HS. Elagolix: a promising oral GnRH antagonist for endometriosis-associated pain. Oncotarget. 2017;8:99219–20.
35. Symons LK, Miller JE, Kay VR, Marks RM, Liblik K, Koti M, Tayade C. The immunopathophysiology of endometriosis. Trends Mol Med. 2018;24:748–62.
36. Halme J, Hammond MG, Hulka JF, Raj SG, Talbert LM. Retrograde menstruation in healthy women and in patients with endometriosis. Obstet Gynecol. 1984;64:151–4.
37. Kvaskoff M, Mu F, Terry KL, Harris HR, Poole EM, Farland L, Missmer SA. Endometriosis: a high-risk population for major chronic diseases? Hum Reprod Update. 2015;21:500–16.
38. Bungum HF, Vestergaard C, Knudsen UB. Endometriosis and type 1 allergies/immediate type hypersensitivity: a systematic review. Eur J Obstet Gynecol Reprod Biol. 2014;179:209–15.
39. Capobianco A, Rovere-Querini P. Endometriosis, a disease of the macrophage. Front Immunol. 2013;4:9.
40. Han SJ, Jung SY, Wu S-P, et al. Estrogen receptor β modulates apoptosis complexes and the Inflammasome to drive the pathogenesis of endometriosis. Cell. 2015;163:960–74.
41. Fox C, Morin S, Jeong J-W, Scott RT, Lessey BA. Local and systemic factors and implantation: what is the evidence? Fertil Steril. 2016;105:873–84.
42. Miller JE, Ahn SH, Monsanto SP, Khalaj K, Koti M, Tayade C. Implications of immune dysfunction on endometriosis associated infertility. Oncotarget. 2016;8(4):7138–47. https://doi.org/10.18632/oncotarget.12577.
43. Kao LC, Germeyer A, Tulac S, Lobo S, Yang JP, Taylor RN, Osteen K, Lessey BA, Giudice LC. Expression profiling of endometrium from women with endometriosis reveals candidate genes for disease-based implantation failure and infertility. Endocrinology. 2003;144:2870–81.
44. Gashaw I, Hastings JM, Jackson KS, Winterhager E, Fazleabas AT. Induced endometriosis in the baboon (Papio anubis) increases the expression of the proangiogenic factor CYR61 (CCN1) in eutopic and ectopic endometria. Biol Reprod. 2006;74:1060–6.
45. Afshar Y, Hastings J, Roqueiro D, Jeong J-W, Giudice LC, Fazleabas AT. Changes in Eutopic endometrial gene expression during the progression of experimental endometriosis in the baboon, Papio Anubis. Biol Reprod. 2013;88:44.

46. Ahn SH, Khalaj K, Young SL, Lessey BA, Koti M, Tayade C. Immune-inflammation gene signatures in endometriosis patients. Fertil Steril. 2016;106:1420–1431.e7. https://doi.org/10.1016/j.fertnstert.2016.07.005.
47. Tamaresis JS, Irwin JC, Goldfien GA, Rabban JT, Burney RO, Nezhat C, DePaolo LV, Giudice LC. Molecular classification of endometriosis and disease stage using high-dimensional genomic data. Endocrinology. 2014;155:4986–99.
48. Kopelman A, Girão MJBC, Bonetti TCS, Carvalho CV, da Silva IDCG, Schor E. Analysis of gene expression in the endocervical epithelium of women with deep endometriosis. Reprod Sci. 2016;23:1269–74.
49. Nikoo S, Ebtekar M, Jeddi-Tehrani M, Shervin A, Bozorgmehr M, Vafaei S, Kazemnejad S, Zarnani A-H. Menstrual blood-derived stromal stem cells from women with and without endometriosis reveal different phenotypic and functional characteristics. Mol Hum Reprod. 2014;20:905–18.
50. Barragan F, Irwin JC, Balayan S, et al. Human endometrial fibroblasts derived from mesenchymal progenitors inherit progesterone resistance and acquire an inflammatory phenotype in the endometrial niche in endometriosis1. Biol Reprod. 2016;94:118. https://doi.org/10.1095/biolreprod.115.136010.
51. Fassbender A, Verbeeck N, Börnigen D, et al. Combined mRNA microarray and proteomic analysis of eutopic endometrium of women with and without endometriosis. Hum Reprod. 2012;27:2020–9.
52. Garcia-Velasco JA, Fassbender A, Ruiz-Alonso M, Blesa D, D'Hooghe T, Simon C. Is endometrial receptivity transcriptomics affected in women with endometriosis? A pilot study. Reprod Biomed Online. 2015;31:647–54.
53. Brunham RC, Gottlieb SL, Paavonen J. Pelvic inflammatory disease. N Engl J Med. 2015;372:2039–48.
54. Gentilini D, Perino A, Vigano P, Chiodo I, Cucinella G, Vignali M, Di Blasio AM, Busacca M. Gene expression profiling of peripheral blood mononuclear cells in endometriosis identifies genes altered in non-gynaecologic chronic inflammatory diseases. Hum Reprod. 2011;26:3109–17.
55. Salmeri FM, Laganà AS, Sofo V, Triolo O, Sturlese E, Retto G, Pizzo A, D'Ascola A, Campo S. Behavior of tumor necrosis factor-α and tumor necrosis factor receptor 1/tumor necrosis factor receptor 2 system in mononuclear cells recovered from peritoneal fluid of women with endometriosis at different stages. Reprod Sci. 2015;22:165–72.
56. Suryawanshi S, Huang X, Elishaev E, et al. Complement pathway is frequently altered in endometriosis and endometriosis-associated ovarian cancer. Clin Cancer Res. 2014;20:6163–74.
57. Borrelli GM, Carvalho KI, Kallas EG, Mechsner S, Baracat EC, Abrão MS. Chemokines in the pathogenesis of endometriosis and infertility. J Reprod Immunol. 2013;98:1–9.
58. Ahn SH, Monsanto SP, Miller C, Singh SS, Thomas R, Tayade C. Pathophysiology and immune dysfunction in endometriosis. Biomed Res Int. 2015;2015:1. https://doi.org/10.1155/2015/795976.
59. Podgaec S, Abrao MS, Dias JA, Rizzo LV, de Oliveira RM, Baracat EC. Endometriosis: an inflammatory disease with a Th2 immune response component. Hum Reprod. 2007;22:1373–9.
60. Chegini N, Roberts M, Ripps B. Differential expression of interleukins (IL)-13 and IL-15 in ectopic and eutopic endometrium of women with endometriosis and normal fertile women. Am J Reprod Immunol. 2003;49:75–83.
61. Beste MT, Pfäffle-Doyle N, Prentice EA, Morris SN, Lauffenburger DA, Isaacson KB, Griffith LG. Molecular network analysis of endometriosis reveals a role for c-Jun–regulated macrophage activation. Sci Transl Med. 2014;6:222ra16.
62. Bakhtiarizadeh MR, Hosseinpour B, Shahhoseini M, Korte A, Gifani P. Weighted gene coexpression network analysis of endometriosis and identification of functional modules associated with its main hallmarks. Front Genet. 2018;9:453.
63. Ahn SH, Singh V, Tayade C. Biomarkers in endometriosis: challenges and opportunities. Fertil Steril. 2017;107:523–32.

64. McArdel SL, Terhorst C, Sharpe AH. Roles of CD48 in regulating immunity and tolerance. Clin Immunol. 2016;164:10–20.
65. Takamura M, Koga K, Izumi G, Hirata T, Harada M, Hirota Y, Hiraike O, Fujii T, Osuga Y. Simultaneous detection and evaluation of four subsets of CD4+ T lymphocyte in lesions and peripheral blood in endometriosis. Am J Reprod Immunol. 2015;74:480–6.
66. Olkowska-Truchanowicz J, Bocian K, Maksym RB, Bialoszewska A, Wlodarczyk D, Baranowski W, Zabek J, Korczak-Kowalska G, Malejczyk J. CD4+ CD25+ FOXP3+ regulatory T cells in peripheral blood and peritoneal fluid of patients with endometriosis. Hum Reprod. 2013;28:119–24.
67. Tariverdian N, Siedentopf F, Rücke M, Blois SM, Klapp BF, Kentenich H, Arck PC. Intraperitoneal immune cell status in infertile women with and without endometriosis. J Reprod Immunol. 2009;80:80–90.
68. Paula R, Oliani AH, Vaz-Oliani DCM, D'Ávila SCGP, Oliani SM, Gil CD. The intricate role of mast cell proteases and the annexin A1-FPR1 system in abdominal wall endometriosis. J Mol Histol. 2015;46:33–43.
69. Binda MM, Donnez J, Dolmans M-M. Targeting mast cells: a new way to treat endometriosis. Expert Opin Ther Targets. 2017;21:67–75.
70. Jeung I, Cheon K, Kim M-R, Jeung I, Cheon K, Kim M-R. Decreased cytotoxicity of peripheral and peritoneal natural killer cell in endometriosis. Biomed Res Int. 2016;2016:1–6.
71. Ota H, Igarashi S. Expression of major histocompatibility complex class II antigen in endometriotic tissue in patients with endometriosis and adenomyosis. Fertil Steril. 1993;60:834–8.
72. Sobalska-Kwapis M, Smolarz B, Słomka M, et al. New variants near RHOJ and C2, HLA-DRA region and susceptibility to endometriosis in the Polish population—The genome-wide association study. Eur J Obstet Gynecol Reprod Biol. 2017;217:106–12.
73. Cavalli G, Hayashi M, Jin Y, et al. MHC class II super-enhancer increases surface expression of HLA-DR and HLA-DQ and affects cytokine production in autoimmune vitiligo. Proc Natl Acad Sci U S A. 2016;113:1363–8.
74. Wild RA, Shivers CA. Antiendometrial antibodies in patients with endometriosis. Am J Reprod Immunol Microbiol. 1985;8:84–6.
75. Fernández-Shaw S, Hicks BR, Yudkin PL, Kennedy S, Barlow DH, Starkey PM. Anti-endometrial and anti-endothelial auto-antibodies in women with endometriosis. Hum Reprod. 1993;8:310–5.
76. Lee K-S, Baek D-W, Kim K-H, Shin B-S, Lee D-H, Kim J-W, Hong Y-S, Bae Y-S, Kwak J-Y. IL-10-dependent down-regulation of MHC class II expression level on monocytes by peritoneal fluid from endometriosis patients. Int Immunopharmacol. 2005;5:1699–712.
77. Na Y-J, Jin J-O, Lee M-S, Song M-G, Lee K-S, Kwak J-Y. Peritoneal fluid from endometriosis patients switches differentiation of monocytes from dendritic cells to macrophages. J Reprod Immunol. 2008;77:63–74.
78. McGranahan N, Rosenthal R, Hiley CT, et al. Allele-specific HLA loss and immune escape in lung cancer evolution. Cell. 2017;171:1259–1271.e11.
79. Campos GB, Marques LM, Rezende IS, Barbosa MS, Abrão MS, Timenetsky J. Mycoplasma genitalium can modulate the local immune response in patients with endometriosis. Fertil Steril. 2018;109:549–560.e4.
80. Yeo SG, Won YS, Lee HY, Kim YI, Lee J-W, Park DC. Increased expression of pattern recognition receptors and nitric oxide synthase in patients with endometriosis. Int J Med Sci. 2013;10:1199–208.
81. Hayashi C, Chishima F, Sugitani M, Ichikawa G, Nakazawa-Watanabe T, Sugita K, Suzuki M, Nemoto N, Yamamoto T. Relationship between toll-like receptor-4 and mPGES-1 gene expression in local lesions of endometriosis patients. Am J Reprod Immunol. 2013;69:231–9.
82. Chang C-M, Wang M-L, Lu K-H, Yang Y-P, Juang C-M, Wang P-H, Hsu R-J, Yu M-H, Chang C-C. Integrating the dysregulated inflammasome-based molecular functionome in the malignant transformation of endometriosis-associated ovarian carcinoma. Oncotarget. 2018;9:3704–26.

83. Tao XJ, Sayegh RA, Isaacson KB. Increased expression of complement component 3 in human ectopic endometrium compared with the matched eutopic endometrium. Fertil Steril. 1997;68:460–7.
84. Kajihara H, Yamada Y, Kanayama S, Furukawa N, Noguchi T, Haruta S, Yoshida S, Sado T, Oi H, Kobayashi H. New insights into the pathophysiology of endometriosis: from chronic inflammation to danger signal. Gynecol Endocrinol. 2011;27:73–9.
85. Khan KN, Kitajima M, Hiraki K, Yamaguchi N, Katamine S, Matsuyama T, Nakashima M, Fujishita A, Ishimaru T, Masuzaki H. Escherichia coli contamination of menstrual blood and effect of bacterial endotoxin on endometriosis. Fertil Steril. 2010;94:2860–3-3.
86. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. Nat Immunol. 2010;11:785–97.
87. Reis ES, Mastellos DC, Ricklin D, Mantovani A, Lambris JD. Complement in cancer: untangling an intricate relationship. Nat Rev Immunol. 2018;18:5–18.
88. Ricklin D, Lambris JD. Complement in immune and inflammatory disorders: pathophysiological mechanisms. J Immunol. 2013;190:3831–8.
89. Rekker K, Saare M, Eriste E, et al. High-throughput mRNA sequencing of stromal cells from endometriomas and endometrium. Reproduction. 2017;154:93–100.
90. Aslan C, Ak H, Askar N, Ozkaya AB, Ergenoglu AM, Yeniel AO, Akdemir A, Aydin HH. Overexpression of complement C5 in endometriosis. Clin Biochem. 2014;47:496–8.
91. Wu J, Xie H, Yao S, Liang Y. Macrophage and nerve interaction in endometriosis. J Neuroinflammation. 2017;14:53.
92. Bałkowiec M, Maksym RB, Włodarski PK. The bimodal role of matrix metalloproteinases and their inhibitors in etiology and pathogenesis of endometriosis (Review). Mol Med Rep. 2018;18:3123–36.
93. Wu Y, Kajdacsy-Balla A, Strawn E, Basir Z, Halverson G, Jailwala P, Wang Y, Wang X, Ghosh S, Guo S-W. Transcriptional characterizations of differences between eutopic and ectopic endometrium. Endocrinology. 2006;147:232–46.
94. Desai O, Winkler J, Minasyan M, Herzog EL. The role of immune and inflammatory cells in idiopathic pulmonary fibrosis. Front Med. 2018;5:43.
95. Gieseck RL, Wilson MS, Wynn TA. Type 2 immunity in tissue repair and fibrosis. Nat Rev Immunol. 2017;18:62. https://doi.org/10.1038/nri.2017.90.
96. Tang PM-K, Nikolic-Paterson DJ, Lan H-Y. Macrophages: versatile players in renal inflammation and fibrosis. Nat Rev Nephrol. 2019;15(3):144–58.
97. Guo S-W. Cancer driver mutations in endometriosis: variations on the major theme of fibrogenesis. Reprod Med Biol. 2018;17:369–97.
98. Treloar SA, O'Connor DT, O'Connor VM, Martin NG. Genetic influences on endometriosis in an Australian twin sample. sueT@qimr.edu.au. Fertil Steril. 1999;71:701–10.
99. Saha R, Pettersson HJ, Svedberg P, Olovsson M, Bergqvist A, Marions L, Tornvall P, Kuja-Halkola R. Heritability of endometriosis. Fertil Steril. 2015;104:947–52.
100. Nyholt DR, Low S-K, Anderson CA, et al. Genome-wide association meta-analysis identifies new endometriosis risk loci. Nat Genet. 2012;44:1355–9.
101. Uno S, Zembutsu H, Hirasawa A, Takahashi A, Kubo M, Akahane T, Aoki D, Kamatani N, Hirata K, Nakamura Y. A genome-wide association study identifies genetic variants in the CDKN2BAS locus associated with endometriosis in Japanese. Nat Genet. 2010;42:707–10.
102. Uimari O, Rahmioglu N, Nyholt DR, Vincent K, Missmer SA, Becker C, Morris AP, Montgomery GW, Zondervan KT. Genome-wide genetic analyses highlight mitogen-activated protein kinase (MAPK) signaling in the pathogenesis of endometriosis. Hum Reprod. 2017;32:780–93.
103. Rahmioglu N, Macgregor S, Drong AW, et al. Genome-wide enrichment analysis between endometriosis and obesity-related traits reveals novel susceptibility loci. Hum Mol Genet. 2015;24:1185–99.
104. Pagliardini L, Gentilini D, Vigano' P, Panina-Bordignon P, Busacca M, Candiani M, Di Blasio AM. An Italian association study and meta-analysis with previous GWAS confirm

WNT4, CDKN2BAS and FN1 as the first identified susceptibility loci for endometriosis. J Med Genet. 2013;50:43–6.
105. Gajbhiye R, McKinnon B, Mortlock S, Mueller M, Montgomery G. Genetic variation at chromosome 2q13 and its potential influence on endometriosis susceptibility through effects on the IL-1 family. Reprod Sci. 2018;25:1307–17.
106. Sapkota Y, Steinthorsdottir V, Morris AP, et al. Meta-analysis identifies five novel loci associated with endometriosis highlighting key genes involved in hormone metabolism. Nat Commun. 2017;8:15539.
107. Zondervan KT, Rahmioglu N, Morris AP, Nyholt DR, Montgomery GW, Becker CM, Missmer SA. Beyond endometriosis genome-wide association study: from genomics to phenomics to the patient. Semin Reprod Med. 2016;34:242–54.
108. Sapkota Y, Low S-K, Attia J, et al. Association between endometriosis and the interleukin 1A (IL1A) locus. Hum Reprod. 2015;30:239–48.
109. Chae W-J, Bothwell ALM. Canonical and non-canonical Wnt signaling in immune cells. Trends Immunol. 2018;39:830–47.

Chapter 11
Gene Expression in Endometriosis

Niraj Joshi, Ren-Wei Su, and Asgerally Fazleabas

Introduction

Endometriosis is an inflammatory gynecological condition which is characterized by the presence of ectopic endometrial tissue outside of uterine cavity including peritoneal wall, ovaries, fallopian tubes, and bladder [1–3]. Endometriosis is generally categorized into three subtypes: (i) superficial peritoneal (SUP), (ii) endometrioma (OMA), and (iii) deeply infiltrating endometriosis (DIE) [4]. Superficial peritoneal (SUP) endometriosis refers to lesions that are found on the surface of the peritoneum in the peritoneal cavity. Endometrioma is a large fluid-filled cyst localized on the ovaries, and DIE is defined by the penetration of lesions into the underlying tissue to a depth greater than 5 mm. Endometriosis is associated with infertility and pelvic pain. In general, up to 50% of women with endometriosis are infertile and >70% of women suffer from pelvic pain, respectively [5–7]. In spite of years of research, we still do not have a good understanding as to how endometriotic lesions are able to survive in the hostile ectopic environment and of the genetic and epigenetic changes they acquire to enable their persistent existence at ectopic sites. Moreover, the presence of ectopic endometrial tissue also alters the gene signature in the eutopic endometrium as evidenced by abnormal, heavy, and

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painful menstruation and compromised fertility. These data suggest that the ectopic lesions can induce major changes in the eutopic gene expression pattern in women with endometriosis.

Theories on Endometriosis Pathogenesis

Although the existence of this enigmatic disease has been known for more than a century, and despite intense research, the exact pathogenesis of endometriosis and its origin remain highly elusive. There are multiple theories that have been proposed regarding the etiology and pathogenesis of endometriosis. The most widely accepted one is Sampson's theory of retrograde menstruation [8]. According to this theory, retrograde menstruation results in the reflux of viable endometrial tissue through the fallopian tubes into the peritoneal cavity where it may implant at ectopic locations and develop lesions. This theory is supported by the evidence that women affected with endometriosis have greater volumes of refluxed blood during menses compared to controls, and lesions are localized surrounding the terminal ends of fimbriae of fallopian tubes. Additionally, the nonhuman primate model of endometriosis developed by others and us utilizes a similar method which consists of depositing the autologous menstrual effluent into the peritoneal cavity of these animals (details are discussed in the following section of this chapter) [9, 10]. Although Sampson's hypothesis of retrograde menstruation is widely accepted, it is important to note that up to 90% of women experience retrograde menstruation, but only 10–15% of these women develop the disease [9], suggesting the involvement of other factors and molecular mechanisms responsible for the pathogenesis of endometriosis [11].

The inflammatory environment of the peritoneal cavity that is caused by refluxed endometrial cells or other pathological conditions favors the implantation, proliferation, and invasion of ectopic endometrium to contribute to the establishment and development of endometriosis [12]. This may explain the large difference in the percentage of women who experience retrograde menstruation versus the women who develop endometriosis. Endometrial stem/progenitor cell implantation is another expansion of the retrograde menstruation theory, according to which the endometrial epithelial progenitor cells and mesenchymal stem-cell-like cells together with their niche cells are seeded into the peritoneum via retrograde menstruation and contribute to the establishment of ectopic implants. Stem cells have been found to be present in the endometrial basalis as well as the functionalis [13], and an increase in the transport of bone-marrow-derived stem cells to the uterus during inflammation has been reported [14]. As these cells are present in menstrual blood, they may also be present in the refluxed menstrual tissue and implant into the peritoneal cavity [13, 15].

Additionally, there are also other theories for endometriosis pathogenesis which do not support the hypothesis that the disease originates from the eutopic endometrium. This is based on the observation of endometriosis in patients without an

endometrium, such as patients with Mayer–Rokitansky–Küster–Hauser syndrome [16]. Further, the coelomic metaplasia theory suggests that the underlying mesothelium of the ovary may undergo transformation into the endometrial tissue under the influence of estrogen. The in vitro 3D culture model further supports this theory: when both ovarian surface epithelium and ovarian stromal cells are cultured in the presence of estradiol in a three-dimensional collagen gel lattice, the ovarian surface epithelium cells formed a lumen structure, surrounded by endometrial stromal cells with an epithelial mesenchymal structure [17]. This theory is particularly relevant to OMA [18]. In reality, endometriotic lesions may develop from a combination of some or all of these theories.

Nonhuman Primate Endometriosis Models

A major limitation to understanding the pathophysiology of endometriosis is the extremely long duration from the initiation of the disease until the onset of symptoms, and the diagnosis which takes on average of 8–11 years. Therefore, to study the mechanisms of disease onset and early development, animal models in which the onset of disease can be exactly controlled are necessary [19]. Menstruating primates can develop spontaneous endometriosis although detection rates are generally reported as being low. Spontaneous disease with lesions resembling those in women has been reported in cynomolgus monkeys, macaques, baboons, and other nonhuman primates [9, 19–22]. Several nonhuman primates have been used for the development of experimental endometriosis, including Japanese macaque, pig-tailed macaque, rhesus monkeys, and baboons [1]. Endometriosis has also been successfully induced in cynomolgus monkeys [23] and the nonmenstruating common marmoset monkey [24]. The use of nonhuman primates is advantageous for the study of endometriosis because they are phylogenetically similar to humans, and nonhuman primate models allow the evaluation of disease pathogenesis as well as exploring therapeutic targets for the treatment of endometriosis.

Among all the available nonhuman primate models, the baboon (*Papio anubis*) is the most favorable because of its size, similar reproductive anatomy to human, and other advantages [1, 19, 25]. Using baboons, others and we have established reproducible protocols to induce lesions in the peritoneum based on autologous inoculation of menstrual tissue [1, 25–27]. Following inoculation with autologous menstrual tissues for two consecutive menstrual cycles, baboons develop peritoneal lesions that are similar to those seen in humans. The majority of the lesions found 1 month after tissue inoculation are red lesions, which frequently change color during the disease progression. In contrast, blue lesions remain consistently blue, while white lesions are evident in the later stages of the disease process and often regressed [28]. After the induction of endometriosis in baboons, a laparoscopic procedure is performed at different time points to study the disease progression, and an endometrectomy is performed to harvest the eutopic endometrial tissues. In another study,

deep nodules have been induced in the baboon model with results suggesting that migration of endometriotic glandular epithelial cells, especially those cells along the invasion front play an important role in the invasion process of deep lesions in this species [29].

Progesterone Resistance in Endometriosis

Progesterone action is crucial for the normal functioning of the female reproductive tract, and it exerts its action through progesterone receptors (PGR) [30–32]. Progesterone regulates various gene networks (Fig. 11.1) in endometrium including vital epithelial–stromal interactions required for optimal decidual response and implantation [33–41], which are indispensable, for the establishment of pregnancy [31, 42]. Sequential analysis of the same animals during disease progression in our baboon model demonstrates an early disease insult and a transitory dominance of an estrogenic phenotype, however, as the disease progresses, a progesterone-resistant phenotype becomes evident [43]. Progesterone-dependent gene signature is altered due to compromised progesterone signaling in women with endometriosis [44–49]. Although a number of attempts have been made to analyze the differential expression of the progesterone receptor in the eutopic endometrium of women with and without endometriosis, the data remains highly inconsistent and nonconclusive [50–57]. Further, evidence from genetic and epigenetic studies strongly advocates for the notion that the eutopic endometrium of patients with endometriosis responds differently to circulating progesterone compared to the nondiseased endometrium [47, 48, 58–65]. This observation has been further confirmed in our baboon model of induced endometriosis [43, 46, 66–68]. Many genes that are regulated by progesterone and have been demonstrated to play key roles in decidualization are decreased in women with endometriosis, such as cyclic AMP-responsive element-binding protein 3-like protein 1 (CREB3L1) [69], cysteine-rich secretory protein LCCL domain 2 (CRISPLD2) [70], nuclear receptor subfamily 2 group F member 2 (NR2F2), and the gene that codes COUP-TFII [35, 71, 72]. Taken together, these reports support the notion of compromised progesterone signaling in the eutopic endometrium of women with endometriosis. The term progesterone resistance is used to describe the altered response of endometrial tissue to circulating progesterone leading to dysregulated progesterone-regulated gene networks affecting vital female reproductive tract functions [57, 58, 66, 73, 74].

Homeobox A10 (HOXA10) is one of the well-documented progesterone target genes in the context of uterine biology and endometriosis (Fig. 11.1). In mice, targeted deletion of HOXA10 leads to severe decidualization defects [75]. In human, HOXA10 is expressed in both endometrial epithelial and stromal cells and is regulated by progesterone [76–78]. The expression of HOXA10 peaks at the time of embryo implantation and is necessary for decidualization [79, 80]. Decidualized stromal cells secrete various proteins, including PRL and IGFBP1 [81], which are considered markers of decidualization. HOXA10 upregulates IGFBP1 promoter

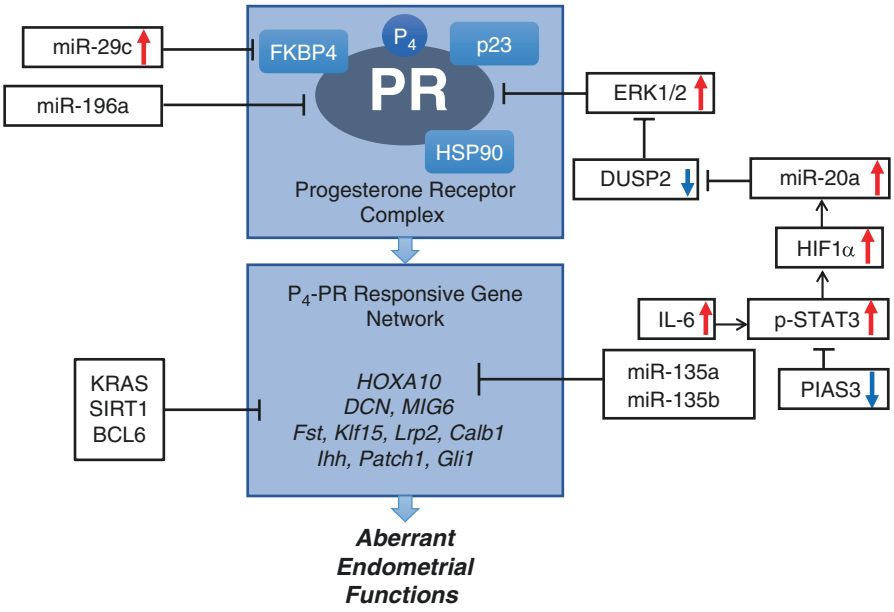

Fig. 11.1 Proposed mechanism of progesterone resistance in endometriosis. Optimal progesterone signaling is critical for normal endometrial function. Aberrant microRNA expression affects the progesterone receptor complex. In addition, inflammation (IL-6) and dysregulated signaling pathways (STAT3, ERK, and KRAS) further contribute to the dysregulated progesterone signaling affecting progesterone-regulated gene networks leading to aberrant endometrial function as a consequence of endometriosis

activity together with FOXO1, a key transcription factor in early decidualization response [82], in baboon endometrial stromal cells [83]. In the baboon model, a decrease in HOXA10 transcript levels has been observed after 3, 6, 12, and 16 months of disease induction, which reaches statistical significance at 12 and 16 months. HOXA10 protein levels are decreased in both the epithelial and stromal cells of the endometrium [78]. Further, laparoscopic endometrioma resection increases peri-implantation endometrial HOXA10 expression, suggesting a strong correlation of HOXA10 expression with endometriosis [84]. Other than altered progesterone signaling, there are other regulators of HOXA10 expression in endometriosis. Multiple studies reported hypermethylation of the HOXA10 promoter region in the eutopic endometrium of women and baboons with endometriosis [78, 85–88]. Interestingly, the methylation level of HOXA10 is higher in eutopic endometrium compared to ectopic endometrium [87]. The fact that HOXA10 mRNA and protein levels in cultured stromal cells from women with endometriosis are significantly increased after the DNMT inhibitor 5-AZA treatment further confirms the negative regulation of HOXA10 by DNA hypermethylation [89]. In addition, HOXA10 reduction in endometriosis has been suggested along with lower acetylation and higher methylation of H3K9 as well as higher incorporation of MeCP2 on the HOXA10 gene promoter [90]. Further, increased expression of miRNAs such as

miR-135a and miR135-b have been reported to play a role in decreasing HOXA10 expression in eutopic endometrium of women with endometriosis [91, 92].

MicroRNAs (miRNAs) are small, single-stranded noncoding RNA molecules of approximately 22 nucleotides in length that are transcribed from miRNA loci that function as repressors of gene function through mRNA cleavage and translational repression [93]. Multiple studies suggest that miRNAs play a role in both benign and malignant diseases of the female reproductive tract and abnormal expression levels of miRNA have been observed in several disease conditions [65, 94–103]. Studies on miRNA expression support the hypothesis that miRNAs are involved in endometriosis [60, 65, 102–105], but the function of aberrant miRNAs expression in the pathogenesis of endometriosis remains unclear [101, 105]. Interestingly, our microRNA array performed on the eutopic endometrium of baboons before and 3 months after the induction of endometriosis identified the altered expression of several miRNAs (miR-451, 141, 29c, 21, 424, 19b, 200a, and 181a) [106]. Our data suggest that the presence of ectopic lesions in baboons causes changes in eutopic endometrial miRNA expression as early as 3 months postinduction of the disease, and some of these changes may persist throughout the course of the disease. These miRNAs are also reported to be altered in women with endometriosis [65, 102, 107–111] and suggested as an important utility of the nonhuman primate model to study this enigmatic disease. The higher level of miR-194-3p represses the progesterone receptor and decidualization in eutopic endometrium from women with endometriosis compared to women without disease [112]. Overexpression of miR-196a activates the MEK/ERK signal and represses the progesterone receptor and decidualization in eutopic endometrium from women with endometriosis [113]. The higher level of miR-135a and miR-135b in eutopic endometrium of endometriosis are proven to negatively regulate HOXA10 expression [91, 92]. Overexpression of miR-542-3p has also been reported, and it attenuates the differentiating capacity of endometriotic stromal cells [114], and the downregulation of miR-543 in the midsecretory phase is associated with endometriosis-related infertility [115].

TargetScan analysis of miR-29c targets revealed that this miRNA directly targets the 3' UTR of FKBP4 [116, 117]. FKBP4 serves as a co-chaperone to optimize progesterone receptor function, and the absence or decrease of FKBP4 results in a diminished progesterone response leading to a blunted decidualization response and implantation defects in the endometrium of mice, baboons, and humans [118–120]. Given the importance of FKBP4 expression in regulating progesterone signaling, we suggest that the increase in miR-29c leading to decreased FKBP4 (Fig. 11.1) during the window of uterine receptivity as a consequence of endometriosis contributes to the observed progesterone resistance in women with endometriosis [6, 66]. Interestingly, the association with endometriosis has been further confirmed when the miR-29c expression is decreased, and FKBP4 expression is restored in eutopic tissues following laparoscopic ablation of the endometriotic lesion in the patients with deep infiltrative endometriosis undergoing assisted reproductive technology for the treatment of endometriosis induced infertility. These in vivo observations have been further characterized by a series of in vitro experiments. We have confirmed this observation in HuF cells using luciferase constructs

containing binding sites for miR-29c in the 3' UTR of FKBP4 mRNA. Both women and baboons with endometriosis have a blunted decidualization response [59, 65, 78, 121, 122]. In vitro transfection of miR-29c mimics into human uterine fibroblast significantly decreases the progesterone-regulated genes such as Decorin, MIG-6, and HOXA10 and showed compromised decidualization response as evidenced by the low expression of IGFBP1 and PRL transcripts compared to nontargeting negative controls. In a separate study, Hawkins and coworkers have reported that miR-29c is overexpressed in women with ovarian endometriosis and this increase affects specific extracellular matrix genes leading to dysregulation of uterine function including an aberrant decidualization response [65]. These data further support our observation that miR-29c plays an important role in endometriosis-induced progesterone resistance.

Recent studies on the epigenetic alterations provide new insights into our understanding of endometriosis. More than 40,000 CpGs have been identified as being differentially methylated in endometriosis [123]. Further, methylation has been associated with the regulation of PGRB and ESR2 [124, 125]. A DNA methylome study that focused on DNA methylation patterns of eutopic endometrium from women with or without endometriosis in different phases of menstrual cycle has demonstrated that altered endometrial DNA methylation in endometriosis is most prominent in the midsecretory phase (peak progesterone). This phase is when stromal cells are about to differentiate into decidual cells, and the disruption of the normal pattern of cycle-dependent DNA methylation changes may also contribute to decidualization defects [63]. DNA methylation changes are associated with altered gene expression relevant to endometrial function/dysfunction, including cell proliferation, inflammation/immune response, angiogenesis, and steroid hormone response, all of which are important for decidualization [63]. Differences in the DNA methylome between eutopic endometrium from disease-free women and ectopic endometrium from endometriotic patients have also been reported. Among the genes that are significantly differently methylated between endometriotic and healthy cells, there are a large number of transcription factors, many of which have been implicated in the pathology of endometriosis and decidualization [126].

In other studies, genes including HOXA10 [89, 127], GATA2/6 [126], and HAND2 [61] are also regulated by DNA methylation in endometriosis. These gene pathways along with PGR and ESR are essential for the processes of decidualization and implantation. GATA2 regulates key genes necessary for the hormone-driven differentiation of healthy stromal cells, but is hypermethylated and repressed in endometriotic cells, whereas GATA6, which is hypomethylated and abundant in endometriotic cells, blocks hormone sensitivity, represses GATA2, and induces markers of endometriosis when expressed in healthy endometrial cells. Silencing GATA2 or overexpressing GATA6 in normal endometrial stromal cells results in a decreased response to progesterone and in vitro decidualization. However, correction of GATA expression in endometriotic stromal cells does not rescue decidualization, indicating that additional factors that are critical to decidualization are altered in endometriosis [126].

Inflammation is strongly associated with endometriosis [6]. However, we have limited understanding of the interplay between inflammation and steroid hormone signaling, particularly in relation to the aberrant progesterone signaling. Recently, we have performed a multiplex array (Eve Technologies) to measure the inflammatory cytokines in plasma samples obtained from women with and without endometriosis and reported that IL-1α, IL-6, and IL-17 levels are significantly higher in the plasma obtained from women with endometriosis [68]. IL-6 is known to activate JAK and Ras signaling leading to KRAS activation and promotes ectopic lesion formation [128, 129]. This study also proves that KRAS and SIRT1 protein expression are significantly elevated in proliferative and secretory eutopic endometrium obtained from women with endometriosis. Transcriptional repressor BCL6 involves B-cell development and tumorigenesis, plays a role in the recruitment of SIRT1, and is overexpressed in baboons and women with endometriosis (Fig. 11.1). Further, using a uterine-specific KRAS activation mouse model, we have also shown that abnormal KRAS activation leads to enhanced expression of SIRT1 and negatively influences PGR target genes (*Fst*, *Klf15*, *Lrp2*, *Calb1*, *Ihh*, *Patch1*, and *Cli1*) without altering PGR expression [68]. These compelling data provide evidence that aberrant KRAS activation along with increased SIRT1 and BCL6 expression leads to progesterone resistance linked with enhanced inflammation in endometriosis. Additionally, Grandi et al. have documented that inflammatory cytokine (TNFα and IL-1β) treatment to endometrial stromal cells has a detrimental effect on PGR expression, thereby suggesting that inflammation can contribute to creating a progesterone-resistant environment [130].

Although progesterone resistance is one of the major hallmarks of this disease, estrogen dominance is also one of the primary features of endometriosis. There is an intricate relationship between the estrogen-dominant and progesterone-resistant stage of endometriosis which is very difficult to explore in the human and other endometriosis animal models, except for the nonhuman primate model of endometriosis. We have previously shown that during first 3–4 months following the inoculation of the menstrual tissue into the peritoneal cavity of the baboons the endometrium undergoes a state of estrogen dominance followed by progesterone resistance by 5–7 months of disease existence [1, 43, 66]. Similar to eutopic endometrium, ectopic endometrium of endometriosis patients undergoes proliferation and growth in response to estrogens [6]. The alterations of estrogen signaling have also been associated with the disease [131]. The ectopic tissue has been consistently shown to have a higher level of estrogen receptors (ESR1 and ESR2) expression than the eutopic endometrium. Specifically, ESR2 expression is much higher in ectopic tissues partially due to hypomethylation of its promoter [19, 124, 132]. The overexpression of ESR2 then, in turn, suppresses ESR1 expression and diminishes estradiol ESR1-mediated induction of the progesterone receptor in ectopic endometrium [124]. This mechanism is thought to contribute to the resistance to selective actions of progesterone in endometriotic tissues [48]. Further, ESR2 has been shown to drive the pathogenesis of endometriosis by modulating apoptotic complexes and inflammasome in an ESR2 null mouse endometriosis model [133]. Estradiol is produced from the known steroidogenic organs, such as the ovary, as well as locally by

the endometriotic implants. In our baboon model of endometriosis, we have demonstrated that the disease develops in two distinct phases: Phase I is invasive and dependent on ovarian steroids, and Phase II is an active phase of the disease that is characterized by endogenous estrogen biosynthesis as a consequence of aromatase expression in these lesions [19, 134]. Significant increases in the expression of aromatase in the eutopic endometrium of women with endometriosis compared to women without the disease, and the aromatase CYP19A1 is the key of the estradiol biosynthesis in endometriotic lesions [121]. Inhibition of aromatase activity in the baboon endometriosis model is able to limit the progression of SUP endometriosis [135]. On the other hand, the lack of HSD17B2, the enzyme that can inactivate estradiol, also contributes to the higher local concentrations of estradiol in both women and the baboon [2, 134]. Physiologically, estrogen induces endometrial proliferation, and a postovulatory increase of progesterone level triggers the inhibition of estrogen and stimulation of endometrial decidualization [136, 137].

Decidualization Impairment in Endometriosis

About 30–50% of patients with endometriosis have trouble getting pregnant [7] which might be due to the prevalence of an inflammatory condition, dominance of estrogen, and resistant to progesterone which leads to impaired endometrial receptivity [138]. Treatment of endometriosis has been shown to be beneficial for future fertility and improves pregnancy outcomes [139, 140]. Although early studies suggest the primary defect associated with endometriosis may be ovarian dysfunction and oocyte quality [141], the more recent studies have also suggested that impaired endometrial receptivity also contributes to endometriosis-associated infertility or subfertility [142]. In the nonhuman primate model of endometriosis, the peripheral and endometrial population of T-regulatory cells have also been shown to be altered in animals with disease [143]. Many of endometrial biomarkers, including estrogen and progesterone receptors, have been reported to be differentially expressed in the endometrium of women with endometriosis compared to women without disease [138], suggesting endometrial receptivity is affected by endometriosis.

Decidualization is a process in which endometrial stromal fibroblasts transdifferentiate into large, epithelioid-like decidual cells. Decidualized stromal cells secrete various proteins, including PRL and IGFBP1 [81], which are considered markers of decidualization. Failure to mount an appropriate decidualization response is thought to be a cause of infertility, subfertility, or recurrent miscarriages [144]. Endometriosis-associated infertility has been focused on the defects in decidualization. Endometrial stromal cells from women with endometriosis exhibit a reduced response to in vitro decidualization, which further supports the theory that abnormalities within the endometrium, as well as progesterone resistance, are responsible for subfertility caused by the disease [122, 145, 146]. Using our well-established experimental baboon model, we demonstrate that stromal cells from the eutopic endometrium of baboons with endometriosis expressed significantly lower levels of

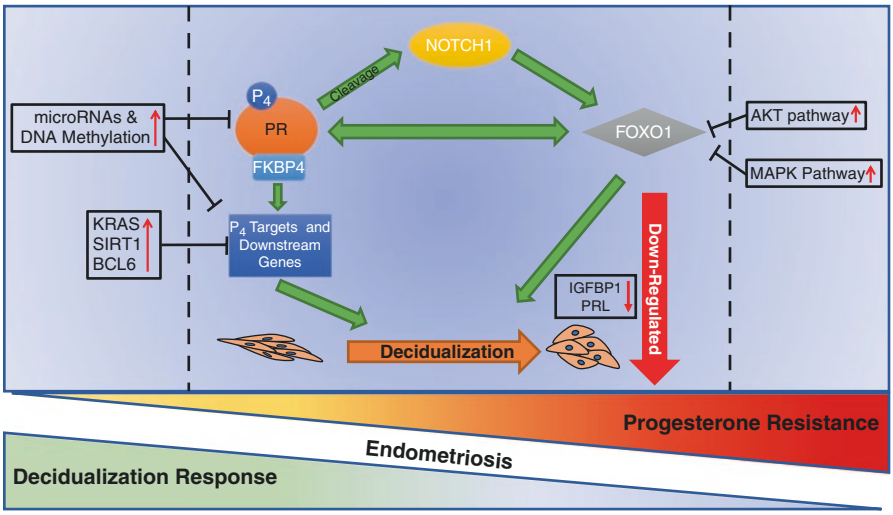

Fig. 11.2 Overview of the altered decidualization response and observed progesterone resistance in endometriosis. Presence of endometriotic lesions influences the altered microRNA expression and aberrant epigenetic changes. In addition, dysregulated NOTCH, AKT, and MAPK signaling along with progesterone resistance contribute to a decrease in FOXO1 leading to decidualization defects, as measured by the decrease in expression levels of the decidualization marker genes IGFBP1 and PRL, in women with endometriosis

IGFBP1 than disease-free animals in response to estradiol, medroxyprogesterone acetate, and dibutyryl cAMP [78] (Fig. 11.2).

NOTCH1 is a gene that is critical for initiating decidualization in both mouse and human uterine stromal cells [147, 148]. We have shown that progesterone-mediated cleavage of the NOTCH1 receptor is critical for endometrial stromal cell decidualization in primates [147]. We have also reported that NOTCH signaling could regulate PGR expression mediated by PU.1, thereby regulating progesterone signaling and contributing to progesterone resistance [113]. We have further shown that the expression of many NOTCH signaling pathway members including receptors, ligands, and targets are decreased in the eutopic endometrium of women and baboons with endometriosis compared to disease-free females [146]. In vitro-cultured primary endometrial stromal cells from women with the disease also express lower levels of NOTCH signaling associated with impaired decidualization [146]. FOXO1–PR interaction is crucial for cell cycle regulation and differentiation of human endometrial stromal cells for an optimal decidualization response in the eutopic endometrium. FOXO1 interacts with PR to regulate their targets (Fig. 11.2). FOXO1 is functionally required for the binding of PR to genomic targets such as IRF4, which has been recently shown to be an essential regulator of decidualization [149, 150]. We have further demonstrated the downstream of NOTCH signaling in endometriosis-related decidualization impairment is FOXO1, a key regulator and early responder to decidualization [83, 149, 151], together with its targets LEFTY2, BCL2L11, IGFBP1, and PRL [146]. Further, we suggest that the NOTCH signaling

is critical for the initiation of decidualization. Interestingly, siRNA-mediated knockdown of NOTCH1 in stromal cells leads to compromised expression of IGFBP1 and PRL following the in vitro decidualization [146]. The impaired NOTCH1–FOXO1 pathway during decidualization is involved with progesterone resistance in endometriosis. We have previously reported that progesterone is associated with NOTCH1 cleavage, which releases the NOTCH1 intracellular domain (N1ICD) and translocates it into the nucleus to activate transcription of target genes [147]. NOTCH1 expression in endometriosis follows a *yin–yang* effect of expression. As mentioned above, NOTCH1 expression is decreased in eutopic endometrium; however, its expression is increased in endometriotic lesions (unpublished data). This observation regarding the increased expression of NOTCH1 in ectopic lesions is further supported by a recent report suggesting that dysregulated NOTCH1 activation in endometriotic lesions leads to progesterone resistance in endometriosis [152]. We have also confirmed that the hostile peritoneal inflammatory environment induces NOTCH1 expression in ectopic lesions and creating a progesterone-resistant and estrogen-dominant microenvironment which promotes the development of endometriotic lesions (unpublished data).

Impaired decidualization has not only been reported in the eutopic endometrium, but also in the ectopic endometrium in endometriosis [149, 153]. The AKT signaling pathway inactivates FOXO1 via phosphorylation and inhibits nuclear localization of FOXO1 [153]. Activation of the AKT pathway is suppressed during decidualization in human endometrial stromal cells [154], and increased activation of PI3K/AKT in stromal cells from ectopic endometrium impairs its response to in vitro decidualization. In contrast, inhibition of PI3K and AKT increases nuclear FOXO1 accumulation and increases IGFBP1 expression in response to in vitro decidualization [153] (Fig. 11.2). PI3K/AKT has also been shown to affect estrogen signaling via activation of ESR1 and downregulation of ESR2 [155, 156], resulting in enhanced estrogen action. Alternatively, the AKT pathway may also affect progesterone action via downregulating PGR expression in stromal cells derived from endometriosis [157]. In the baboon model, a number of PI3K/AKT pathway genes are altered as early as 1 month after disease induction [43].

The MAPK pathway is a classical signaling pathway which is involved in diverse biological functions and is dysregulated in various pathological conditions including endometriosis [158–163]. This pathway plays an important role to bridge the extracellular environmental stimuli to fundamental intracellular responses and is subdivided into three families: (i) ERK, (ii) p38, and (iii) JNK [162]. Endometriosis is an inflammatory disease [6, 68]. ERK is predominantly activated by inflammation and growth factors, whereas p38 and JNK are primarily activated via stress and inflammation [158]. Further, enhanced phospho-ERK (p-ERK) expression is documented in stromal cells from women with endometriosis suggesting aberrant MAPK–ERK signaling in endometriosis [164–167]. In another study that conducted comprehensive profiling of gene expression differences between ectopic and eutopic endometrium from women with endometriosis, many regulators of the MAPK signaling pathway are altered [168]. The increased ERK1/2 activity in eutopic endometrial stromal cells from women with endometriosis has been shown

to inhibit the response of stromal cells to cAMP, a key inductor of decidualization [169]. FOXO1, the essential regulator and early responder to decidualization, can be phosphorylated and its function can be modified by ERK and p38 MAPK pathways [48]. Treatment with an ERK1/2 inhibitor significantly decreased the expression of known decidualization marker genes, suggesting C/EBPβ as a downstream target of ERK1/2 [170]. Zhou et al. have performed microarray analysis for both microRNAs and mRNAs on RNA extracted from eutopic endometrium of infertile women with mild/minimal endometriosis and disease-free controls [113]. Their data suggest that increased levels of miR-196a positively regulate the p-MEK/p-ERK leading to dysregulated PGR protein levels resulting in abnormal decidualization. This study also confirms that inhibition of miR-196a could restore the progesterone response and decidualization [113]. DUSP2 plays an important role to inactivate MAPK signaling. DUSP2 expression is transcriptionally suppressed by HIF1A resulting in activation of ERK and p-38 [171]. In a subsequent study, the same group also suggests that HIF1A-induced miR-20a expression is responsible for the diminished expression of DUSP2 resulting in a constitutively active MAPK/ERK signaling pathway in women with endometriosis [172]. Collectively, these studies suggest that increased expression of miR-196a and miR-20a positively influences the activation of the MAPK signaling pathway and may contribute to the observed dysregulated progesterone-modulated gene networks in the women with endometriosis. We have previously reported abnormal activation of STAT3 due to increased phosphorylation of STAT3, which along with IL-6 stabilizes HIF1A. This observation of p-STAT3 activation has been confirmed by a series of in vitro experiment and was further validated in eutopic endometrium obtained from baboons with induced endometriosis and human clinical samples [173]. Additionally, to understand the molecular mechanisms responsible for aberrant STAT3 activation, in a subsequent study we attempted to explore the role of PIAS3, a protein inhibitor of activated STAT3, in the context of endometriosis. Protein expression of PIAS3 is significantly reduced and inversely correlated with the p-STAT3 levels in the eutopic endometrium of women with endometriosis compared to disease-free controls. Interestingly, induction of endometriosis in baboons results in a marked reduction in PIAS3 expression during the disease progression [174]. The in vitro data from the same study also revealed that INF-γ lowers the PIAS3 protein expression and enhances CXCL10-mediated p-STAT3 levels in both 12Z and Ishikawa endometrial cell lines [174]. In addition, it has been reported that STAT5 [144, 175], an inhibitor of STAT3, is induced by progesterone and lack of proper progesterone action could further favor the aberrant STAT3 signaling (Figs. 11.1 and 11.2) in endometriosis [173].

In the light of studies mentioned above, it is conceivable that inflammation and aberrant expression of microRNAs, both in eutopic endometrium and ectopic lesions, play a pivotal role in regulating progesterone-regulated gene networks, thereby contributing to progesterone resistance, a hallmark of endometriosis. Further, very little information is available to explain the delicate switch between observed estrogen dominance and progesterone resistance during disease progression. This is because the diagnosis of endometriosis could take an average 8–11 years and by that time the disease is already established and would have caused adequate

damage to the uterus. Understanding the early events of the disease can be overcome by utilizing the baboon nonhuman primate model of endometriosis. In baboon model of induced endometriosis, each animal serves its own control which enables us to follow the disease progression and obtain eutopic endometrium at multiple time points to temporally study the genetic, epigenetic, cellular, and molecular changes occurring in the eutopic endometrium due to the presence of ectopic lesions. This would also enable us to focus on deciphering mechanisms associated with the onset of progesterone resistance in endometriosis and serve as a powerful tool to enhance our understanding of the molecular mechanisms that result in this disorder. Despite a number of studies, our present understanding of about endometriosis-induced progesterone resistance remains a puzzle and warrants more studies that are comprehensive and utilizes appropriate in vitro and in vivo models including nonhuman primate models.

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References

1. Braundmeier AG, Fazleabas AT. The non-human primate model of endometriosis: research and implications for fecundity. Mol Hum Reprod. 2009;15(10):577–86.
2. Giudice LC, Kao LC. Endometriosis Lancet. 2004;364(9447):1789–99.
3. Eskenazi B, Warner ML. Epidemiology of endometriosis. Obstet Gynecol Clin North Am. 1997;24(2):235–58.
4. Chapron C, Bourret A, Chopin N, Dousset B, Leconte M, Amsellem-Ouazana D, et al. Surgery for bladder endometriosis: long-term results and concomitant management of associated posterior deep lesions. Hum Reprod. 2010;25(4):884–9.
5. Giudice LC. Clinical practice. Endometriosis. N Engl J Med. 2010;362(25):2389–98.
6. Bulun SE. Endometriosis. N Engl J Med. 2009;360(3):268–79.
7. Missmer SA, Hankinson SE, Spiegelman D, Barbieri RL, Marshall LM, Hunter DJ. Incidence of laparoscopically confirmed endometriosis by demographic, anthropometric, and lifestyle factors. Am J Epidemiol. 2004;160(8):784–96.
8. Sampson JA. Peritoneal endometriosis, due to the menstrual dissemination of endometrial tissue into the peritoneal cavity. Am J Obstet Gynecol. 1927;15(1):101–10.
9. D'Hooghe TM. Clinical relevance of the baboon as a model for the study of endometriosis. Fertil Steril. 1997;68(4):613–25.
10. Hastings JM, Fazleabas AT. A baboon model for endometriosis: implications for fertility. Reprod Biol Endocrinol. 2006;4 Suppl 1:S7.
11. McKinnon BD, Bertschi D, Wanner J, Bersinger NA, Mueller MD. Hormonal contraceptive use and the prevalence of endometriotic lesions at different regions within the peritoneal cavity. Biomed Res Int. 2014;2014:590950.
12. Ruiz A, Ruiz L, Colon-Caraballo M, Torres-Collazo BJ, Monteiro JB, Bayona M, et al. Pharmacological blockage of the CXCR4-CXCL12 axis in endometriosis leads to contrasting effects in proliferation, migration, and invasion. Biol Reprod. 2018;98(1):4–14.
13. Hapangama DK, Drury J, Da Silva L, Al-Lamee H, Earp A, Valentijn AJ, et al. Abnormally located SSEA1+/SOX9+ endometrial epithelial cells with a basalis-like phenotype in the

eutopic functionalis layer may play a role in the pathogenesis of endometriosis. Hum Reprod. 2019;34(1):56–68.
14. Du H, Taylor HS. Contribution of bone marrow-derived stem cells to endometrium and endometriosis. Stem Cells. 2007;25(8):2082–6.
15. Gargett CE, Schwab KE, Deane JA. Endometrial stem/progenitor cells: the first 10 years. Hum Reprod Update. 2016;22(2):137–63.
16. Elliott JE, Abduljabar H, Morris M. Presurgical management of dysmenorrhea and endometriosis in a patient with Mayer-Rokitansky-Kuster-Hauser syndrome. Fertil Steril. 2011;96(2):e86–9.
17. Matsuura K, Ohtake H, Katabuchi H, Okamura H. Coelomic metaplasia theory of endometriosis: evidence from in vivo studies and an in vitro experimental model. Gynecol Obstet Invest. 1999;47 Suppl 1:18–20; discussion 20–2.
18. Nisolle M, Donnez J. Peritoneal endometriosis, ovarian endometriosis, and adenomyotic nodules of the rectovaginal septum are three different entities. Fertil Steril. 1997;68(4):585–96.
19. Fazleabas AT, Brudney A, Gurates B, Chai D, Bulun S. A modified baboon model for endometriosis. Ann N Y Acad Sci. 2002;955:308–17; discussion 40–2, 96–406
20. Nishimoto-Kakiuchi A, Netsu S, Matsuo S, Hayashi S, Ito T, Okabayashi S, et al. Characteristics of histologically confirmed endometriosis in cynomolgus monkeys. Hum Reprod. 2016;31(10):2352–9.
21. Ami Y, Suzaki Y, Goto N. Endometriosis in cynomolgus monkeys retired from breeding. J Vet Med Sci. 1993;55(1):7–11.
22. Zondervan K, Cardon L, Desrosiers R, Hyde D, Kemnitz J, Mansfield K, et al. The genetic epidemiology of spontaneous endometriosis in the rhesus monkey. Ann N Y Acad Sci. 2002;955:233–8; discussion 93–5, 396–406.
23. Schenken RS, Asch RH, Williams RF, Hodgen GD. Etiology of infertility in monkeys with endometriosis: measurement of peritoneal fluid prostaglandins. Am J Obstet Gynecol. 1984;150(4):349–53.
24. Einspanier A, Lieder K, Bruns A, Husen B, Thole H, Simon C. Induction of endometriosis in the marmoset monkey (*Callithrix jacchus*). Mol Hum Reprod. 2006;12(5):291–9.
25. Fazleabas AT. A baboon model for inducing endometriosis. Methods Mol Med. 2006;121:95–9.
26. D'Hooghe TM, Kyama CM, Chai D, Fassbender A, Vodolazkaia A, Bokor A, et al. Nonhuman primate models for translational research in endometriosis. Reprod Sci. 2009;16(2):152–61.
27. D'Hooghe TM, Bambra CS, Raeymaekers BM, De Jonge I, Lauweryns JM, Koninckx PR. Intrapelvic injection of menstrual endometrium causes endometriosis in baboons (*Papio cynocephalus* and *Papio anubis*). Am J Obstet Gynecol. 1995;173(1):125–34.
28. Harirchian P, Gashaw I, Lipskind ST, Braundmeier AG, Hastings JM, Olson MR, et al. Lesion kinetics in a non-human primate model of endometriosis. Hum Reprod. 2012;27(8):2341–51.
29. Donnez O, Orellana R, Van Kerk O, Dehoux JP, Donnez J, Dolmans MM. Invasion process of induced deep nodular endometriosis in an experimental baboon model: similarities with collective cell migration? Fertil Steril. 2015;104(2):491–7. e2
30. Patel B, Elguero S, Thakore S, Dahoud W, Bedaiwy M, Mesiano S. Role of nuclear progesterone receptor isoforms in uterine pathophysiology. Hum Reprod Update. 2015;21(2):155–73.
31. Wetendorf M, DeMayo FJ. Progesterone receptor signaling in the initiation of pregnancy and preservation of a healthy uterus. Int J Dev Biol. 2014;58(2–4):95–106.
32. Ruiz-Alonso M, Blesa D, Simon C. The genomics of the human endometrium. Biochim Biophys Acta. 2012;1822(12):1931–42.
33. Szwarc MM, Hai L, Gibbons WE, Peavey MC, White LD, Mo Q, et al. Human endometrial stromal cell decidualization requires transcriptional reprogramming by PLZF. Biol Reprod. 2018;98(1):15–27.

34. Wang X, Wu SP, DeMayo FJ. Hormone dependent uterine epithelial-stromal communication for pregnancy support. Placenta. 2017;60(Suppl 1):S20–S6.
35. Li X, Large MJ, Creighton CJ, Lanz RB, Jeong JW, Young SL, et al. COUP-TFII regulates human endometrial stromal genes involved in inflammation. Mol Endocrinol. 2013;27(12):2041–54.
36. Jeong JW, Lee HS, Lee KY, White LD, Broaddus RR, Zhang YW, et al. Mig-6 modulates uterine steroid hormone responsiveness and exhibits altered expression in endometrial disease. Proc Natl Acad Sci U S A. 2009;106(21):8677–82.
37. Bhurke AS, Bagchi IC, Bagchi MK. Progesterone-regulated endometrial factors controlling implantation. Am J Reprod Immunol. 2016;75(3):237–45.
38. Hantak AM, Bagchi IC, Bagchi MK. Role of uterine stromal-epithelial crosstalk in embryo implantation. Int J Dev Biol. 2014;58(2–4):139–46.
39. Pawar S, Starosvetsky E, Orvis GD, Behringer RR, Bagchi IC, Bagchi MK. STAT3 regulates uterine epithelial remodeling and epithelial-stromal crosstalk during implantation. Mol Endocrinol. 2013;27(12):1996–2012.
40. Li Q, Kannan A, Das A, Demayo FJ, Hornsby PJ, Young SL, et al. WNT4 acts downstream of BMP2 and functions via beta-catenin signaling pathway to regulate human endometrial stromal cell differentiation. Endocrinology. 2013;154(1):446–57.
41. Wetendorf M, DeMayo FJ. The progesterone receptor regulates implantation, decidualization, and glandular development via a complex paracrine signaling network. Mol Cell Endocrinol. 2012;357(1–2):108–18.
42. Wu SP, Li R, DeMayo FJ. Progesterone receptor regulation of uterine adaptation for pregnancy. Trends Endocrinol Metab. 2018;29(7):481–91.
43. Afshar Y, Hastings J, Roqueiro D, Jeong JW, Giudice LC, Fazleabas AT. Changes in eutopic endometrial gene expression during the progression of experimental endometriosis in the baboon, *Papio anubis*. Biol Reprod. 2013;88(2):44.
44. Lessey BA, Young SL. Homeostasis imbalance in the endometrium of women with implantation defects: the role of estrogen and progesterone. Semin Reprod Med. 2014;32(5):365–75.
45. Young SL, Lessey BA. Progesterone function in human endometrium: clinical perspectives. Semin Reprod Med. 2010;28(1):5–16.
46. Joshi NR, Miyadahira EH, Afshar Y, Jeong JW, Young SL, Lessey BA, et al. Progesterone resistance in endometriosis is modulated by the altered expression of microRNA-29c and FKBP4. J Clin Endocrinol Metab. 2017;102(1):141–9.
47. Barragan F, Irwin JC, Balayan S, Erikson DW, Chen JC, Houshdaran S, et al. Human endometrial fibroblasts derived from mesenchymal progenitors inherit progesterone resistance and acquire an inflammatory phenotype in the endometrial niche in endometriosis. Biol Reprod. 2016;94(5):118.
48. Burney RO, Talbi S, Hamilton AE, Vo KC, Nyegaard M, Nezhat CR, et al. Gene expression analysis of endometrium reveals progesterone resistance and candidate susceptibility genes in women with endometriosis. Endocrinology. 2007;148(8):3814–26.
49. Evans J, Salamonsen LA, Winship A, Menkhorst E, Nie G, Gargett CE, et al. Fertile ground: human endometrial programming and lessons in health and disease. Nat Rev Endocrinol. 2016;12(11):654–67.
50. Colon-Caraballo M, Garcia M, Mendoza A, Flores I. Human endometriosis tissue microarray reveals site-specific expression of estrogen receptors, progesterone receptor, and Ki67. Appl Immunohistochem Mol Morphol. 2018;27(7):491–500.
51. Prentice A, Randall BJ, Weddell A, McGill A, Henry L, Horne CH, et al. Ovarian steroid receptor expression in endometriosis and in two potential parent epithelia: endometrium and peritoneal mesothelium. Hum Reprod. 1992;7(9):1318–25.
52. Igarashi TM, Bruner-Tran KL, Yeaman GR, Lessey BA, Edwards DP, Eisenberg E, et al. Reduced expression of progesterone receptor-B in the endometrium of women with endometriosis and in cocultures of endometrial cells exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin. Fertil Steril. 2005;84(1):67–74.

53. Bukulmez O, Hardy DB, Carr BR, Word RA, Mendelson CR. Inflammatory status influences aromatase and steroid receptor expression in endometriosis. Endocrinology. 2008;149(3):1190–204.
54. Gentilini D, Vigano P, Vignali M, Busacca M, Panina-Bordignon P, Caporizzo E, et al. Endometrial stromal progesterone receptor-A/progesterone receptor-B ratio: no difference between women with and without endometriosis. Fertil Steril. 2010;94(4):1538–40.
55. Bedaiwy MA, Dahoud W, Skomorovska-Prokvolit Y, Yi L, Liu JH, Falcone T, et al. Abundance and localization of progesterone receptor isoforms in endometrium in women with and without endometriosis and in peritoneal and ovarian endometriotic implants. Reprod Sci. 2015;22(9):1153–61.
56. Wolfler MM, Kuppers M, Rath W, Buck VU, Meinhold-Heerlein I, Classen-Linke I. Altered expression of progesterone receptor isoforms A and B in human eutopic endometrium in endometriosis patients. Ann Anat. 2016;206:1–6.
57. McKinnon B, Mueller M, Montgomery G. Progesterone resistance in endometriosis: an acquired property? Trends Endocrinol Metab. 2018;29(8):535–48.
58. Al-Sabbagh M, Lam EW, Brosens JJ. Mechanisms of endometrial progesterone resistance. Mol Cell Endocrinol. 2012;358(2):208–15.
59. Aghajanova L, Tatsumi K, Horcajadas JA, Zamah AM, Esteban FJ, Herndon CN, et al. Unique transcriptome, pathways, and networks in the human endometrial fibroblast response to progesterone in endometriosis. Biol Reprod. 2011;84(4):801–15.
60. Zhao L, Gu C, Ye M, Zhang Z, Li L, Fan W, et al. Integration analysis of microRNA and mRNA paired expression profiling identifies deregulated microRNA-transcription factor-gene regulatory networks in ovarian endometriosis. Reprod Biol Endocrinol. 2018;16(1):4.
61. Yotova I, Hsu E, Do C, Gaba A, Sczabolcs M, Dekan S, et al. Epigenetic alterations affecting transcription factors and signaling pathways in stromal cells of endometriosis. PLoS One. 2017;12(1):e0170859.
62. Warren LA, Shih A, Renteira SM, Seckin T, Blau B, Simpfendorfer K, et al. Analysis of menstrual effluent: diagnostic potential for endometriosis. Mol Med. 2018;24(1):1.
63. Houshdaran S, Nezhat CR, Vo KC, Zelenko Z, Irwin JC, Giudice LC. Aberrant endometrial DNA methylome and associated gene expression in women with endometriosis. Biol Reprod. 2016;95(5):93.
64. Bulun SE, Monsivais D, Kakinuma T, Furukawa Y, Bernardi L, Pavone ME, et al. Molecular biology of endometriosis: from aromatase to genomic abnormalities. Semin Reprod Med. 2015;33(3):220–4.
65. Hawkins SM, Creighton CJ, Han DY, Zariff A, Anderson ML, Gunaratne PH, et al. Functional microRNA involved in endometriosis. Mol Endocrinol. 2011;25(5):821–32.
66. Fazleabas AT. Progesterone resistance in a baboon model of endometriosis. Semin Reprod Med. 2010;28(1):75–80.
67. Yoo JY, Jeong JW, Fazleabas AT, Tayade C, Young SL, Lessey BA. Protein inhibitor of activated STAT3 (PIAS3) is Down-regulated in eutopic endometrium of women with endometriosis. Biol Reprod. 2016;95(1):11.
68. Yoo JY, Kim TH, Fazleabas AT, Palomino WA, Ahn SH, Tayade C, et al. KRAS activation and over-expression of SIRT1/BCL6 contributes to the pathogenesis of endometriosis and progesterone resistance. Sci Rep. 2017;7(1):6765.
69. Ahn JI, Yoo JY, Kim TH, Kim YI, Ferguson SD, Fazleabas AT, et al. cAMP-response element-binding 3-like protein 1 (CREB3L1) is required for decidualization and its expression is decreased in women with endometriosis. Curr Mol Med. 2016;16(3):276–87.
70. Yoo JY, Shin H, Kim TH, Choi WS, Ferguson SD, Fazleabas AT, et al. CRISPLD2 is a target of progesterone receptor and its expression is decreased in women with endometriosis. PLoS One. 2014;9(6):e100481.
71. Kurihara I, Lee DK, Petit FG, Jeong J, Lee K, Lydon JP, et al. COUP-TFII mediates progesterone regulation of uterine implantation by controlling ER activity. PLoS Genet. 2007;3(6):e102.

72. Lee DK, Kurihara I, Jeong JW, Lydon JP, DeMayo FJ, Tsai MJ, et al. Suppression of ERalpha activity by COUP-TFII is essential for successful implantation and decidualization. Mol Endocrinol. 2010;24(5):930–40.
73. Bulun SE, Cheng YH, Yin P, Imir G, Utsunomiya H, Attar E, et al. Progesterone resistance in endometriosis: link to failure to metabolize estradiol. Mol Cell Endocrinol. 2006;248(1–2):94–103.
74. Aghajanova L, Velarde MC, Giudice LC. Altered gene expression profiling in endometrium: evidence for progesterone resistance. Semin Reprod Med. 2010;28(1):51–8.
75. Benson G, Lim H, Paria B, Satokata I, Dey S, Maas R, et al. Mechanisms of reduced fertility in Hoxa-10 mutant mice: uterine homeosis and loss of maternal Hoxa-10 expression. Development. 1996;122:2687–96.
76. Taylor HS, Arici A, Olive D, Igarashi P. HOXA10 is expressed in response to sex steroids at the time of implantation in the human endometrium. J Clin Invest. 1998;101(7):1379–84.
77. Daftary GS, Taylor HS. Endocrine regulation of HOX genes. Endocr Rev. 2006;27(4):331–55.
78. Kim JJ, Taylor HS, Lu Z, Ladhani O, Hastings JM, Jackson KS, et al. Altered expression of HOXA10 in endometriosis: potential role in decidualization. Mol Hum Reprod. 2007;13(5):323–32.
79. Godbole GB, Modi DN, Puri CP. Regulation of homeobox A10 expression in the primate endometrium by progesterone and embryonic stimuli. Reproduction. 2007;134(3):513–23.
80. Lu Z, Hardt J, Kim JJ. Global analysis of genes regulated by HOXA10 in decidualization reveals a role in cell proliferation. Mol Hum Reprod. 2008;14(6):357–66.
81. Gellersen B, Brosens IA, Brosens JJ. Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. Semin Reprod Med. 2007;25(6):445–53.
82. Kim JJ, Buzzio OL, Li S, Lu Z. Role of FOXO1A in the regulation of insulin-like growth factor-binding protein-1 in human endometrial cells: interaction with progesterone receptor. Biol Reprod. 2005;73(4):833–9.
83. Kim JJ, Taylor H, Akbas G, Foucher I, Trembleau A, Jaffe R, et al. Regulation of insulin-like growth factor binding protein-1 promoter activity by FKHR and HOXA10 in primate endometrial cells. Biol Reprod. 2003;68(1):24–30.
84. Celik O, Unlu C, Otlu B, Celik N, Caliskan E. Laparoscopic endometrioma resection increases peri-implantation endometrial HOXA-10 and HOXA-11 mRNA expression. Fertil Steril. 2015;104(2):356–65.
85. Wu Y, Halverson G, Basir Z, Strawn E, Yan P, Guo SW. Aberrant methylation at HOXA10 may be responsible for its aberrant expression in the endometrium of patients with endometriosis. Am J Obstet Gynecol. 2005;193(2):371–80.
86. Szczepanska M, Wirstlein P, Luczak M, Jagodzinski PP, Skrzypczak J. Reduced expression of HOXA10 in the midluteal endometrium from infertile women with minimal endometriosis. Biomed Pharmacother. 2010;64(10):697–705.
87. Andersson KL, Bussani C, Fambrini M, Polverino V, Taddei GL, Gemzell-Danielsson K, et al. DNA methylation of HOXA10 in eutopic and ectopic endometrium. Hum Reprod. 2014;29(9):1906–11.
88. Ji F, Yang X, He Y, Wang H, Aili A, Ding Y. Aberrant endometrial DNA methylome of homeobox A10 and catechol-O-methyltransferase in endometriosis. J Assist Reprod Genet. 2017;34(3):409–15.
89. Lu H, Yang X, Zhang Y, Lu R, Wang X. Epigenetic disorder may cause downregulation of HOXA10 in the eutopic endometrium of fertile women with endometriosis. Reprod Sci. 2013;20(1):78–84.
90. Samadieh Y, Favaedi R, Ramezanali F, Afsharian P, Aflatoonian R, Shahhoseini M. Epigenetic dynamics of HOXA10 gene in infertile women with endometriosis. Reprod Sci. 2018; https://doi.org/10.1177/1933719118766255.
91. Petracco R, Grechukhina O, Popkhadze S, Massasa E, Zhou Y, Taylor HS. MicroRNA 135 regulates HOXA10 expression in endometriosis. J Clin Endocrinol Metab. 2011;96(12):E1925–33.

92. Mirabutalebi SH, Karami N, Montazeri F, Fesahat F, Sheikhha MH, Hajimaqsoodi E, et al. The relationship between the expression levels of miR-135a and HOXA10 gene in the eutopic and ectopic endometrium. Int J Reprod Biomed. 2018;16(8):501–6.
93. Du T, Zamore PD. microPrimer: the biogenesis and function of microRNA. Development. 2005;132(21):4645–52.
94. Zhu XM, Han T, Sargent IL, Yin GW, Yao YQ. Differential expression profile of microRNAs in human placentas from preeclamptic pregnancies vs normal pregnancies. Am J Obstet Gynecol. 2009;200(6):661.e1–7.
95. Chung TK, Cheung TH, Huen NY, Wong KW, Lo KW, Yim SF, et al. Dysregulated microRNAs and their predicted targets associated with endometrioid endometrial adenocarcinoma in Hong Kong women. Int J Cancer. 2009;124(6):1358–65.
96. Wang T, Zhang X, Obijuru L, Laser J, Aris V, Lee P, et al. A micro-RNA signature associated with race, tumor size, and target gene activity in human uterine leiomyomas. Genes Chromosomes Cancer. 2007;46(4):336–47.
97. Marsh EE, Lin Z, Yin P, Milad M, Chakravarti D, Bulun SE. Differential expression of microRNA species in human uterine leiomyoma versus normal myometrium. Fertil Steril. 2008;89(6):1771–6.
98. Nagaraja AK, Creighton CJ, Yu Z, Zhu H, Gunaratne PH, Reid JG, et al. A link between mir-100 and FRAP1/mTOR in clear cell ovarian cancer. Mol Endocrinol. 2010;24(2):447–63.
99. Creighton CJ, Benham AL, Zhu H, Khan MF, Reid JG, Nagaraja AK, et al. Discovery of novel microRNAs in female reproductive tract using next generation sequencing. PLoS One. 2010;5(3):e9637.
100. Zhang L, Volinia S, Bonome T, Calin GA, Greshock J, Yang N, et al. Genomic and epigenetic alterations deregulate microRNA expression in human epithelial ovarian cancer. Proc Natl Acad Sci U S A. 2008;105(19):7004–9.
101. Ohlsson Teague EM, Van der Hoek KH, Van der Hoek MB, Perry N, Wagaarachchi P, Robertson SA, et al. MicroRNA-regulated pathways associated with endometriosis. Mol Endocrinol. 2009;23(2):265–75.
102. Burney RO, Hamilton AE, Aghajanova L, Vo KC, Nezhat CN, Lessey BA, et al. MicroRNA expression profiling of eutopic secretory endometrium in women with versus without endometriosis. Mol Hum Reprod. 2009;15(10):625–31.
103. Teague EM, Print CG, Hull ML. The role of microRNAs in endometriosis and associated reproductive conditions. Hum Reprod Update. 2010;16(2):142–65.
104. Haikalis ME, Wessels JM, Leyland NA, Agarwal SK, Foster WG. MicroRNA expression pattern differs depending on endometriosis lesion type. Biol Reprod. 2018;98(5):623–33.
105. Rekker K, Tasa T, Saare M, Samuel K, Kadastik U, Karro H, et al. Differentially-expressed miRNAs in ectopic stromal cells contribute to endometriosis development: the plausible role of miR-139-5p and miR-375. Int J Mol Sci. 2018;19(12):E3789.
106. Joshi NR, Su RW, Chandramouli GV, Khoo SK, Jeong JW, Young SL, et al. Altered expression of microRNA-451 in eutopic endometrium of baboons (*Papio anubis*) with endometriosis. Hum Reprod. 2015;30(12):2881–91.
107. Aghajanova L, Giudice LC. Molecular evidence for differences in endometrium in severe versus mild endometriosis. Reprod Sci. 2011;18(3):229–51.
108. Filigheddu N, Gregnanin I, Porporato PE, Surico D, Perego B, Galli L, et al. Differential expression of microRNAs between eutopic and ectopic endometrium in ovarian endometriosis. J Biomed Biotechnol. 2010;2010:369549.
109. Hull ML, Nisenblat V. Tissue and circulating microRNA influence reproductive function in endometrial disease. Reprod Biomed Online. 2013;27(5):515–29.
110. Nothnick WB. MicroRNAs and endometriosis: distinguishing drivers from passengers in disease pathogenesis. Semin Reprod Med. 2017;35(2):173–80.
111. Gilabert-Estelles J, Braza-Boils A, Ramon LA, Zorio E, Medina P, Espana F, et al. Role of microRNAs in gynecological pathology. Curr Med Chem. 2012;19(15):2406–13.

112. Pei T, Liu C, Liu T, Xiao L, Luo B, Tan J, et al. miR-194-3p represses the progesterone receptor and decidualization in eutopic endometrium from women with wndometriosis. Endocrinology. 2018;159(7):2554–62.
113. Zhou M, Fu J, Xiao L, Yang S, Song Y, Zhang X, et al. miR-196a overexpression activates the MEK/ERK signal and represses the progesterone receptor and decidualization in eutopic endometrium from women with endometriosis. Hum Reprod. 2016;31(11):2598–608.
114. Sultana S, Kajihara T, Mizuno Y, Sato T, Oguro T, Kimura M, et al. Overexpression of microRNA-542-3p attenuates the differentiating capacity of endometriotic stromal cells. Reprod Med Biol. 2017;16(2):170–8.
115. Yang P, Wu Z, Ma C, Pan N, Wang Y, Yan L. Endometrial miR-543 is downregulated during the implantation window in women with endometriosis-related infertility. Reprod Sci. 2018;26(7):900–8. https://doi.org/10.1177/1933719118799199.
116. Agarwal V, Bell GW, Nam JW, Bartel DP. Predicting effective microRNA target sites in mammalian mRNAs. Elife. 2015;4 https://doi.org/10.7554/eLife.05005.
117. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. 2009;19(1):92–105.
118. Tranguch S, Wang H, Daikoku T, Xie H, Smith DF, Dey SK. FKBP52 deficiency-conferred uterine progesterone resistance is genetic background and pregnancy stage specific. J Clin Invest. 2007;117(7):1824–34.
119. Jackson KS, Brudney A, Hastings JM, Mavrogianis PA, Kim JJ, Fazleabas AT. The altered distribution of the steroid hormone receptors and the chaperone immunophilin FKBP52 in a baboon model of endometriosis is associated with progesterone resistance during the window of uterine receptivity. Reprod Sci. 2007;14(2):137–50.
120. Yang H, Zhou Y, Edelshain B, Schatz F, Lockwood CJ, Taylor HS. FKBP4 is regulated by HOXA10 during decidualization and in endometriosis. Reproduction. 2012;143(4):531–8.
121. Aghajanova L, Hamilton A, Kwintkiewicz J, Vo KC, Giudice LC. Steroidogenic enzyme and key decidualization marker dysregulation in endometrial stromal cells from women with versus without endometriosis. Biol Reprod. 2009;80(1):105–14.
122. Klemmt PA, Carver JG, Kennedy SH, Koninckx PR, Mardon HJ. Stromal cells from endometriotic lesions and endometrium from women with endometriosis have reduced decidualization capacity. Fertil Steril. 2006;85(3):564–72.
123. Koike N, Higashiura Y, Akasaka J, Uekuri C, Ito F, Kobayashi H. Epigenetic dysregulation of endometriosis susceptibility genes (Review). Mol Med Rep. 2015;12(2):1611–6.
124. Xue Q, Lin Z, Cheng YH, Huang CC, Marsh E, Yin P, et al. Promoter methylation regulates estrogen receptor 2 in human endometrium and endometriosis. Biol Reprod. 2007;77(4):681–7.
125. Wu Y, Strawn E, Basir Z, Halverson G, Guo SW. Promoter hypermethylation of progesterone receptor isoform B (PR-B) in endometriosis. Epigenetics. 2006;1(2):106–11.
126. Dyson MT, Roqueiro D, Monsivais D, Ercan CM, Pavone ME, Brooks DC, et al. Genome-wide DNA methylation analysis predicts an epigenetic switch for GATA factor expression in endometriosis. PLoS Genet. 2014;10(3):e1004158.
127. Kulp JL, Mamillapalli R, Taylor HS. Aberrant HOXA10 methylation in patients with common gynecologic disorders: implications for reproductive outcomes. Reprod Sci. 2016;23(4):455–63.
128. Brooks GD, McLeod L, Alhayyani S, Miller A, Russell PA, Ferlin W, et al. IL6 trans-signaling promotes KRAS-driven lung carcinogenesis. Cancer Res. 2016;76(4):866–76.
129. Cheng CW, Licence D, Cook E, Luo F, Arends MJ, Smith SK, et al. Activation of mutated K-ras in donor endometrial epithelium and stroma promotes lesion growth in an intact immunocompetent murine model of endometriosis. J Pathol. 2011;224(2):261–9.
130. Grandi G, Mueller MD, Papadia A, Kocbek V, Bersinger NA, Petraglia F, et al. Inflammation influences steroid hormone receptors targeted by progestins in endometrial stromal cells from women with endometriosis. J Reprod Immunol. 2016;117:30–8.

131. Bulun SE, Monsavais D, Pavone ME, Dyson M, Xue Q, Attar E, et al. Role of estrogen receptor-beta in endometriosis. Semin Reprod Med. 2012;30(1):39–45.
132. Smuc T, Hevir N, Ribic-Pucelj M, Husen B, Thole H, Rizner TL. Disturbed estrogen and progesterone action in ovarian endometriosis. Mol Cell Endocrinol. 2009;301(1–2):59–64.
133. Han SJ, Jung SY, Wu SP, Hawkins SM, Park MJ, Kyo S, et al. Estrogen receptor beta modulates apoptosis complexes and the inflammasome to drive the pathogenesis of endometriosis. Cell. 2015;163(4):960–74.
134. Bulun SE, Zeitoun KM, Takayama K, Sasano H. Estrogen biosynthesis in endometriosis: molecular basis and clinical relevance. J Mol Endocrinol. 2000;25(1):35–42.
135. Langoi D, Pavone ME, Gurates B, Chai D, Fazleabas A, Bulun SE. Aromatase inhibitor treatment limits progression of peritoneal endometriosis in baboons. Fertil Steril. 2013;99(3):656–62.e3.
136. Li Q, Kannan A, DeMayo FJ, Lydon JP, Cooke PS, Yamagishi H, et al. The antiproliferative action of progesterone in uterine epithelium is mediated by Hand2. Science. 2011;331(6019):912–6.
137. Lee K, Jeong J, Kwak I, Yu CT, Lanske B, Soegiarto DW, et al. Indian hedgehog is a major mediator of progesterone signaling in the mouse uterus. Nat Genet. 2006;38(10):1204–9.
138. Lessey BA, Kim JJ. Endometrial receptivity in the eutopic endometrium of women with endometriosis: it is affected, and let me show you why. Fertil Steril. 2017;108(1):19–27.
139. Marcoux S, Maheux R, Berube S. Laparoscopic surgery in infertile women with minimal or mild endometriosis. Canadian Collaborative Group on Endometriosis. N Engl J Med. 1997;337(4):217–22.
140. Jacobson TZ, Barlow DH, Koninckx PR, Olive D, Farquhar C. Laparoscopic surgery for subfertility associated with endometriosis. Cochrane Database Syst Rev. 2002;(4):CD001398.
141. Simon C, Gutierrez A, Vidal A. de los Santos MJ, Tarin JJ, Remohi J, et al. Outcome of patients with endometriosis in assisted reproduction: results from in-vitro fertilization and oocyte donation. Hum Reprod. 1994;9(4):725–9.
142. Prapas Y, Goudakou M, Matalliotakis I, Kalogeraki A, Matalliotaki C, Panagiotidis Y, et al. History of endometriosis may adversely affect the outcome in menopausal recipients of sibling oocytes. Reprod Biomed Online. 2012;25(5):543–8.
143. Braundmeier A, Jackson K, Hastings J, Koehler J, Nowak R, Fazleabas A. Induction of endometriosis alters the peripheral and endometrial regulatory T cell population in the non-human primate. Hum Reprod. 2012;27(6):1712–22.
144. Maruyama T, Yoshimura Y. Molecular and cellular mechanisms for differentiation and regeneration of the uterine endometrium. Endocr J. 2008;55(5):795–810.
145. Minici F, Tiberi F, Tropea A, Orlando M, Gangale MF, Romani F, et al. Endometriosis and human infertility: a new investigation into the role of eutopic endometrium. Hum Reprod. 2008;23(3):530–7.
146. Su RW, Strug MR, Joshi NR, Jeong JW, Miele L, Lessey BA, et al. Decreased Notch pathway signaling in the endometrium of women with endometriosis impairs decidualization. J Clin Endocrinol Metab. 2015;100(3):E433–42.
147. Afshar Y, Miele L, Fazleabas AT. Notch1 is regulated by chorionic gonadotropin and progesterone in endometrial stromal cells and modulates decidualization in primates. Endocrinology. 2012;153(6):2884–96.
148. Afshar Y, Jeong JW, Roqueiro D, DeMayo F, Lydon J, Radtke F, et al. Notch1 mediates uterine stromal differentiation and is critical for complete decidualization in the mouse. FASEB J. 2012;26(1):282–94.
149. Takano M, Lu Z, Goto T, Fusi L, Higham J, Francis J, et al. Transcriptional cross talk between the forkhead transcription factor forkhead box O1A and the progesterone receptor coordinates cell cycle regulation and differentiation in human endometrial stromal cells. Mol Endocrinol. 2007;21(10):2334–49.
150. Vasquez YM, Mazur EC, Li X, Kommagani R, Jiang L, Chen R, et al. FOXO1 is required for binding of PR on IRF4, novel transcriptional regulator of endometrial stromal decidualization. Mol Endocrinol. 2015;29(3):421–33.

151. Kim JJ, Fazleabas AT. Uterine receptivity and implantation: the regulation and action of insulin-like growth factor binding protein-1 (IGFBP-1), HOXA10 and forkhead transcription factor-1 (FOXO-1) in the baboon endometrium. Reprod Biol Endocrinol. 2004;2:34.
152. Brown DM, Lee HC, Liu S, Quick CM, Fernandes LM, Simmen FA, et al. Notch-1 signaling activation and progesterone receptor expression in ectopic lesions of women with endometriosis. J Endocr Soc. 2018;2(7):765–78.
153. Yin X, Pavone ME, Lu Z, Wei J, Kim JJ. Increased activation of the PI3K/AKT pathway compromises decidualization of stromal cells from endometriosis. J Clin Endocrinol Metab. 2012;97(1):E35–43.
154. Yoshino O, Osuga Y, Hirota Y, Koga K, Yano T, Tsutsumi O, et al. Akt as a possible intracellular mediator for decidualization in human endometrial stromal cells. Mol Hum Reprod. 2003;9(5):265–9.
155. Campbell RA, Bhat-Nakshatri P, Patel NM, Constantinidou D, Ali S, Nakshatri H. Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance. J Biol Chem. 2001;276(13):9817–24.
156. Sanchez M, Sauve K, Picard N, Tremblay A. The hormonal response of estrogen receptor beta is decreased by the phosphatidylinositol 3-kinase/Akt pathway via a phosphorylation-dependent release of CREB-binding protein. J Biol Chem. 2007;282(7):4830–40.
157. Eaton JL, Unno K, Caraveo M, Lu Z, Kim JJ. Increased AKT or MEK1/2 activity influences progesterone receptor levels and localization in endometriosis. J Clin Endocrinol Metab. 2013;98(12):E1871–9.
158. McKinnon BD, Kocbek V, Nirgianakis K, Bersinger NA, Mueller MD. Kinase signalling pathways in endometriosis: potential targets for non-hormonal therapeutics. Hum Reprod Update. 2016;22(3):382–403.
159. Uimari O, Rahmioglu N, Nyholt DR, Vincent K, Missmer SA, Becker C, et al. Genome-wide genetic analyses highlight mitogen-activated protein kinase (MAPK) signaling in the pathogenesis of endometriosis. Hum Reprod. 2017;32(4):780–93.
160. Mormile R, Vittori G. MAPK signaling pathway and endometriosis: what is the link? Arch Gynecol Obstet. 2013;287(4):837–8.
161. Zhou WD, Chen QH, Chen QX. The action of p38 MAP kinase and its inhibitors on endometriosis. Yao Xue Xue Bao. 2010;45(5):548–54.
162. Yoshino O, Osuga Y, Hirota Y, Koga K, Hirata T, Harada M, et al. Possible pathophysiological roles of mitogen-activated protein kinases (MAPKs) in endometriosis. Am J Reprod Immunol. 2004;52(5):306–11.
163. Kim EK, Choi EJ. Pathological roles of MAPK signaling pathways in human diseases. Biochim Biophys Acta. 2010;1802(4):396–405.
164. Matsuzaki S, Darcha C. Co-operation between the AKT and ERK signaling pathways may support growth of deep endometriosis in a fibrotic microenvironment in vitro. Hum Reprod. 2015;30(7):1606–16.
165. Yotova IY, Quan P, Leditznig N, Beer U, Wenzl R, Tschugguel W. Abnormal activation of Ras/Raf/MAPK and RhoA/ROCKII signalling pathways in eutopic endometrial stromal cells of patients with endometriosis. Hum Reprod. 2011;26(4):885–97.
166. Ngo C, Nicco C, Leconte M, Chereau C, Arkwright S, Vacher-Lavenu MC, et al. Protein kinase inhibitors can control the progression of endometriosis in vitro and in vivo. J Pathol. 2010;222(2):148–57.
167. Leconte M, Nicco C, Ngo C, Arkwright S, Chereau C, Guibourdenche J, et al. Antiproliferative effects of cannabinoid agonists on deep infiltrating endometriosis. Am J Pathol. 2010;177(6):2963–70.
168. Wu Y, Kajdacsy-Balla A, Strawn E, Basir Z, Halverson G, Jailwala P, et al. Transcriptional characterizations of differences between eutopic and ectopic endometrium. Endocrinology. 2006;147(1):232–46.
169. Velarde MC, Aghajanova L, Nezhat CR, Giudice LC. Increased mitogen-activated protein kinase kinase/extracellularly regulated kinase activity in human endometrial stromal fibro-

blasts of women with endometriosis reduces 3′,5′-cyclic adenosine 5′-monophosphate inhibition of cyclin D1. Endocrinology. 2009;150(10):4701–12.
170. Lee CH, Kim TH, Lee JH, Oh SJ, Yoo JY, Kwon HS, et al. Extracellular signal-regulated kinase 1/2 signaling pathway is required for endometrial decidualization in mice and human. PLoS One. 2013;8(9):e75282.
171. Wu MH, Lin SC, Hsiao KY, Tsai SJ. Hypoxia-inhibited dual-specificity phosphatase-2 expression in endometriotic cells regulates cyclooxygenase-2 expression. J Pathol. 2011;225(3):390–400.
172. Lin SC, Wang CC, Wu MH, Yang SH, Li YH, Tsai SJ. Hypoxia-induced microRNA-20a expression increases ERK phosphorylation and angiogenic gene expression in endometriotic stromal cells. J Clin Endocrinol Metab. 2012;97(8):E1515–23.
173. Kim BG, Yoo J-Y, Kim TH, Shin J-H, Langenheim JF, Ferguson SD, et al. Aberrant activation of signal transducer and activator of transcription-3 (STAT3) signaling in endometriosis. Hum Reprod. 2015;30(5):1069–78.
174. Yoo J-Y, Jeong J-W, Fazleabas AT, Tayade C, Young SL, Lessey BA. Protein inhibitor of activated STAT3 (PIAS3) is Down-regulated in eutopic endometrium of women with endometriosis1. Biol Reprod. 2016;95(1):11, 1–7–, 1–7.
175. Walker SR, Nelson EA, Yeh JE, Pinello L, Yuan G-C, Frank DA. STAT5 outcompetes STAT3 to regulate the expression of the oncogenic transcriptional modulator BCL6. Mol Cell Biol. 2013;33(15):2879–90.

Chapter 12
MicroRNA and Endometriosis

Swati Agrawal and Christian M. Becker

Endometriosis is a disease common in women of reproductive age, and it is defined by the presence of endometrial glands and stroma outside the uterine cavity. Worldwide, it is estimated to affect 10% women of the reproductive age group, and the prevalence rises to 30–50% in patients with infertility and 20% in patients with chronic pelvic pain [1–4]. There is no correlation between the severity of symptoms and the extent of endometriosis. The primary clinical presentation of the disease includes symptoms like severe chronic pelvic pain, dysmenorrhoea, dyspareunia, and infertility. The symptoms are often debilitating, causing enormous physical and mental suffering and unfortunately, no treatment has been found to relieve the symptoms or cure the disease completely. Moreover, diagnosis mainly relies on surgery. Therefore, it is crucial to elucidate the aberrant pathological process responsible for the pathogenesis of this enigmatic disease.

The Need for a Biomarker

Multiple studies have demonstrated a significantly long lag phase between the onset of symptoms of endometriosis and its diagnosis [5, 6]. The lag phase is found to be between 8 and 12 years in developed countries [7]. This may partially be due to the scarcity of findings on clinical examination and a lack of noninvasive tests to diagnose endometriosis. The diagnosis can only be established by direct visualization of the lesions during laparoscopic surgery and histological confirmation of

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endometriosis in biopsied lesions [5]. Several potential biomarkers have been tested, but none of them has proven to be sufficiently sensitive and specific for clinical use [8]. The biomarkers can not only be used to screen and diagnose the disease but can also be useful in classification and therefore treatment stratification purposes, possibly avoiding unnecessary surgical and medical treatments associated with side effects, morbidity, and potential mortality. This might also help us in evaluating the risk of recurrence of the disease and therefore alter secondary prevention strategies. Finally, early diagnosis may also influence decision making regarding fertility issues. Thus, the identification of a robust, reliable, easily applicable biomarker – or a panel of these – presents an enormous unmet clinical need.

MicroRNAs (miRNAs)

Considerable evidence suggests the role of genetic and epigenetic factors in the development and progression of endometriosis, and the implicated genetic loci typically reside in intergenic regions that regulate gene expression [9–11]. The hormonal milieu, immune response, and environmental factors also play a significant role. Microarray analyses have identified multiple differentially expressed messenger RNAs (mRNAs) in eutopic and ectopic endometrial tissues of women with the disease and normal endometrium of women without the disease [12].

An important class of molecules that are involved in gene expression regulation is miRNAs. miRNAs are noncoding molecules of RNA which are single-stranded, ≈ 21–25 nucleotides long. They are highly conserved RNAs that bind to their complementary mRNA and regulate gene expression by acting as negative regulators of translation and degradation of mRNA and mRNA silencing. In addition, miRNAs are involved in many cardinal body processes, such as cell division, development, proliferation, differentiation, immune reaction, signal transduction, cell adaptation, and apoptosis [13, 14]. However, very intricate regulatory networks involving a combination of several miRNAs, mRNAs, and transcription factors are responsible for the final translational activity, and thus the effect on the final protein synthesis depends on the network pathway [15]. miRNAs are cell messengers (cell-to-cell communication system) secreted from cells contained in extracellular vesicles and carry information between cells. The role of tissue or circulating miRNAs has been studied in various reproductive tract disorders like uterine fibroids [16, 17], endometrial adenocarcinoma [18], ovarian adenocarcinoma [19–21], and endometriosis.

In 1993, Lee et al., and Wightman et al., independently, discovered miRNAs and their mechanism of action in *Caenorhabditis elegans* [22, 23]. All the miRNA sequences and annotation can be assessed online from a vast repository named miRBase. The latest miRBase 22 has 38,589 entries from 271 different organisms, representing hairpin precursor microRNAs, which produce a total of 48,860 different mature miRNA sequences [24]. There are other miRNA databases specific to humans detailing miRNAs distinct to various tissues (e.g., miRmine – Human miRNA Expression Database [25], miRGator [26], the Human miRNA Tissue Atlas

[27]). miRNAs bind to the 3′ untranslated region (3'UTR) to recognize the target mRNA with the help of nucleotides from position 2 to 7 called "RNA seed" [28]. miRNAs are known to show cooperativity in gene regulation which means that one miRNA can bind with many target genes and one target gene can be inhibited by various miRNAs [29, 30]. More than 60% of human protein-coding genes have conserved sites for miRNA binding and considering some nonconserved sites too, and so a significant portion of the human genome is regulated by miRNAs [31].

Biogenesis of miRNAs

The biogenesis of miRNA is very intricately regulated and plays a crucial role in gene regulatory networks (Fig. 12.1). Any aberration has the potential to cause various disorders including most chronic diseases, metabolic disorders, and cancers. In recent years, many miRNAs have been known to influence the pathogenesis of various diseases. Their number and proportion largely determine the degree of expression of the target mRNA. Most miRNAs are intragenic, transcribed from genes in the intronic region of coding or noncoding transcripts, and fewer are coded from exons [32]. They may be present in small clusters of two to six genes located in close proximity. The genes can be present with the corresponding mRNA codons, thus transcribing together and regulating the transcription of the target gene in a coupled manner, or they can be transcribed independently of the host gene regulated by their promoter [32, 33]. The miRNA genes are regulated by one or more promoters and transcribed mostly by RNA polymerase II (Pol II) to generate primary miRNAs (pri-miRNAs) which are further processed to generate precursor miRNAs (pre-miRNAs), and some are transcribed by Pol III [34]. The pri-miRNAs have long hairpin looped structures are capped at the 5′ end, polyadenylated, and spliced [35]. This helps their recognition by DiGeorge Syndrome Critical Region 8 (DGCR8), and double-stranded (ds) RNase III enzyme, DROSHA to form Microprocessor complex [36–38]. DROSHA cleaves pri-miRNA duplex toward the base of stem-loop to release an approximately 70 nucleotides long hairpin-shaped precursor miRNA (pre-miRNA), which has two nucleotides overhanging on the 3′ end of the pre-miRNA. Pre-miRNAs once generated are then transported from the nucleus to the cytoplasm by a shuttler, exportin-5 (XPO5)/RanGTP complex in an energy-dependent process [39, 40]. In the cytoplasm, RNase III endonuclease enzyme, Dicer cuts away the terminal loop at the 3′ and 5′ ends leaving an asymmetrical mature miRNA duplex [41, 42]. The duplex is then unwound by helicase enzyme to form two miRNAs, one of which usually gets degraded. One or both the strands of a mature miRNA binds to the Argonaute (AGO) family of proteins in an ATP-dependent manner and gets incorporated into the RNA-induced silencing complex (RISC) [43]. The proportion of 5′ or 3′ strands loaded on AGO varies widely due to thermodynamic instability depending on the cellular microenvironment. miRNA then guides the complex to bind to target mRNA at the 3′ untranslated region (UTR) and cause gene silencing either by translational repression or mRNA deadenylation and decapping [43, 44].

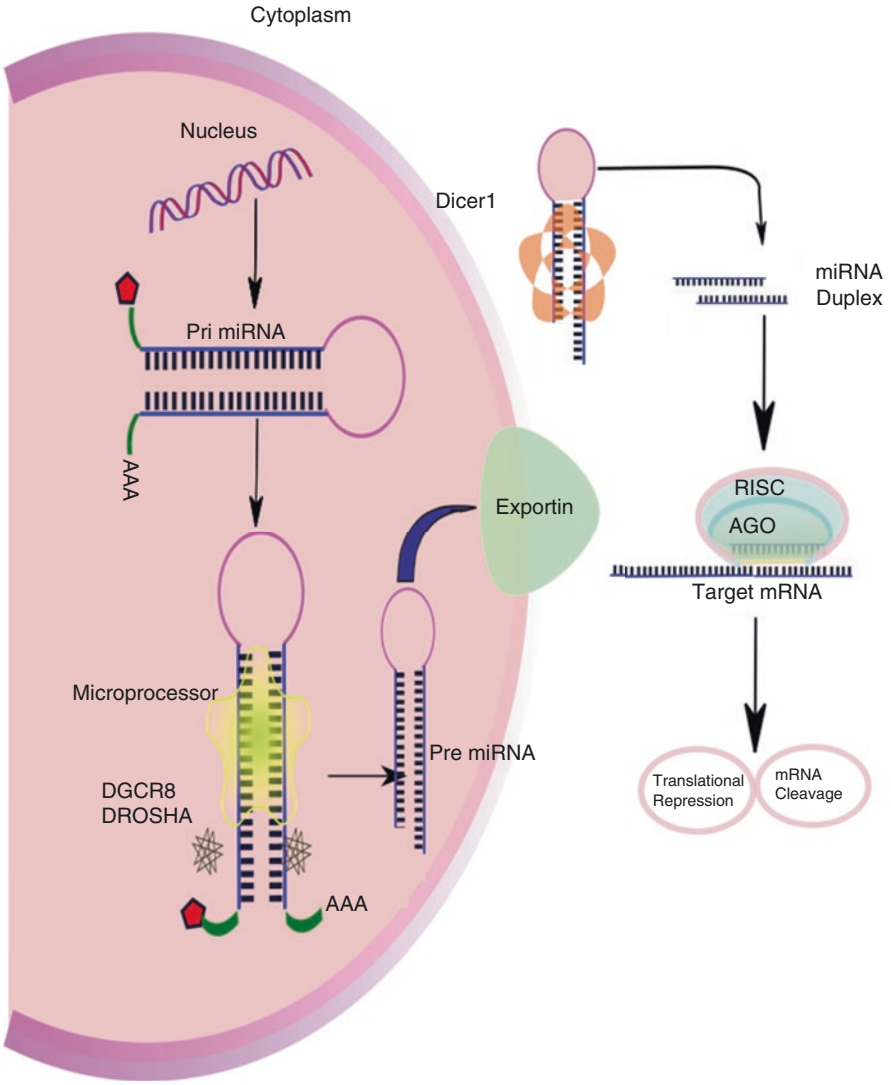

Fig. 12.1 Overview of miRNA biogenesis and function. (Source: Author' work published in Agrawal et al. [65])

miRNAs as Biomarkers

Identifying new protein-based biomarkers is very challenging due to their high complexity and post-translational modifications. miRNAs being less complex, tissue-specific, and without any known post-processing, modifications surpassed these challenges as biomarkers [45]. miRNAs are intracellular in origin and are

secreted into the circulation [46], chaperoned by carriers like AGO2, nucleophosmin 1 (NPM1), and high-density lipoproteins (HDL), or packed into vesicles such as extracellular vesicles, exosomes, or apoptotic bodies (Vickers et al. 2011; Turchinovich et al. 2011). This binding of circulating miRNAs prevents them from degradation by ribonucleases (RNases) present in blood and other biofluids. So, as opposed to the cellular ones, extracellular miRNAs are highly stable and survive deleterious conditions such as extremes of pH and temperature [47]. The cell-free, circulating miRNAs are then taken up by the recipient cells, where they repress translation. They can act as autocrine, paracrine, or endocrine modulators of cellular function. They function as signaling molecules facilitating cross-talk between cells. The fact that miRNAs are secreted from the cells into the circulation makes them a prospective noninvasive biomarker in various body fluids. However, finding out the levels of miRNA is very challenging due to their small size, dissimilar hybridization properties secondary to variable GC content, making their detection very exigent and requiring robust, precise, and highly sensitive techniques for detection [48]. The approach to detect DNA and RNA has been well established and validated, but techniques and protocols for miRNA analysis yet need to be optimized.

Challenges in miRNA Studies in Endometriosis

There are numerous pre-analytical variables which need serious consideration before studying miRNA expression. The majority of laboratory errors arise in the pre-analytical phase, and rigorous methodology needs to be implemented to minimize these inaccuracies [49, 50]. The expression of miRNA in an individual is dynamic and influenced by an array of factors like age, ethnicity, physiological stage of the body, presence of various diseases, smoking, and various other external factors. The tissues for isolating miRNAs in endometriosis are obtained from laparoscopic surgeries and so are very heterogeneous containing the surrounding healthy tissue which could be peritoneum, normal ovarian tissue, any other adjacent tissue, inflammatory cells, or epithelial cells. This may mask the disease-specific miRNA dysregulation and can lead to false or inconsistent results. The various human miRNA databases suggest a high variability in miRNAs in different tissues. The levels of multiple miRNAs are also highly influenced by the different phases of the menstrual cycle and previous studies have found a significant difference in the miRNA expression profile in endometrial and ovarian tissue in the different phases [51–55]. However, another article in endometriotic tissues contradicted, as they found a similar expression of miRNA in all cycle phases [56].

Another factor which can confound the study is the presence of other chronic inflammatory and autoimmune diseases. Endometriosis is a chronic inflammatory disease, and miRNAs specific for endometriosis arise from endometrial cells and are released in circulation contained in exosomes or bound to proteins. Many of these dysregulated miRNAs target mRNAs involved in inflammation. Similar miRNAs have been proved to be significantly dysregulated in various other inflammatory and

autoimmune disorders [57–60]. Regrettably, most of the studies on tissue and circulating miRNAs in endometriosis have not taken care of the pre-analytical variables [61–64].

miRNA Profiling Studies in Endometriosis

It is imperative to elucidate the pathophysiology of the disease in order to find a reliable biomarker. Many studies have been done recently demonstrating the cardinal role of miRNAs in the pathogenesis of endometriosis. They include studies investigating the dysregulation of particular candidate miRNAs and high-throughput studies to explore the changes in miRNA levels associated with the disease (Table 12.1). A number of miRNAs have been identified to be dysregulated in eutopic and ectopic endometrium, endometriomas, peritoneal fluid, and blood in the patients with the disease [65]. The levels of the miRNAs have been found to vary with tissue identified and the various phases of the menstrual cycle [51, 52]. Many of the studies have not specified the menstrual cycle phase in which the tissues were collected. In the literature, the differentially expresses miRNAs in various tissues differ widely, with minimal overlap. Even the scarce overlap may be an overestimation as most of the analyses do not involve strand specification (5-p or 3-p) of miRNA. This also sheds light on the very complex molecular changes in endometriosis. Also, the role of most of the dysregulated miRNAs is unclear in the pathogenesis of endometriosis due to the lack of knowledge on their target miRNAs and confounding downstream validations. miR-200 family is the most dysregulated miRNA, the levels being decreased in 7 out of 28 studies in endometriotic tissues, which will be discussed subsequently.

The Role of miRNAs in the Pathogenesis of Endometriosis

Endometriosis is a benign disease, although ectopic endometrium possesses many characteristics of malignant cells like invasiveness, high proliferation rate, and metastasis. Several dysregulated miRNAs play an array of roles in the pathogenesis of endometriosis including decreased apoptosis, increased cell proliferation, angiogenesis, invasion and viability, and epithelial-mesenchymal transition (EMT). Here, we will discuss EMT regulated by miR-200b family which is the most common miRNA found to be differentially expressed in endometriosis (Fig. 12.2). In the embryo, endometrial cells are derived from intermediate mesoderm of the primitive germ cell layers after mesenchymal-epithelial transition (MET). According to the prevalent theory, the epithelial cells in pelvic endometriosis originate from retrograde menstruation of the endometrial cells. Due to their origin, these are particularly prone to revert to their mesenchymal state. This transition of epithelial cells to mesenchymal cells and subsequent cell migration is thus proposed to be one of the

Table 12.1 List of miRNAs dysregulated in endometriotic tissues as found in various studies after validation by qRT-PCR

S. No	Author	Sample size	Sample type	Dysregulated miRNA
1	Ohlsson Teague et al. (2009) [66]	Cases-7 (microarray) Cases-8 (qRT-PCR)	Eutopic and ectopic endometrium	Up➔ miR-99a, miR-126, miR-145 Down➔ miR-141, miR-200b, miR-424
2	Burney et al. (2009) [67]	Cases-4 Controls-3	Eutopic endometrium cases and controls	Down➔ miR-34c-5p, miR-34b-5p, miR-9-5p, miR-9-3p
3	Filigheddu et al. (2010) [68]	Cases-16 (3:microarray; 13:qRT-PCR)	Eutopic and ectopic in endometrium	Up➔ miR-202 Down➔ miR-200a, miR-200b, miR-200c, miR-182
4	Hawkins et al. (2011) [63]	Cases-18 (10-microarray; 8-qRT-PCR) Controls-20 (11:microarray; 9:qRT-PCR)	Endometrioma and control endometrium	Up➔ miR-29c, miR-100, miR-193a-3p, miR-193a-5p, miR-202, miR-485-3p, miR-509-3-5p, miR-574-3p, miR-708, miR-720 Down➔ miR-10a, miR-34c-5p, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-375, miR-429, miR-449b, miR-504, miR-873
5	Petracco et al. (2011) [69]	Cases-32 Controls-50	Eutopic endometrium	Up➔ miR-135a and miR-135b
6	Ramon et al. (2011) [70]	Cases-58 Control-38	Ovarian endometrioma, eutopic endometrium, peritoneal lesions	Endometrioma vs control endometrium Up➔ miR-21, miR-125a, miR-222 Down➔ miR-17-5p, miR-20a Peritoneal implants vs endometrioma Down➔ miR-125a, miR-222
7	Dai et al. (2012) [71]	Cases-12 Controls-12	Ovarian endometriomas and eutopic endometrium	Down➔ miR-199a
8	Lin et al. (2012) [72]	Cases-31 Controls-17 12 pairs of eutopic and ectopic endometrium	Ovarian endometriomas, ectopic and eutopic endometrium	Up➔ miR-20a
9	Liu et al. (2012) [73]	Cases-31 Control-27 (ovarian endometriomas)	Ovarian endometriomas, ectopic, and eutopic endometrium	Down➔ miR-126

(continued)

Table 12.1 (continued)

S. No	Author	Sample size	Sample type	Dysregulated miRNA
10	Abe et al. (2013) [74]	Cases-26 Controls-18	Ovarian endometrioma, eutopic endometrium, and normal endometrium	Down➔ miR-196b
11	Shen et al. (2013) [75]	Cases-23 Controls-15	Ectopic and eutopic endometrium	Down➔ miR-23a, miR-23b
12	Laudanski et al. (2013) [76]	Cases-21 Controls-25	Endometrium cases vs. controls Cases were with ovarian endometriosis	Up➔ miR-24, miR-885-5p Down➔ let-7b, miR-26b, miR-185, miR-142-3p, miR-29b, miR-483-5p, miR-144-5p, miR-145-3p, miR-629-3p, miR-222-5p, miR-497, miR-675, miR-106b-3p
13	Shi et al. (2014) [77]	Cases-20 Controls-20	Ectopic and eutopic endometrium	Down➔ miR-183, 215, 363
14	Braza-Boïls et al. (2014) [78]	Cases-51 Controls-32	Eutopic endometrium, ovarian endometrioma	Eutopic endometrium cases vs control endometrium Down➔ miR-202-3p, miR-424-5p, 449b-3p, miR-556-3p Endometriomas vs control endometrium Up➔ miR-29c, miR-138, miR-202-3p, miR-373-3p, miR-411-5p Down➔ miR-449b-3p
15	Zheng et al. (2014) [79]	Cases-11 Controls-22	Eutopic endometrium cases and controls, paired eutopic and ectopic endometrium controls	Up➔ miR-143, miR-145
16	Zhao et al. (2014) [80]	Cases-40 (ovarian endometriosis) Controls-20 (benign ovarian tumor)	Ovarian tissue	Up➔ miR-20a
17	Saare et al. (2014) [56]	Cases-24 Controls-24	Peritoneal endometriotic [24] and adjacent healthy area [24]. Eutopic endometrium (9 pts. with endometriosis and 8 pts. w/o endometriosis)	Eutopic endometrium (cases vs controls): Up➔ miR-200a, b, miR-141 Endometriotic lesions in peritoneum: Up➔ miR-449a, miR-34c, miR-200a, b, miR-141

(continued)

Table 12.1 (continued)

S. No	Author	Sample size	Sample type	Dysregulated miRNA
18	Graham et al. (2015) [81]	Cases-30	Eutopic endometrium ($n = 30$) and endometriotic lesion ($n = 43$)	Up➔ miR-451
19	Chen et al. (2015) [82]	Cases-57 Controls-44	Eutopic endometrium	Down➔ miR-93, 106a
20	Zhang et al. (2015) [83]	Ectopic endometrium-45, eutopic endometrium-25, normal endometrium-26	Ectopic endometrium, eutopic endometrium, normal endometrium	Up➔ miR-202
21	Dong et al. (2015) [84]	Healthy ovarian tissue-12, endometrioma-12	Healthy ovarian tissue and endometrioma	Up➔ miR-91
22	Long et al. (2015) [85]	Ectopic endometrium-20, eutopic endometrium-20, normal endometrium-10	Ectopic endometrium, eutopic endometrium, normal endometrium	Down➔ miR-29c
23	Yang et al. (2016) [86]	Cases-32 Controls-19	Normal endometrium, eutopic endometrium	Up➔ miR-16-5p, miR-106b-5p, miR-145-5p, miR-21-5p Down➔ miR-200b, miR-200c, miR-15a-5p, miR-19b-1-5p, miR-146a-5p, miR-126
24	Nothnick et al. (2017) [87]	Ectopic endometrium-41, eutopic endometrium-41	Ectopic endometrium, eutopic endometrium	Up➔ miR-451-a
25	Liang et al. (2017) [88]	Cases-27 Controls-12	Ectopic endometrium, eutopic endometrium	Down➔ miR-200c
26	Zhao et al. (2018) [89]	Ectopic endometrium-22, eutopic endometrium-22	Ectopic endometrium, eutopic endometrium	Up➔ miR-615-3p Down➔miR-34c-5p, miR-182-5p, miR-449b-5p, miR-200a-3p, miR-106a-5p
27	Liu et al. (2018) [90]	Endometrioma-19, eutopic endometrium-19, normal endometrium-35	Endometrioma, eutopic endometrium from cases and controls	Down➔ miR-449b-3p
28	Rekker et al. (2018) [91]	Eutopic endometrium-4, endometrioma-4	Endometrioma vs. eutopic endometrium	In endometrioma Up➔ miR-139-5p Down➔ miR-375

Up➔ indicates upregulated miRNAs
Down➔ indicates downregulated miRNAs

critical processes in initiating endometriosis [92, 93]. In EMT, cells of epithelial type undergo a transition to a mesenchymal phenotype, which is then capable of breaching the basement membrane and reach distant sites. The cardinal feature in EMT is the loss of epithelial markers involved in cohesion like E-cadherin (epithelial cadherin), Desmoplakin, Mucin-1, occludin, and claudin, and the gain of mesenchymal markers involved in migration and invasion such as N-cadherin (neural cadherin), smooth-muscle actin, vimentin, and fibronectin [94, 95]. Studies have found that cells in peritoneal endometriosis are E-cadherin negative compared to the cells in the eutopic endometrium [96, 97]. The expression of E-cadherin is decreased by various transcriptional factors like the Snail family of zinc-finger transcription factors (Snail, Slug, and Smuc), the δEF1 (Elongation Factor) family of two-handed zinc-finger factors including Zinc finger E-box-binding homeobox 1 (ZEB1) and 2 (ZEB2), and SIP1 (Smad-interacting protein 1), which in turn act as EMT-activators. It has been found that the miR-200 family and ZEB1, ZEB2, and SIP1 reciprocally regulate each other in a negative feedback loop which governs EMT maintaining cells either in an epithelial or mesenchymal state [98, 99] (Fig. 12.2). Thus, downregulation of miR-200b induces EMT by upregulating ZEB1 and ZEB2. Moreover, P53 by inducing miR-34 a, b, c can downregulate Snail, and conversely, Snail and ZEB1 can transcriptionally repress miR-34 a, b, c [100, 101]. In an experiment conducted on Madin Darby canine kidney (MDCK) epithelial cells, TGF-β1 treatment induced a morphological change with the loss of cohesion decreased expression of E-cadherin and elevated markers of mesenchymal origin like fibronectin, ZEB1, and ZEB2 [102]. This was associated with a significant downregulation in the miR-200 family, strongly pointing toward their role in EMT.

The signals stimulating endometriosis are proposed to be hypoxia and estrogen [93]. Hypoxia results in the upregulation of hypoxia-inducible factors (HIFs), the overexpression of which is observed in endometriotic tissues. The authors hypothesized keeping in line with the retrograde menstruation theory that during implantation, endometrial cells might be adapted to hypoxia and hypoxia induces EMT enhancing the invasiveness of the cells which contribute to the development of ectopic lesions. Angiogenesis during this process is stimulated by vascular endothelial growth factor (VEGF) which also has a role in enhancing EMT. Several other factors including reactive oxygen species, lysyl oxidases, and NFκB and Notch signaling pathway also contribute to EMT.

Endometriosis is an estrogen-dependent disease, and it has been found that endometriotic lesions exhibit higher levels of estradiol than the normal endometrium. Estrogen acts through its receptors: estrogen receptor (ER), ERα and ERβ. It has been thought that ERα binds directly to hepatocyte growth factor (HGF) promoter to stimulate EMT in the endometrial cells and also upregulates the Snail family of zinc-finger transcription factors.

As stated earlier, ectopic endometrial cells have a high proliferation rate which could be due to dysregulation at various steps. One of the transcription factors, SOX-6 is found to decrease in ectopic endometrium as compared to the normal endometrium (Zhang et al. 2015), and this protein regulates the cell cycle at various stages through p21, Rb, and cyclin D1. The same authors found that the levels of p21 and SOX6 were decreased, while Rb and cyclin D1 levels were increased in

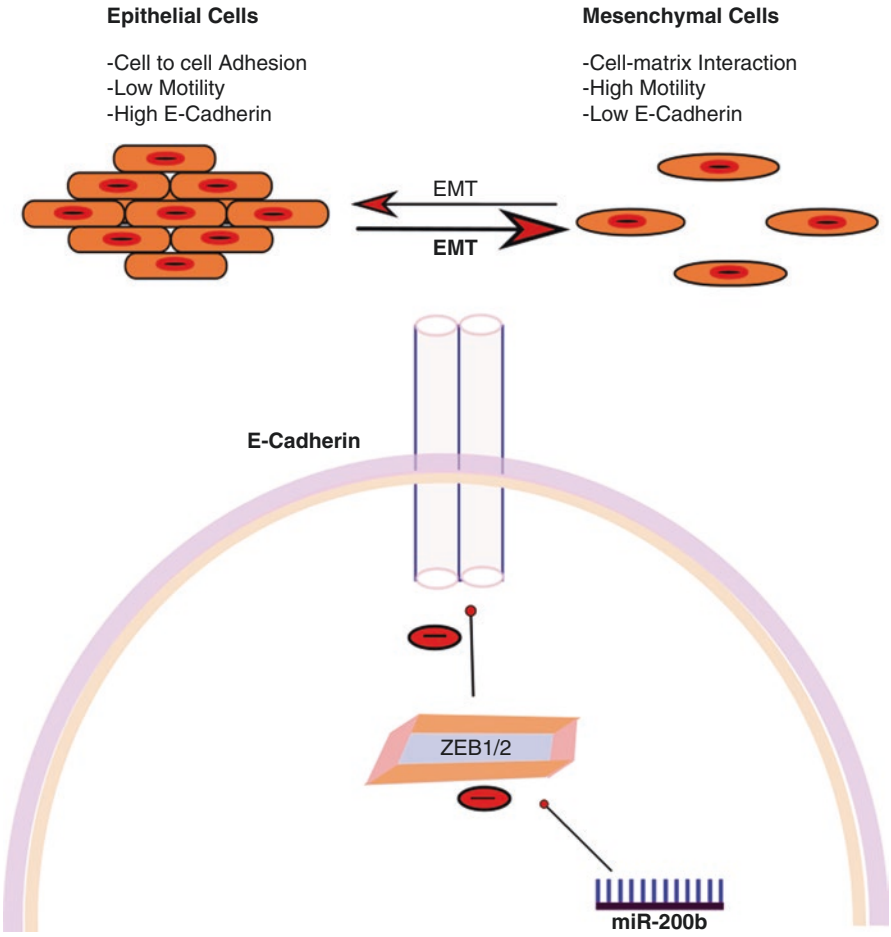

Fig. 12.2 The role of miR-200b in the pathogenesis of endometriosis. (Source: Author' work published in Agrawal et al. [65])

ectopic endometrial tissue. The miRNA targeting SOX6, miR-202 has been shown to be upregulated in ectopic endometrial tissue [83]. Recently, a study has proposed using microRNA Let-7b for the treatment of endometriosis using a murine model [103]. The treatment has shown to decrease the expression of various genes known to promote endometriosis and also reduced the size of the endometriotic lesions.

Conclusion

Among the epigenetic players, miRNAs have emanated as powerful regulators of gene expression, and are investigated as diagnostic biomarkers for various diseases. The minimal concordance rate among the studies could be due to the lack of

standardization in the study protocols, like sample collection, processing, various techniques used, and normalization controls. The importance of standard operating procedures (SOPs) cannot be undermined. World Endometriosis Research Foundation (WERF) has launched a global initiative Endometriosis Phenome and Biobanking Harmonisation Project (EPHect), to develop unanimity in sample collection and data recording in patients with endometriosis, which will facilitate comprehensive, global, internationally collaborative robust research in endometriosis. Adhering to the SOPs would serve to minimize the pre-analytical errors. There is an absolute requirement of similar SOPs for studying RNA expression to develop unanimity among the studies. Only then would it be possible to establish a panel of miRNAs which could diagnose endometriosis noninvasively, with sufficient sensitivity and specificity. Pending these, miRNAs could prove valuable not only in assessing prognosis and monitoring treatment but also as major therapeutic targets in endometriosis.

References

1. Giudice LC, Kao LC. Endometriosis. Lancet (London, England). 2004;364(9447):1789–99.
2. Guo S-W, Wang Y. The prevalence of endometriosis in women with chronic pelvic pain. Gynecol Obstet Investig. 2006;62(3):121–30.
3. Meuleman C, Vandenabeele B, Fieuws S, Spiessens C, Timmerman D, D'Hooghe T. High prevalence of endometriosis in infertile women with normal ovulation and normospermic partners. Fertil Steril. 2009;92(1):68–74.
4. Zondervan KT, Becker CM, Koga K, Missmer SA, Taylor RN, Viganò P. Endometriosis. Nat Rev Dis Prim. 2018;4(1):9.
5. Kennedy S, Bergqvist A, Chapron C, D'Hooghe T, Dunselman G, Greb R, et al. ESHRE guideline for the diagnosis and treatment of endometriosis. Hum Reprod. 2005;20(10):2698–704.
6. Nnoaham KE, Hummelshoj L, Webster P, D'Hooghe T, de Cicco NF, de Cicco NC, et al. Impact of endometriosis on quality of life and work productivity: a multicenter study across ten countries. Fertil Steril. 2011;96(2):366–373.e8.
7. Hadfield R, Mardon H, Barlow D, Kennedy S. Delay in the diagnosis of endometriosis: a survey of women from the USA and the UK. Hum Reprod. 1996;11(4):878–80.
8. May KE, Conduit-Hulbert SA, Villar J, Kirtley S, Kennedy SH, Becker CM. Peripheral biomarkers of endometriosis: a systematic review. Hum Reprod Update. 2010;16(6):651–74.
9. Rahmioglu N, Nyholt DR, Morris AP, Missmer SA, Montgomery GW, Zondervan KT. Genetic variants underlying risk of endometriosis: insights from meta-analysis of eight genome-wide association and replication datasets. Hum Reprod Update. 2014;20(5):702–16.
10. Zondervan KT, Rahmioglu N, Morris AP, Nyholt DR, Montgomery GW, Becker CM, et al. Beyond endometriosis genome-wide association study: from genomics to phenomics to the patient. Semin Reprod Med. 2016;34(4):242–54.
11. Hsiao K-Y, Wu M-H, Tsai S-J. Epigenetic regulation of the pathological process in endometriosis. Reprod Med Biol. 2017;16(4):314–9.
12. Ohlsson Teague EMC, Van der Hoek KH, Van der Hoek MB, Perry N, Wagaarachchi P, Robertson SA, et al. MicroRNA-regulated pathways associated with endometriosis. Mol Endocrinol. 2009;23(2):265–75.
13. van Rooij E, Sutherland LB, Qi X, Richardson JA, Hill J, Olson EN. Control of stress-dependent cardiac growth and gene expression by a microRNA. Science. 2007;316(5824):575–9.
14. Guarnieri DJ, DiLeone RJ. MicroRNAs: a new class of gene regulators. Ann Med. 2008;40(3):197–208.

15. Bartel DP, Lee R, Feinbaum R. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116:281–97.
16. Wang T, Zhang X, Obijuru L, Laser J, Aris V, Lee P, et al. A micro-RNA signature associated with race, tumor size, and target gene activity in human uterine leiomyomas. Genes Chromosomes Cancer. 2007;46(4):336–47.
17. Marsh EE, Lin Z, Yin P, Milad M, Chakravarti D, Bulun SE. Differential expression of microRNA species in human uterine leiomyoma versus normal myometrium. Fertil Steril. 2008;89(6):1771–6.
18. Chung TK, Cheung TH, Huen NY, Wong KW, Lo KW, Yim SF, et al. Dysregulated microRNAs and their predicted targets associated with endometrioid endometrial adenocarcinoma in Hong Kong women. Int J Cancer. 2009;124(6):1358–65.
19. Zhang L, Volinia S, Bonome T, Calin GA, Greshock J, Yang N, et al. Genomic and epigenetic alterations deregulate microRNA expression in human epithelial ovarian cancer. Proc Natl Acad Sci U S A. 2008;105(19):7004–9.
20. Creighton CJ, Fountain MD, Yu Z, Nagaraja AK, Zhu H, Khan M, et al. Molecular profiling uncovers a p53-associated role for microRNA-31 in inhibiting the proliferation of serous ovarian carcinomas and other cancers. Cancer Res. 2010;70(5):1906–15.
21. Nagaraja AK, Creighton CJ, Yu Z, Zhu H, Gunaratne PH, Reid JG, et al. A link between mir-100 and FRAP1/mTOR in clear cell ovarian cancer. Mol Endocrinol. 2010;24(2):447–63.
22. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell. 1993;75(5):843–54.
23. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell. 1993;75(5):855–62.
24. Kozomara A, Birgaoanu M, Griffiths-Jones S. miRBase: from microRNA sequences to function. Nucleic Acids Res. 2018;47(D1):D155–62.
25. Panwar B, Omenn GS, Guan Y. miRmine: a database of human miRNA expression profiles. Bioinformatics. 2017;33(10):1554–60.
26. Lee B, Ryu D, Chang H, Choi I, Jang I, Ko M, et al. miRGator v3.0: a microRNA portal for deep sequencing, expression profiling and mRNA targeting. Nucleic Acids Res. 2012;41(D1):D252–7.
27. Meese E, Ludwig N, Leidinger P, Rheinheimer S, Becker K, Backes C, et al. Distribution of miRNA expression across human tissues. Nucleic Acids Res. 2016;44(8):3865–77.
28. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009;136(2):215–33.
29. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell. United States. 2005;120:15–20.
30. Grun D, Wang Y-L, Langenberger D, Gunsalus KC, Rajewsky N. microRNA target predictions across seven Drosophila species and comparison to mammalian targets. PLoS Comput Biol. 2005;1(1):e13.
31. Friedman RC, Farh KK-H, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. 2009;19(1):92–105.
32. Rodriguez A, Griffiths-Jones S, Ashurst JL, Bradley A. Identification of mammalian microRNA host genes and transcription units. Genome Res. 2004;14(10A):1902–10.
33. Baskerville S, Bartel DP. Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. RNA. 2005;11(3):241–7.
34. Lee Y, Kim M, Han J, Yeom K-H, Lee S, Baek SH, et al. MicroRNA genes are transcribed by RNA polymerase II. EMBO J. 2004;23(20):4051–60.
35. Cai X, Hagedorn CH, Cullen BR. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA. 2004;10(12):1957–66.
36. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, et al. The nuclear RNase III Drosha initiates microRNA processing. Nature. 2003;425(6956):415–9.
37. Gregory RI, Yan K-P, Amuthan G, Chendrimada T, Doratotaj B, Cooch N, et al. The microprocessor complex mediates the genesis of microRNAs. Nature. 2004;432(7014):235–40.

38. Denli AM, Tops BBJ, Plasterk RHA, Ketting RF, Hannon GJ. Processing of primary microRNAs by the microprocessor complex. Nature. 2004;432(7014):231–5.
39. Yi R, Qin Y, Macara IG, Cullen BR. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev. 2003;17(24):3011–6.
40. Lund E, Guttinger S, Calado A, Dahlberg JE, Kutay U. Nuclear export of microRNA precursors. Science. 2004;303(5654):95–8.
41. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature. 2001;409(6818):363–6.
42. Park J-E, Heo I, Tian Y, Simanshu DK, Chang H, Jee D, et al. Dicer recognizes the 5′ end of RNA for efficient and accurate processing. Nature. 2011;475(7355):201–5.
43. Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R. Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell. 2005;123(4):631–40.
44. Zamore PD, Tuschl T, Sharp PA, Bartel DP. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell. 2000;101(1):25–33.
45. Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, et al. The microRNA spectrum in 12 body fluids. Clin Chem. 2010;56(11):1733–41.
46. Skog J, Wurdinger T, van Rijn S, Meijer D, Gainche L, Sena-Esteves M, et al. Glioblastoma microvesicles transport RNA and protein that promote tumor growth and provide diagnostic biomarkers. Nat Cell Biol. 2008;10(12):1470–6.
47. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A. 2008;105(30):10513–8.
48. Blondal T, Jensby Nielsen S, Baker A, Andreasen D, Mouritzen P, Wrang Teilum M, et al. Assessing sample and miRNA profile quality in serum and plasma or other biofluids. Methods. 2013;59(1):S1–6.
49. Bonini P, Plebani M, Ceriotti F, Rubboli F. Errors in laboratory medicine. Clin Chem. 2002;48(5):691–8.
50. Becker N, Lockwood CM. Pre-analytical variables in miRNA analysis. Clin Biochem. 2013;46(10–11):861–8.
51. Kuokkanen S, Chen B, Ojalvo L, Benard L, Santoro N, Pollard JW. Genomic profiling of microRNAs and messenger RNAs reveals hormonal regulation in microRNA expression in human endometrium. Biol Reprod. 2010;82(4):791–801.
52. Ruiz-Alonso M, Blesa D, Simón C. The genomics of the human endometrium. Biochim Biophys Acta Mol basis Dis. 2012;1822(12):1931–42.
53. Braza-Boils A, Mari-Alexandre J, Gilabert J, Sanchez-Izquierdo D, Espana F, Estelles A, et al. MicroRNA expression profile in endometriosis: its relation to angiogenesis and fibrinolytic factors. Hum Reprod. 2014;29(5):978–88.
54. Sha A-G, Liu J-L, Jiang X-M, Ren J-Z, Ma C-H, Lei W, et al. Genome-wide identification of micro-ribonucleic acids associated with human endometrial receptivity in natural and stimulated cycles by deep sequencing. Fertil Steril. 2011;96(1):150–155.e5.
55. Altmae S, Martinez-Conejero JA, Esteban FJ, Ruiz-Alonso M, Stavreus-Evers A, Horcajadas JA, et al. MicroRNAs miR-30b, miR-30d, and miR-494 regulate human endometrial receptivity. Reprod Sci. 2013;20(3):308–17.
56. Saare M, Rekker K, Laisk-Podar T, Soritsa D, Roost AM, Simm J, et al. High-throughput sequencing approach uncovers the miRNome of peritoneal endometriotic lesions and adjacent healthy tissues. PLoS One. 2014;9(11):e112630.
57. Sweeney TE, Suliman HB, Hollingsworth JW, Piantadosi CA. Differential regulation of the PGC family of genes in a mouse model of Staphylococcus aureus sepsis. PLoS One. 2010;5(7):e11606.
58. De Faria O, Moore CS, Kennedy TE, Antel JP, Bar-Or A, Dhaunchak AS. MicroRNA dysregulation in multiple sclerosis. Front Genet. 2012;3:311.
59. Kumar M, Nerurkar VR. Integrated analysis of microRNAs and their disease related targets in the brain of mice infected with West Nile virus. Virology. 2014;452-453:143–51.
60. Andersen HH, Duroux M, Gazerani P. MicroRNAs as modulators and biomarkers of inflammatory and neuropathic pain conditions. Neurobiol Dis. 2014;71:159–68.

61. Zubrzycka A, Zubrzycki M, Janecka A, Zubrzycka M, Zhu L, Sun L, et al. Circulating microRNAs as potential biomarkers for endometriosis. Bronson R, editor. PLoS One. 2015;6(4):278A.
62. Ramon LA, Braza-Boils A, Gilabert J, Chirivella M, Espana F, Estelles A, et al. microRNAs related to angiogenesis are dysregulated in endometrioid endometrial cancer. Hum Reprod. 2012;27:3036–45.
63. Hawkins SM, Creighton CJ, Han DY, Zariff A, Anderson ML, Gunaratne PH, et al. Functional MicroRNA involved in endometriosis. Mol Endocrinol. 2011;25(5):821–32.
64. Mari-Alexandre J, Garcia-Oms J, Barcelo-Molina M, Gilabert-Aguilar J, Estelles A, Braza-Boils A, et al. MicroRNAs and angiogenesis in endometriosis. Thromb Res. 2015;135:S38–40.
65. Agrawal S, Tapmeier T, Rahmioglu N, Kirtley S, Zondervan K, Becker C. The miRNA mirage: how close are we to finding a non-invasive diagnostic biomarker in endometriosis? A systematic review. Int J Mol Sci. 2018;19(2):599.
66. Ohlsson Teague EMC, Van der Hoek KH, Van der Hoek MB, Perry N, Wagaarachchi P, Robertson SA, et al. MicroRNA-regulated pathways associated with endometriosis. Mol Endocrinol. 2009;23(2):265–75.
67. Burney RO, Hamilton AE, Aghajanova L, Vo KC, Nezhat CN, Lessey BA, et al. MicroRNA expression profiling of eutopic secretory endometrium in women with versus without endometriosis. Mol Hum Reprod. 2009;15(10):625–31.
68. Filigheddu N, Gregnanin I, Porporato PE, Surico D, Perego B, Galli L, et al. Differential expression of micrornas between eutopic and ectopic endometrium in ovarian endometriosis. J Biomed Biotechnol. 2010;2010:369549.
69. Petracco R, Grechukhina O, Popkhadze S, Massasa E, Zhou Y, Taylor HS. MicroRNA 135 regulates HOXA10 expression in endometriosis. J Clin Endocrinol Metab. 2011;96(12):E1925–33.
70. Ramon LA, Braza-Boils A, Gilabert-Estelles J, Gilabert J, Espana F, Chirivella M, et al. microRNAs expression in endometriosis and their relation to angiogenic factors. Hum Reprod. 2011;26(5):1082–90.
71. Dai L, Gu L, Di W. MiR-199a attenuates endometrial stromal cell invasiveness through suppression of the IKKbeta/nf-kappab pathway and reduced interleukin-8 expression. Mol Hum Reprod. 2012;18(3):136–45.
72. Lin S-C, Wang C-C, Wu M-H, Yang S-H, Li Y-H, Tsai S-J. Hypoxia-induced microRNA-20a expression increases ERK phosphorylation and angiogenic gene expression in endometriotic stromal cells. J Clin Endocrinol Metab. 2012;97(8):E1515–23.
73. Liu S, Gao S, Wang XY, Wang DB. Expression of miR-126 and Crk in endometriosis: miR-126 may affect the progression of endometriosis by regulating Crk expression. Arch Gynecol Obstet. 2012;285(4):1065–72.
74. Abe W, Nasu K, Nakada C, Kawano Y, Moriyama M, Narahara H, et al. MiR-196b targets c-myc and Bcl-2 expression, inhibits proliferation and induces apoptosis in endometriotic stromal cells. Hum Reprod. 2013;28(3):750–61.
75. Shen L, Yang S, Huang W, Xu W, Wang Q, Song Y, et al. MicroRNA23a and MicroRNA23b deregulation derepresses SF-1 and upregulates estrogen signaling in ovarian endometriosis. J Clin Endocrinol Metab. 2013;98(4):1575–82.
76. Laudanski P, Charkiewicz R, Kuzmicki M, Szamatowicz J, Charkiewicz A, Niklinski J. MicroRNAs expression profiling of eutopic proliferative endometrium in women with ovarian endometriosis. Reprod Biol Endocrinol. 2013;11:78.
77. Shi X-Y, Lin G, Jie C, Guo X-R, Shi Y-L. Downregulation of miR-183 inhibits apoptosis and enhances the invasive potential of endometrial stromal cells in endometriosis. Int J Mol Med. 2014;33(1):59–67.
78. Braza-Boils A, Mari-Alexandre J, Gilabert J, Sanchez-Izquierdo D, Espana F, Estelles A, et al. MicroRNA expression profile in endometriosis: its relation to angiogenesis and fibrinolytic factors. Hum Reprod. 2014;29(5):978–88.
79. Zheng B, Xue X, Zhao Y, Chen J, Xu C-Y, Duan P. The differential expression of microRNA-143,145 in endometriosis. Iran J Reprod Med. 2014;12(8):555–60.

80. Zhao M, Tang Q, Wu W, Xia Y, Chen D, Wang X. miR-20a contributes to endometriosis by regulating NTN4 expression. Mol Biol Rep. 2014;41(9):5793–7.
81. Graham A, Falcone T, Nothnick WB. The expression of microRNA-451 in human endometriotic lesions is inversely related to that of macrophage migration inhibitory factor (MIF) and regulates MIF expression and modulation of epithelial cell survival. Hum Reprod. 2015;30(3):642–52.
82. Lv X, Chen P, Liu W. Down regulation of MiR-93 contributes to endometriosis through targeting MMP3 and VEGFA. Am J Cancer Res. 2015;5(5):1706–17.
83. Zhang D, Li Y, Tian J, Zhang H, Wang S. MiR-202 promotes endometriosis by regulating SOX6 expression. Int J Clin Exp Med. 2015;8(10):17757–64.
84. Dong M, Yang P, Hua F. MiR-191 modulates malignant transformation of endometriosis through regulating TIMP3. Med Sci Monit. 2015;21:915–20.
85. Long M, Wan X, La X, Gong X, Cai X, Long M, et al. MiR-29c is downregulated in the ectopic endometrium and exerts its effects on endometrial cell proliferation, apoptosis and invasion by targeting c-Jun. Int J Mol Med. 2015;35(4):1119–25.
86. Yang RQ, Teng H, Xu XH, Liu SY, Wang YH, Guo FJ, et al. Microarray analysis of microRNA deregulation and angiogenesis-related proteins in endometriosis. Genet Mol Res. 2016;15(2):7826–33.
87. Nothnick WB, Falcone T, Joshi N, Fazleabas AT, Graham A. Serum miR-451a levels are significantly elevated in women with endometriosis and recapitulated in baboons (papio anubis) with experimentally-induced disease. Reprod Sci. 2017;24(8):1195–202.
88. Liang Z, Chen Y, Zhao Y, Xu C, Zhang A, Zhang Q, et al. miR-200c suppresses endometriosis by targeting MALAT1 in vitro and in vivo. Stem Cell Res Ther. 2017;8(1):251.
89. Zhao L, Gu C, Ye M, Zhang Z, Li L, Fan W, et al. Integration analysis of microRNA and mRNA paired expression profiling identifies deregulated microRNA-transcription factor-gene regulatory networks in ovarian endometriosis. Reprod Biol Endocrinol. 2018;16(1):4.
90. Liu Y, Chen J, Zhu X, Tang L, Luo X, Shi Y. Role of miR-449b-3p in endometriosis via effects on endometrial stromal cell proliferation and angiogenesis. Mol Med Rep. 2018;18(3):3359–65.
91. Rekker K, Tasa T, Saare M, Samuel K, Kadastik Ü, Karro H, et al. Differentially-expressed miRNAs in ectopic stromal cells contribute to endometriosis development: the plausible role of miR-139-5p and miR-375. Int J Mol Sci. 2018;19(12):3789.
92. Matsuzaki S, Darcha C. Epithelial to mesenchymal transition-like and mesenchymal to epithelial transition-like processes might be involved in the pathogenesis of pelvic endometriosis. Hum Reprod. 2012;27(3):712–21.
93. Yang Y-M, Yang W-X. Epithelial-to-mesenchymal transition in the development of endometriosis. Oncotarget. 2017;8(25):41679–89.
94. Nieto MA. Epithelial plasticity: a common theme in embryonic and cancer cells. Science. 2013;342(6159):1234850.
95. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol. 2014;15(3):178–96.
96. Gaetje R, Kotzian S, Herrmann G, Baumann R, Starzinski-Powitz A. Nonmalignant epithelial cells, potentially invasive in human endometriosis, lack the tumor suppressor molecule E-cadherin. Am J Pathol. 1997;150(2):461–7.
97. Zeitvogel A, Baumann R, Starzinski-Powitz A. Identification of an invasive, N-cadherin-expressing epithelial cell type in endometriosis using a new cell culture model. Am J Pathol. 2001;159(5):1839–52.
98. Korpal M, Lee ES, Hu G, Kang Y. The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. J Biol Chem. 2008;283(22):14910–4.
99. Gregory-Bass RC, Olatinwo M, Xu W, Matthews R, Stiles JK, Thomas K, et al. Prohibitin silencing reverses stabilization of mitochondrial integrity and chemoresistance in ovarian cancer cells by increasing their sensitivity to apoptosis. Int J Cancer. 2008;122(9):1923–30.

100. Kim NH, Kim HS, Li X-Y, Lee I, Choi H-S, Kang SE, et al. A p53/miRNA-34 axis regulates Snail1-dependent cancer cell epithelial-mesenchymal transition. J Cell Biol. 2011;195(3):417–33.
101. Siemens H, Jackstadt R, Hunten S, Kaller M, Menssen A, Gotz U, et al. miR-34 and SNAIL form a double-negative feedback loop to regulate epithelial-mesenchymal transitions. Cell Cycle. 2011;10(24):4256–71.
102. Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol. 2008;10(5):593–601.
103. Sahin C, Mamillapalli R, Yi KW, Taylor HS. microRNA Let-7b: a novel treatment for endometriosis. J Cell Mol Med. 2018;22(11):5346–53.

Chapter 13
Metabolic and Nutritional Impact on Endometrial Gene Expression and Reproductive Disorder

Lianghui Diao, Songchen Cai, Jinli Ding, and Yong Zeng

The Metabolic Pathways Involved in Endometrial Receptivity, Decidualization, and Placentation

A normal menstrual cycle can be divided into three phases: the menstruation, the proliferative phase, and the secretory phase. The endometrium in the secretory phase which corresponds to the luteal phase of the ovarian cycle holds the potential for trophoblasts adhesion and invasions. The endometrial stromal cells transform their phenotype from spindle-shaped cells into large, glycogen and lipid droplets-filled decidual cells during the secretory phase, which process is called decidualization. During decidualization, an extensive cross-talk takes place between the stromal cells and immune cells, providing a nutritive and immune-privileged matrix essential for embryo implantation and placental development [1]. Given that metabolism shapes distinct cell functions, data from transcriptomics and proteomics suggest that stromal cells and the immune cells might undergo metabolic reprogramming and functionally adapt to the fetal allograft. Inadequate decidualization has been found under the pathological conditions, which is detrimental to placentation and fetal outcome [2], including reduced endometrial receptivity and pregnancy complications.

The concept "Paradox of the fetal allograft" has been accepted as the fundamental theory of reproductive immunology for more than 60 years, and now people understand that pregnancy is more like cancer, but not transplantation [3]. Regardless of the

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theological models, the similarity is that the immune privilege is actively created by the maternal immune system to adapt to the presence of fetal antigens. The immune cells involved in the interaction at the maternal-fetal interface mainly consist of natural killer (NK) cells, macrophages (Mφ), dendritic cells (DCs), and regulatory T cells. NK cells are dominant immune cells in the decidua during pregnancy, which comprise about 70% of the decidual lymphocytes [4]. During a physiological condition of pregnancy, a series of cytokines and chemokines are secreted by decidual NK cells, thus regulating trophoblast invasion and remodeling the uterine spiral arteries [5]. The second largest population, Mφ, accounts for about 20% of the decidual lymphocytes, which can be polarized into M1 or M2 state to adapt to the uterine microenvironment. M2 supports immune tolerance at the maternal-fetal interface and promotes angiogenesis, whereas M1 polarization of Mφ eliminates the pathogens via pattern recognition receptors [6]. DC are specific antigen-presenting cells, which could process antigen and activate naïve T cells. The critical role of DC during pregnancy has been noticed by the conditional ablation of uterine DC (uDC), which results in defects in implantation even in T cell-deficient animals [7]. This suggests that uDC govern their endometrial receptivity independent of immune tolerance at the maternal-fetal interface. Regulatory T cells (Treg) are a subset of T cells that mediate immune tolerance and prevent overwhelming or misguided immune activation [8]. The maternal tolerance mediated by Treg has been demonstrated in a mouse model by adoptive transfer system [9].

In general, at least six metabolic pathways are involved in endometrial receptivity, decidualization, and pregnancy placentation, which include glycolysis, tricarboxylic acid cycle (TCA), pentose phosphate pathway (PPP), fatty acid oxidation (FAO), fatty acid synthesis (FAS), and amino acid metabolism (Fig. 13.1) [10].

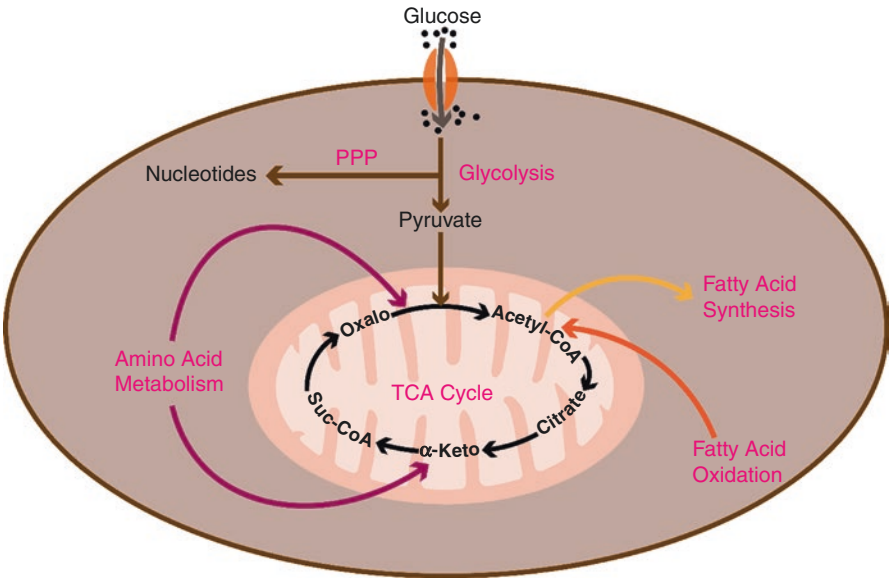

Fig. 13.1 Metabolic pathways involved in endometrial receptivity and decidualization. Abbreviations: PPP pentose phosphate pathway, TCA tricarboxylic acid cycle, Oxalo oxaloacetate, Acetyl-CoA acetyl coenzyme A, α-Keto α-ketoglutarate, Suc-CoA Succinyl-coenzyme A

In rapid proliferating and activated cells, glycolysis is the priority metabolic pathway. In glycolysis, glucose is mainly imported by glucose transporter 1 (GLUT1) for generating the intermediate glucose-6-phosphate (G6P), which is either converted into 6-phosphogluconolactone by glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting enzyme of PPP, or catalyzed by pyruvate kinase (PKM) to provide pyruvate for TCA cycle or lactate production. The function of glycolysis during successful pregnancy is well known for its involvement in stromal cells during decidualization in both rodents and human [11, 12]. In an animal study, knockdown of PKM2 results in defective induction of decidualization markers [13]. Glycolysis could rapidly generate sufficient energy to meet the desired energy demanded for the functional activities of immune cells, such as phagocytosis, cytokine production, and antigen presentation. In the activated immune cells, such as macrophage [14], DCs [15], NK cells [16], T cell subsets including Teff [17, 18], T_H17 [19], T_H1, and T_H2 [18], and B cells [20], glycolysis is enhanced. Moreover, glycolysis promotes the initiation of Treg cell generation, but inhibits Treg differentiation [17, 21]. However, how glycolysis affects the immune adaptations in response to the fetus are still enigmatic [10].

Parallel to glycolysis, PPP is a side branch metabolic pathway that generates NADPH for fatty acid synthesis, ribose 5-phosphate (R5P) for the synthesis of nucleotides, and nucleic acids erythrose 4-phosphate (E4P) for the synthesis of aromatic amino acids. Inhibition of G6PD is detrimental to decidualization and could be rescued by supplying exogenous nucleoside [22]. NADPH and NADPH-dependent function are critical for macrophage differentiation and generation of reactive oxygen species (ROS) together with neutrophils during infection [23, 24]. Moreover, NADPH could also act as an antioxidant to prevent excessive tissue damage [25]. Ribonucleotide reductase (RNR) is induced by progesterone in the stromal cells during decidualization and displays distinct distribution between implantation sites and inter-implantation sites in mouse uterus [26].

TCA cycle exists in most quiescent or nonproliferative cells. It is the critical metabolic pathway that connects the metabolism of amino acid, fatty acid, and carbohydrate by providing nicotinamide adenine dinucleotide (NADH) and precursors for following steps of the metabolic pathways. Accumulation of citrate, an intermediate of TCA cycle in generating fatty acids and precursors for biogenesis, is needed for antigen presentation in activated DCs [17, 27]. Silencing TCA-related genes or inhibiting their activities impairs the generation of nitric oxide (NO), ROS, and prostaglandins, suggesting that TCA is involved in the initiation of inflammation [28]. Hypoxia-inducible factor-1α (HIF-1α), a primary regulator in response to hypoxia, is regulated by TCA cycle metabolites. Immunohistochemistry study showed that HIF-1α protein is undetectable in the proliferative phase but highly expressed through secretory to menstrual phase in human endometrium [29]. Expression profiles study show that isocitrate dehydrogenase 2 (Idh2), the enzyme responsible for the generation of the 2-oxoglutarate from isocitrate, significantly increased in pregnant myometrium than in nonpregnant [30], and a similar phenomenon was found in mice—significantly increased on mid- and late pregnant placenta than in base [31].

The fourth metabolic pathway is fatty acid oxidation (FAO). FAO is preferentially utilized by noninflammatory and tolerogenic immune cells. The effector T cells, such as T_H1, T_H2, and T_H17, show less reliance on FAO than Treg cells [17]. The activation of effector T cells requires decreased lipid oxidation and increased glycolysis and glutaminolysis [32]. Depletion of the rate-limiting enzyme carnitine palmitoyltransferase I (CPT1) either by RNA interference or etomoxir reduced the expression levels of decidualization markers in cultured ESCs [33]. Meanwhile, when the mice were treated with FAO inhibitors, for example, ranolazine, the decidualization was impaired, and the number of pups was reduced [33].

The fifth metabolic pathway is fatty acid synthesis (FAS), which is involved in the inflammatory response and regulates the generation of innate and adaptive immune cell subsets. In vivo study showed that macrophage induced by LPS exhibited an enhanced lipid synthesis activity [34]. Moreover, the type of synthesized fatty acid may govern the cytokine production by T cells, e.g., saturated fatty acids have been shown to promote the synthesis of pro-inflammatory cytokines [35]. An immunohistochemical study showed that in the cycling endometrium, expression of fatty acid synthase is associated with the proliferation and differentiation of the stromal cells [36].

The sixth metabolic pathway is amino acid metabolism. Amino acids provide building blocks of protein biosynthesis. So far, glutamine, arginine, and tryptophan have been studied most extensively for their roles in immune responses. Cell proliferation and cytokine production are impaired by depleting glutamine in T cells, suggesting that glutamine metabolism could regulate T cell activation [37]. Arginine is also involved in T cell proliferation and function. Limiting of arginine leads to a reduction of T cell proliferation and mTOR signaling, accompanied by induction of forkhead box P3 (FOXP3) expression, which is the Treg-specific transcription factor [38]. Tryptophan has been reported to be broken down by enzymes induced by immune activation, such as indoleamine 2,3-dioxygenase (IDO), the rate-limiting enzyme present in Mφ and DCs [39]. Enzyme activity of IDO could be enhanced by IFN-γ, which is a critical cytokine for innate and adaptive immunity [40]. T cell response could be regulated by APC via IDO expression and tryptophan catabolism [41], and a direct effect of tryptophan catabolism mediated by IDO has been demonstrated in vitro [42]. Besides, the importance of IDO in pregnancy has been demonstrated in a murine model that tryptophan catabolism mediated by IDO in maternal T cells is required for maternal tolerance and prevention of allogeneic fetal rejection [43] (Table 13.1).

The abovementioned six metabolic pathways should not be seen as mutually exclusive or restricted to specific cell types. The requirements of the "metabolic reprogramming"' for changing demands provide potential targets for therapeutic intervention. One of the prototypical examples is known as "Warburg" effect in highly proliferated cancer cells that favor glycolysis rather than oxidative phosphorylation pathway even under normoxia microenvironment. The immunometabolism research in cancer sheds light on research for metabolism in human reproduction. However, the current understanding of metabolic plasticity in reproduction is still mysterious.

Table 13.1 Metabolic genes involved in endometrial receptivity, decidualization, and placentation

Genes	function during pregnancy	Publications
GLUT1	Decidualization	[11, 12]
PKM2	Decidualization	[13]
G6PD	Decidualization	[22]
RNR	Decidualization	[26]
HIF1α	Expressed through secretory to menstrual phase, decidualization	[13, 29]
PDK1	Decidualization	[13]
CPT1	Decidualization	[33]
FAS	Proliferation and differentiation of the stromal cells	[36]
idh2	Increased expression in pregnant myometrium and placenta	[30, 31]
IDO	Prevention of allogeneic fetal rejection	[41]

Abbreviations: *GLUT1* Glucose transporter 1, *PKM* Catalyzed by pyruvate kinase, *G6PD* Glucose-6-phosphate dehydrogenase, *RNR* Ribonucleotide reductase, *HIF-1α* Hypoxia-inducible factor-1α, *CPT1* Carnitine palmitoyltransferase I, *FAS* Fatty acid synthase, *NADP+* Isocitrate dehydrogenase 2, *IDH2* Mitochondrial, *IDO* Indoleamine 2,3-dioxygenase

Metabolic and Nutritional Impact on Genetic Vulnerabilities of Endometrial Gene Expression, Decidualization, and Placentation

The majority of early pregnancy losses occur in human is attributed, at least in part, to inadequate endometrial receptivity, insufficient decidualization, and defects in placentation. By the analysis of transcriptome and proteome from receptive and nonreceptive endometrium, a series of biomarkers for endometrial receptivity have been identified [44–46]. Empirical observations indicate that disturbed glucose influx in endometrial cells is correlated to the poor reproductive outcomes; for example, the GLUT1 expression in endometrium was significantly lower in women with idiopathic infertility [47].

The metabolic alterations in obesity and PCOS women reduce endometrial receptivity, which subsequently becomes detrimental to embryonic implantation, and lead to subfertility and miscarriage [48]. Transcriptional profiling data showed that the glycolysis-related genes (such as *PKM2, PDHA,* and *HK1)* were downregulated in the endometrium of PCOS females [49]. Excessive dehydroepiandrosterone (DHEA), a potent inhibitor of G6PD, is observed in more than 50% of women with PCOS [50]. The prevalence of impaired glucose tolerance (IGT) and non-insulin-dependent diabetes (NIDDM) are significantly higher in women with PCOS [51]. The endometrial expression of adhesion molecules, such as αvβ3-integrin; transcription factors, such as Homeobox protein alpha 10 (HOXA-10) and HOXA-11; and decidualization markers, such as insulin-like growth factor (IGFBP-I), were downregulated in PCOS patients during secretory phase [52–55]. Women with high luteinizing hormone (LH), which could be induced by insulin

resistance, showed a significantly higher miscarriage rate than the women with normal LH levels [56]. Besides the malfunction of ovulation and abnormal expression of the decidualization-related marker, higher leptin levels were found in women with PCOS, and a strong correlation existed between leptin level, BMI, and LH level [57]. On the contrary, a lower level of adiponectin was observed in women with PCOS [58, 59].

Pre-eclampsia is a pregnancy disorder characterized by new-onset hypertension and proteinuria at ≥20 weeks of gestation with a high risk of maternal and fetal morbidity and mortality [60]. Inadequate trophoblast invasion and defects in spiral artery remodeling are thought to be constructive for the pathology of pre-eclampsia. Indeed, HIF-1α increases in early gestation and falls around 9 weeks in normal pregnancy [61]. However, over-expressed HIF-1α was found in the placenta of women with pre-eclampsia [62]. HIF-1α-overexpressing pregnant mice showed increased blood pressure and proteinuria [63].

Imbalances of nutrition and metabolites interfere with the metabolic activity of the cells, interfering the function of stromal cells and the immune cells in the endometrium. The mRNA expression of decidualization markers, such as PRL and IGFBP1 is significantly lower in obese women (BMI ≥ 30) compared to lean women (BMI <30) [64], suggesting a reduced ability of decidualization. Consistent with this, diet-induced obesity mice have reduced the size of implantation sites [65]. Besides, endometrial transcriptome profile in immune response and cell adhesion were changed in high-fat diet-induced mice [66]. On the contrary, low levels of leptin, which are usually observed in women with a low BMI (<18.5), also suppress reproductive function, for example, the delay of ovulation [67]. Interestingly, leptin (*ob/ob*)-deficient mice were infertile, and exogenous administration of leptin could reverse the defects in reproduction [68] (Table 13.2).

Direct evidence to unravel the correlation between endometrial receptivity and nutrition comes from the in vitro study. In vitro stimulation by adiponectin significantly alters the endometrial mRNA profile in porcine, which includes genes that involved in embryo implantation, such as integrin, alpha L (*ITGAL*), and mucin 4(*MUC4*), and metabolism of lipids, carbohydrates, and proteins, such as insulin receptor (*INSR*) [69]. Leptin upregulated αvβ3-integrin and promoted cell adhesion in isolated mouse endometrial epithelial cells, which could facilitate blastocyst implantation [70].

In addition to a direct function in reproduction, leptin and adiponectin also affect immune responses. Low level of leptin impairs glucose influx and disturbs the activation, proliferation, and differentiation of T cells. In contrast, overweight women (BMI > 25) and obese women (BMI > 30) are associated with high levels of leptin and low levels of adiponectin that promote the inflammatory response and immune activation. This could be observed by the increased number of pro-inflammatory macrophages, neutrophils, and mast cells, as well as activation of pro-inflammatory T cells [71–73]. The defects of glucose metabolism promote the generation of Tregs [74], leading to the transition into immune tolerance state. Immune tolerance might be beneficial for pregnancy maintenance, but it leads to a higher risk of pathogen invasion [75–77]. Besides, the pro-inflammatory environment is a requisite for blas-

Table 13.2 Reproductive disorders, metabolic syndromes, and endometrial gene expressions

Reproductive disorders	Altered gene expressions	Publications
Idiopathic infertility	GLUT1 ↓	[47]
PCOS	PKM2, PDHA, and HK1 ↓	[49]
PCOS	DHEA ↑	[50]
PCOS	αvβ3-Integrin, HOXA-10, HOXA-11, IGFBP-I↓	[52–55]
PCOS	Leptin↑	[57]
PCOS	Adiponectin↓	[58, 59].
Preeclampsia	HIF-1α↑	[61]
Obese women	IGFBP1↓	[64]

Abbreviations: *GLUT1* Glucose transporter 1, *PKM2* Pyruvate kinase isozymes M2, *PDHA* Pyruvate dehydrogenase (lipoamide) alpha 1, and *HK1* Hexokinase-1, *DHEA* Dehydroepiandrosterone, *IGFBP-I* Insulin-like growth factor, *HOXA-10* Homeobox protein alpha 10, *HIF-1α* Hypoxia-inducible factor-1α, *IGFBP-I* Insulin-like growth factor

tocyst implantation [78, 79]. Hence, the function of the immune cell might be under the regulation of immunometabolism, which is affected by abnormal leptin and adiponectin and may account for the pregnancy complication in women with abnormal BMI. Therefore, overweight or obese women depicted a microenvironment in the endometrium that elevated in the inflammatory response, which may lead to spontaneous abortion and recurrent miscarriage. In contrast, lean women (BMI < 18.5) depicted a microenvironment in the endometrium that elevated immune tolerance, which may lead to repeated implantation failure.

Strategies of Metabolic Intervention on Endometrial Gene Expression and Pregnancy Outcomes

Metabolic manipulations of the immune cells by inhibitors of distinct metabolic pathways have blossomed into a potential therapeutic intervention to induce metabolic reprogramming in distinct immune diseases, such as autoimmunity, allograft rejection, and graft-versus-host disease. The experience for metabolic manipulation in such immune diseases could be drawn by the context of reproduction, as the maternal-fetal interface is under precise regulation of immune cells. However, limited metabolic manipulations have been used in clinical practice due to lack of evidence to depict the direct correlation between the immunometabolism and successful pregnancy, and the vulnerability of fetus to this approach.

Results from clinical trials with inhibitors of metabolic enzymes provide valuable insights for the prospect of metabolic intervention. Blockade of CTLA-4, PD-1, and PD-L1, which could restore glucose in the tumor microenvironment and permit T cell glycolysis, has been used clinically for antitumor treatment [80]. The PKM2 inhibitor, TLN-232, has been tested in a clinical trial for refractory renal cell carcinoma (NCT00422786), and REDD1—and the inhibitor of mTOR—

has been applied in phase 2 clinical trial (NCT00713518) for the treatment of neovascularization in age-related macular degeneration (AMD) patients. The inhibitor of H/K-ATPase, proton pump inhibitor (PPIs), has been conventionally used for the treatment of gastric ulcer [81]. AMP-activated protein kinase (AMPK) is a protein kinase-modulating energy metabolism, and agonists of AMPK, including aspirin, metformin, adiponectin, migration inhibitory factor (MIF), activated protein C, etc. have been used to treat diseases such as cancer, type 2 diabetes, and cardiovascular disease [82].

So far, metformin, an inhibitor of glutamine metabolism, is one of the few inhibitors used in clinical practice. Previously, metformin was a category B drug for pregnant women and restricted in treating type 2 diabetes. Nowadays, it is utilized as the effective ovulation induction agent for PCOS women with its potential effect on glucose absorption, suppressing gluconeogenesis, and reduction of body weight [83]. New evidence suggests that metformin may improve the endometrial receptivity, with a significant increase of IGFBP1 and progestogen-associated endometrial protein (PAEP), and the circulating level of PAEP reflects the endometrial function [84]. Moreover, vascular penetration was increased, and the blood flow of spiral arteries, calculated by resistance index, was decreased in the endometrium of women with PCOS when treated with metformin [85]. Both spontaneous abortion and gestational diabetes alleviate in women with PCOS who received metformin therapy during pregnancy [86, 87].

Orlistat (Xenical) is another metabolic intervention for women with PCOS. It is a potent inhibitor of pancreatic lipase, which is also served as an inhibitor of fatty acid synthase with antitumor properties [88]. Orlistat inhibits proliferation and induces apoptosis of tumor cells [88]. Moreover, it reduces metastasis and angiogenesis in melanomas [89]. In the field of reproduction, one small open-label RCT compared obese anovulatory PCOS patients using metformin or orlistat for 3 months, showing that both metformin and orlistat could improve physiological conditions in patients including weight, ovulation rates, and androgen concentration [83]. Orlistat is a category B drug for pregnant women and data in the Swedish Medical Birth Register during 1998–2011 suggested the safety of orlistat in pregnant women [90]. However, evidence for orlistat in reproduction are sparse, and the use of orlistat seems to depend on their role in lipase instead of fatty acid synthase.

2-Deoxy-D-glucose (2-DG) is a glucose analog, a potent inhibitor of glucose hexokinase [91], which could interfere with glycolysis and glycosylation, thus serving as antitumor medicine in combination with chemotherapy [92]. However, evidence shows that 2-DG blocks ESC decidualization with defects in cellular phenotype and decidual markers [91], which are detrimental for pregnancy and reduce a possibility for the application of 2-DG in the context of reproduction. Besides, administration of 2-DG in mice resulted in immune alterations, as well as hyperglycemia because of hyperphagia [93].

Etomoxir (ETO) is small molecule that interferes with carnitine palmitoyltransferase 1a (CPT1a), thus serving as a potent inhibitor of fatty acid oxidation. As mentioned in the previous part, depletion of CPT1a by ETO impairs the decidualization process in cultured ESCs [33]. However, a higher concentration of ETO

shows off-target effects, such as inhibition of oxidative metabolism and induction of severe oxidative stress in T cells [94].

Instead of regulating metabolism by the inhibitor, manipulating nutrition before and during pregnancy might be a feasible way to modulate the expression of the genes of the endometrium and thus improving the endometrial receptivity. According to the guideline recommended by the World Health Organization (WHO), prepregnancy BMI, age, parity, smoking, and race should be taken into consideration for optimal weight gain during pregnancy [95]. As pregnancy itself is susceptible to infection and limited medical management could be used during pregnancy, the destruction of immune balance by fasting will be detrimental for the pregnancy outcome if the infection takes place. On the contrary, women may develop gestational diabetes, pre-eclampsia, and preterm birth if excessive calorie intake was made during pregnancy.

Evidence from domestic animals shows that short-term and long-term diet controls significantly alter the expression of a large number of genes in the endometrium during the peri-implantation period [96, 97]. The concentration of branched-chain amino acids was significantly higher in the uterine fluid of women with an unhealthy diet [98]. It should be introduced to the public that maintaining healthy nutritional behavior is necessary for a successful pregnancy, and fasting as well as excessive calorie intake should be avoided.

Leptin levels drop in response to starvation or calorie restriction, leading to disturbed glucose influx and interfering the function of Th cells but promoting the generation of Tregs [74, 99]. T cells and macrophages are accumulated in adipose tissue and contribute to the pathogenesis of insulin resistance [73, 100]. The insulin resistance in obesity is associated with a distinct profile of IgG autoantibodies, and B cells resided in adipose tissue could promote insulin resistance via activation of pro-inflammatory macrophages and T cells [101]. To ensure stable glucose level and availability to the fetus, high levels of several diabetogenic hormones, such as resistin and leptin, could be present during normal pregnancies, leading to physiological insulin resistance [102]. Resistin over-expression mice model displayed insulin resistance [103], and the administration of anti-resistin antibody improved blood glucose and insulin levels in mice with diet-induced obesity [104]. Since macrophage could also express resistin, elevated resistin during pregnancy might be contributed by the increased number of pro-inflammatory macrophage, suggesting that insulin resistance is influenced via pro-inflammatory pathways, e.g., IL-6 production which can induce gestational diabetes [105]. However, the association between circulating resistin level and gestational diabetes is still controversial [106]. Therefore, directly interfering the adipokines such as leptin and adiponectin by the administration of insulin and growth hormone might strongly reverse the defects, which is caused by the imbalance of adipokines. It is technically feasible that the injection of leptin in leptin-deficient mice could successfully rescue infertility and implantation failure which has been proven in the animal model [107]. However, controversies exist in the relationship between adipokine and pathology, such as resistin and gestation diabetes as mentioned above.

Summary and Conclusion

The endometrial receptivity is regulated by the crosstalk of stromal and immune cells, which undergo the adaptations in their function. The demand during the functional changes for energy and biosynthesis could be fulfilled by metabolism. The notion that "Warburg effect" in the cancer cell is only a part of the iceberg in metabolic reprogramming is critical progress, which expands and deepens the study of metabolism. One of the inspiring findings is that the metabolic reprogramming is not restricted to cancer cells, thus shedding light on research for metabolism in reproduction. From the evidence and insights currently known in the rodent and human reproduction, metabolic and nutritional impacts contribute to the endometrial gene expression and pathology of reproductive disorders, such as RIF, RM, IUGR, and preterm labor. However, limited knowledge of the metabolic reprogramming has impeded the preclinical and clinical development of metabolic-related inhibitors. Most of the medicines used in reproduction are focused on their roles in controlling the weight of women with PCOS, instead of their role in targeting the metabolic pathway. Metformin is one of few inhibitors used in reproduction to improve ovulation and endometrial receptivity, as well as to reduce early pregnancy loss.

Instead of regulating metabolism by the inhibitors, manipulating nutrition during pregnancy is more suitable and readily accepted. The nutritional condition affects the function of both decidual and immune cells, and impaired decidualization was found in stromal cells of women with malnutrition. Therefore, nutritional intervention should be taken into consideration to ameliorate pregnancy complications. However, more works are needed to unravel the role of nutrition underlying successful mechanisms for reproduction.

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References

1. Gellersen B, Brosens JJ. Cyclic decidualization of the human endometrium in reproductive health and failure. Endocr Rev. 2014;35(6):851–905. https://doi.org/10.1210/er.2014-1045.
2. Cha J, Sun X, Dey SK. Mechanisms of implantation: strategies for successful pregnancy. Nat Med. 2012;18(12):1754–67. https://doi.org/10.1038/nm.3012.

3. Patil R, Patil SA, Beaman KD, Patil SA. Indole molecules as inhibitors of tubulin polymerization: potential new anticancer agents, an update (2013-2015). Future Med Chem. 2016;8(11):1291–316. https://doi.org/10.4155/fmc-2016-0047.
4. Manaster I, Mizrahi S, Goldman-Wohl D, Sela HY, Stern-Ginossar N, Lankry D, et al. Endometrial NK cells are special immature cells that await pregnancy. J Immunol. 2008;181(3):1869–76.
5. Rätsep MT, Felker AM, Kay VR, Tolusso L, Hofmann AP, BA C. Uterine natural killer cells: supervisors of vasculature construction in early decidua basalis. Reproduction. 2015;149(2):R91–R102.
6. Nagamatsu T, Schust DJ. The contribution of macrophages to normal and pathological pregnancies. Am J Reprod Immunol. 2010;63(6):460–71. https://doi.org/10.1111/j.1600-0897.2010.00813.x.
7. Plaks V, Birnberg T, Berkutzki T, Sela S, BenYashar A, Kalchenko V, et al. Uterine DCs are crucial for decidua formation during embryo implantation in mice. J Clin Invest. 2008;118(12):3954–65. https://doi.org/10.1172/JCI36682.
8. Corthay A. How do regulatory T cells work? Scand J Immunol. 2009;70(4):326–36.
9. Aluvihare VR, Kallikourdis M, Betz AG. Regulatory T cells mediate maternal tolerance to the fetus. Nat Immunol. 2004;5(3):266–71. https://doi.org/10.1038/ni1037.
10. Thiele K, Diao L, Arck PC. Immunometabolism, pregnancy, and nutrition. Semin Immunopathol. 2018;40(2):157–74. https://doi.org/10.1007/s00281-017-0660-y.
11. Frolova AI, Moley KH. Quantitative analysis of glucose transporter mRNAs in endometrial stromal cells reveals critical role of GLUT1 in uterine receptivity. Endocrinology. 2011;152(5):2123–8. https://doi.org/10.1210/en.2010-1266.
12. Frolova A, Flessner L, Chi M, Kim ST, Foyouzi-Yousefi N, Moley KHJE. Facilitative glucose transporter type 1 is differentially regulated by progesterone and estrogen in murine and human endometrial stromal cells. Endocrinology. 2008;150(3):1512–20.
13. Zuo RJ, Gu XW, Qi QR, Wang TS, Zhao XY, Liu JL, et al. Warburg-like glycolysis and lactate shuttle in mouse decidua during early pregnancy. J Biol Chem. 2015;290(35):21280–91. https://doi.org/10.1074/jbc.M115.656629.
14. Rodríguez-Prados J-C, Través PG, Cuenca J, Rico D, Aragonés J, Martín-Sanz P, et al. Substrate fate in activated macrophages: a comparison between innate, classic, and alternative activation. J Immunol. 2010;185(1):605–14.
15. Krawczyk CM, Holowka T, Sun J, Blagih J, Amiel E, RJ DB, et al. Toll-like receptor–induced changes in glycolytic metabolism regulate dendritic cell activation. Blood. 2010;115(23):4742–9.
16. Donnelly RP, Loftus RM, Keating SE, Liou KT, Biron CA, Gardiner CM, et al. mTORC1-dependent metabolic reprogramming is a prerequisite for NK cell effector function. J Immunol. 2014;193(9):4477–84.
17. Michalek RD, Gerriets VA, Jacobs SR, Macintyre AN, MacIver NJ, Mason EF, et al. Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4+ T cell subsets. J Immunol. 2011;186(6):3299–303. https://doi.org/10.4049/jimmunol.1003613.
18. Gubser PM, Bantug GR, Razik L, Fischer M, Dimeloe S, Hoenger G, et al. Rapid effector function of memory CD8+ T cells requires an immediate-early glycolytic switch. Nat Immunol. 2013;14(10):1064–72. https://doi.org/10.1038/ni.2687.
19. Shi LZ, Wang R, Huang G, Vogel P, Neale G, Green DR, et al. HIF1alpha-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. J Exp Med. 2011;208(7):1367–76. https://doi.org/10.1084/jem.20110278.
20. Doughty CA, Bleiman BF, Wagner DJ, Dufort FJ, Mataraza JM, Roberts MF, et al. Antigen receptor–mediated changes in glucose metabolism in B lymphocytes: role of phosphatidylinositol 3-kinase signaling in the glycolytic control of growth. Blood. 2006;107(11):4458–65.
21. Angelin A, Gil-de-Gomez L, Dahiya S, Jiao J, Guo L, Levine MH, et al. Foxp3 reprograms t cell metabolism to function in low-glucose, high-lactate environments. Cell Metab. 2017;25(6):1282–93. e7. https://doi.org/10.1016/j.cmet.2016.12.018.

22. Frolova AI, O'neill K, Moley KH. Dehydroepiandrosterone inhibits glucose flux through the pentose phosphate pathway in human and mouse endometrial stromal cells, preventing decidualization and implantation. Mol Endocrinol. 2011;25(8):1444–55.
23. Nguyen GT, Green ER, Mecsas J. Neutrophils to the ROScue: mechanisms of NADPH oxidase activation and bacterial resistance. Front Cell Infect Microbiol. 2017;7:373. https://doi.org/10.3389/fcimb.2017.00373.
24. Xu Q, Choksi S, Qu J, Jang J, Choe M, Banfi B, et al. NADPH oxidases are essential for macrophage differentiation. J Biol Chem. 2016;291(38):20030–41. https://doi.org/10.1074/jbc.M116.731216.
25. Kirsch M, De Groot H. NAD(P)H, a directly operating antioxidant? FASEB J. 2001;15(9):1569–74.
26. Lei W, Feng X-H, Deng W-B, Ni H, Zhang Z-R, Jia B, et al. Progesterone and DNA damage encourage uterine cell proliferation and decidualization through up-regulating ribonucleotide reductase 2 expression during early pregnancy in mice. J Biol Chem. 2012;287(19):15174–92.
27. O'Sullivan D, van der Windt GJ, Huang SC-C, Curtis JD, Chang C-H, Buck MD, et al. Memory CD8+ T cells use cell-intrinsic lipolysis to support the metabolic programming necessary for development. Immunity. 2014;41(1):75–88.
28. Infantino V, Convertini P, Cucci L, Panaro MA, Di Noia MA, Calvello R, et al. The mitochondrial citrate carrier: a new player in inflammation. Biochem J. 2011;438(3):433–6. https://doi.org/10.1042/BJ20111275.
29. Critchley HO, Osei J, Henderson TA, Boswell L, Sales KJ, Jabbour HN, et al. Hypoxia-inducible factor-1alpha expression in human endometrium and its regulation by prostaglandin E-series prostanoid receptor 2 (EP2). Endocrinology. 2006;147(2):744–53. https://doi.org/10.1210/en.2005-1153.
30. Rehman KS, Yin S, Mayhew BA, Word RA, Rainey WE. Human myometrial adaptation to pregnancy: cDNA microarray gene expression profiling of myometrium from non-pregnant and pregnant women. Mol Hum Reprod. 2003;9(11):681–700.
31. Paquette A, Baloni P, Holloman AB, Nigam S, Bammler T, Mao Q, et al. Temporal transcriptomic analysis of metabolic genes in maternal organs and placenta during murine pregnancy. Biol Reprod. 2018;99(6):1255–65. https://doi.org/10.1093/biolre/ioy148.
32. Gerriets VA, Kishton RJ, Nichols AG, Macintyre AN, Inoue M, Ilkayeva O, et al. Metabolic programming and PDHK1 control CD4+ T cell subsets and inflammation. J Clin Invest. 2015;125(1):194–207. https://doi.org/10.1172/JCI76012.
33. Tsai JH, Chi MM, Schulte MB, Moley KH. The fatty acid beta-oxidation pathway is important for decidualization of endometrial stromal cells in both humans and mice. Biol Reprod. 2014;90(2):34. https://doi.org/10.1095/biolreprod.113.113217.
34. Posokhova E, Khoshchenko O, Chasovskikh M, Pivovarova E, M D. Lipid synthesis in macrophages during inflammation in vivo: effect of agonists of peroxisome proliferator activated receptors α and γ and of retinoid X receptors. Biochemistry (Mosc). 2008;73(3):296.
35. Wang C, Yosef N, Gaublomme J, Wu C, Lee Y, Clish CB, et al. CD5L/AIM regulates lipid biosynthesis and restrains Th17 cell pathogenicity. Cell. 2015;163(6):1413–27. https://doi.org/10.1016/j.cell.2015.10.068.
36. Pizer ES, Kurman RJ, Pasternack GR, Kuhajda FP. Expression of fatty acid synthase is closely linked to proliferation and stromal decidualization in cycling endometrium. Int J Gynecol Pathol. 1997;16(1):45–51. https://doi.org/10.1097/00004347-199701000-00008.
37. Carr EL, Kelman A, Wu GS, Gopaul R, Senkevitch E, Aghvanyan A, et al. Glutamine uptake and metabolism are coordinately regulated by ERK/MAPK during T lymphocyte activation. J Immunol. 2010;185(2):1037–44. https://doi.org/10.4049/jimmunol.0903586.
38. Cobbold SP, Adams E, Farquhar CA, Nolan KF, Howie D, Lui KO, et al. Infectious tolerance via the consumption of essential amino acids and mTOR signaling. Proc Natl Acad Sci U S A. 2009;106(29):12055–60. https://doi.org/10.1073/pnas.0903919106.
39. Moffett JR, Namboodiri MA. Tryptophan and the immune response. Immunol Cell Biol. 2003;81(4):247–65. https://doi.org/10.1046/j.1440-1711.2003.t01-1-01177.x.

40. Yoshida R, Imanishi J, Oku T, Kishida T, Hayaishi O. Induction of pulmonary indoleamine 2,3-dioxygenase by interferon. Proc Natl Acad Sci U S A. 1981;78(1):129–32.
41. Munn DH, Shafizadeh E, Attwood JT, Bondarev I, Pashine A, Mellor AL. Inhibition of T cell proliferation by macrophage tryptophan catabolism. J Exp Med. 1999;189(9):1363–72.
42. Lee GK, Park HJ, Macleod M, Chandler P, Munn DH, Mellor AL. Tryptophan deprivation sensitizes activated T cells to apoptosis prior to cell division. Immunology. 2002;107(4):452–60.
43. Munn DH, Zhou M, Attwood JT, Bondarev I, Conway SJ, Marshall B, et al. Prevention of allogeneic fetal rejection by tryptophan catabolism. Science. 1998;281(5380):1191–3.
44. Bourdiec A, Calvo E, Rao C, Akoum A. Transcriptome analysis reveals new insights into the modulation of endometrial stromal cell receptive phenotype by embryo-derived signals interleukin-1 and human chorionic gonadotropin: possible involvement in early embryo implantation. PLoS One. 2013;8(5):e64829.
45. Garrido-Gómez T, Ruiz-Alonso M, Blesa D, Diaz-Gimeno P, Vilella F, C S, et al. Profiling the gene signature of endometrial receptivity: clinical results. Fertil Steril. 2013;99(4):1078–85.
46. Maekawa R, Taketani T, Mihara Y, Sato S, Okada M, Tamura I, et al. Thin endometrium transcriptome analysis reveals a potential mechanism of implantation failure. Reprod Med Biol. 2017;16(2):206–27.
47. Frolova AI, Moley KH. Glucose transporters in the uterus: an analysis of tissue distribution and proposed physiological roles. Reproduction. 2011;142(2):211–20. https://doi.org/10.1530/REP-11-0114.
48. Schulte MM, Tsai JH, Moley KH. Obesity and PCOS: the effect of metabolic derangements on endometrial receptivity at the time of implantation. Reprod Sci. 2015;22(1):6–14. https://doi.org/10.1177/1933719114561552.
49. Kim JY, Song H, Kim H, Kang HJ, Jun JH, Hong SR, et al. Transcriptional profiling with a pathway-oriented analysis identifies dysregulated molecular phenotypes in the endometrium of patients with polycystic ovary syndrome. J Clin Endocrinol Metab. 2009;94(4):1416–26. https://doi.org/10.1210/jc.2008-1612.
50. Azziz R, Black V, Knochenhauer E, Hines G, Boots L. Ovulation after glucocorticoid suppression of adrenal androgens in the polycystic ovary syndrome is not predicted by the basal dehydroepiandrosterone sulfate level. J Clin Endocrinol Metab. 1999;84(3):946–50.
51. Ehrmann DA, Barnes RB, Rosenfield RL, Cavaghan MK, Imperial J. Prevalence of impaired glucose tolerance and diabetes in women with polycystic ovary syndrome. Diabetes Care. 1999;22(1):141–6.
52. Cakmak H, Taylor HS. Implantation failure: molecular mechanisms and clinical treatment. Hum Reprod Update. 2011;17(2):242–53. https://doi.org/10.1093/humupd/dmq037.
53. Peyghambari F, Fayazi M, Amanpour S, Haddadi M, Muhammadnejad S, Muhammadnejad A, et al. Assessment of α4, αv, β1 and β3 integrins expression throughout the implantation window phase in endometrium of a mouse model of polycystic ovarian syndromes. Iran J Reprod Med. 2014;12(10):687.
54. Apparao KB, Lovely LP, Gui Y, Lininger RA, Lessey BA. Elevated endometrial androgen receptor expression in women with polycystic ovarian syndrome. Biol Reprod. 2002;66(2):297–304.
55. Cermik D, Selam B, Taylor HS. Regulation of HOXA-10 expression by testosterone in vitro and in the endometrium of patients with polycystic ovary syndrome. J Clin Endocrinol Metab. 2003;88(1):238–43. https://doi.org/10.1210/jc.2002-021072.
56. Regan L, Owen EJ, Jacobs HS. Hypersecretion of luteinising hormone, infertility, and miscarriage. Lancet. 1990;336(8724):1141–4.
57. Jalilian N, Haghnazari L, Rasolinia S. Leptin and body mass index in polycystic ovary syndrome. Indian J Endocrinol Metab. 2016;20(3):324–8. https://doi.org/10.4103/2230-8210.180005.
58. Shin HY, Lee DC, Lee JW. Adiponectin in women with polycystic ovary syndrome. Korean J Fam Med. 2011;32(4):243–8. https://doi.org/10.4082/kjfm.2011.32.4.243.

59. Toulis KA, Goulis DG, Farmakiotis D, Georgopoulos NA, Katsikis I, Tarlatzis BC, et al. Adiponectin levels in women with polycystic ovary syndrome: a systematic review and a meta-analysis. Hum Reprod Update. 2009;15(3):297–307. https://doi.org/10.1093/humupd/dmp006.
60. Chaiworapongsa T, Chaemsaithong P, Yeo L, Romero R. Pre-eclampsia part 1: current understanding of its pathophysiology. Nat Rev Nephrol. 2014;10(8):466–80. https://doi.org/10.1038/nrneph.2014.102.
61. Tal R. The role of hypoxia and hypoxia-inducible factor-1alpha in preeclampsia pathogenesis. Biol Reprod. 2012;87(6):134. https://doi.org/10.1095/biolreprod.112.102723.
62. Rajakumar A, Brandon HM, Daftary A, Ness R, Conrad KP. Evidence for the functional activity of hypoxia-inducible transcription factors overexpressed in preeclamptic placentae. Placenta. 2004;25(10):763–9. https://doi.org/10.1016/j.placenta.2004.02.011.
63. Tal R, Shaish A, Barshack I, Polak-Charcon S, Afek A, Volkov A, et al. Effects of hypoxia-inducible factor-1α overexpression in pregnant mice: possible implications for preeclampsia and intrauterine growth restriction. Am J Pathol. 2010;177(6):2950–62.
64. Schulte M, Cusumano A, Zhang W, Kettle-Oestreich A, Moley KH. Impaired autophagy contributes to obese patients decreased ability to undergo in vitro decidualization. Article in Fertil Steril. 2017;107(3):e4–5.
65. Rhee JS, Saben JL, Mayer AL, Schulte MB, Asghar Z, Stephens C, et al. Diet-induced obesity impairs endometrial stromal cell decidualization: a potential role for impaired autophagy. Hum Reprod. 2016;31(6):1315–26. https://doi.org/10.1093/humrep/dew048.
66. Cheng Y, Lv Q, Xie B, Yang B, Shan W, Ning C, et al. Estrogen and high-fat diet induced alterations in C57BL/6 mice endometrial transcriptome profile. Endocr Connect. 2018;7(1):36–46. https://doi.org/10.1530/EC-17-0315.
67. Ahima RS, Prabakaran D, Mantzoros C, Qu D, Lowell B, Maratos-Flier E, et al. Role of leptin in the neuroendocrine response to fasting. Nature. 1996;382(6588):250–2. https://doi.org/10.1038/382250a0.
68. Dos Santos E, Serazin V, Morvan C, Torre A, Wainer R, de Mazancourt P, et al. Adiponectin and leptin systems in human endometrium during window of implantation. Fertil Steril. 2012;97(3):771–8. e1. https://doi.org/10.1016/j.fertnstert.2011.12.042.
69. Smolinska N, Szeszko K, Dobrzyn K, Kiezun M, Rytelewska E, Kisielewska K, et al. Transcriptomic analysis of porcine endometrium during implantation after in vitro stimulation by adiponectin. Int J Mol Sci. 2019;20(6):1335. https://doi.org/10.3390/ijms20061335.
70. Yang YJ, Cao YJ, Bo SM, Peng S, Liu WM, Duan EK. Leptin-directed embryo implantation: leptin regulates adhesion and outgrowth of mouse blastocysts and receptivity of endometrial epithelial cells. Anim Reprod Sci. 2006;92(1–2):155–67. https://doi.org/10.1016/j.anireprosci.2005.05.019.
71. Huh JY, Park YJ, Ham M, Kim JB. Crosstalk between adipocytes and immune cells in adipose tissue inflammation and metabolic dysregulation in obesity. Mol Cells. 2014;37(5):365.
72. Yang H, Youm YH, Vandanmagsar B, Ravussin A, Gimble JM, Greenway F, et al. Obesity increases the production of proinflammatory mediators from adipose tissue T cells and compromises TCR repertoire diversity: implications for systemic inflammation and insulin resistance. J Immunol. 2010;185(3):1836–45. https://doi.org/10.4049/jimmunol.1000021.
73. Nishimura S, Manabe I, Nagasaki M, Eto K, Yamashita H, Ohsugi M, et al. CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. Nat Med. 2009;15(8):914–20. https://doi.org/10.1038/nm.1964.
74. Procaccini C, Galgani M, De Rosa V, Matarese G. Intracellular metabolic pathways control immune tolerance. Trends Immunol. 2012;33(1):1–7. https://doi.org/10.1016/j.it.2011.09.002.
75. Gabriel G, Arck PC. Sex, immunity and influenza. J Infect Dis. 2014;209 Suppl 3(suppl_3):S93–9. https://doi.org/10.1093/infdis/jiu020.
76. Engels G, Hierweger AM, Hoffmann J, Thieme R, Thiele S, Bertram S, et al. Pregnancy-related immune adaptation promotes the emergence of highly virulent h1n1 influenza virus

strains in allogenically pregnant mice. Cell Host Microbe. 2017;21(3):321–33. https://doi.org/10.1016/j.chom.2017.02.020.
77. van Riel D, Mittrücker H-W, Engels G, Klingel K, Markert UR, Gabriel G, editors. Influenza pathogenicity during pregnancy in women and animal models. Seminars in immunopathology. Berlin, Heidelberg: Springer; 2016.
78. Mor G, Cardenas I, Abrahams V, Guller SJ. Inflammation and pregnancy: the role of the immune system at the implantation site. Ann N Y Acad Sci. 2011;1221(1):80–7.
79. Dekel N, Gnainsky Y, Granot I, Mor G. Inflammation and implantation. Am J Reprod Immunol. 2010;63(1):17–21. https://doi.org/10.1111/j.1600-0897.2009.00792.x.
80. Kim J. Regulation of immune cell functions by metabolic reprogramming. J Immunol Res. 2018;2018:8605471. https://doi.org/10.1155/2018/8605471.
81. Shin JM, Sachs G. Pharmacology of proton pump inhibitors. Curr Gastroenterol Rep. 2008;10(6):528–34.
82. Goodman M, Liu Z, Zhu P, Li J. AMPK Activators as a drug for diabetes, cancer and cardiovascular disease. Pharm Regul Aff. 2014;3(2) https://doi.org/10.4172/2167-7689.1000118.
83. Metwally M, Amer S, Li TC, Ledger WL. An RCT of metformin versus orlistat for the management of obese anovulatory women. Hum Reprod. 2009;24(4):966–75. https://doi.org/10.1093/humrep/den454.
84. Sakumoto T, Tokunaga Y, Tanaka H, Nohara M, Motegi E, Shinkawa T, et al. Insulin resistance/hyperinsulinemia and reproductive disorders in infertile women. Reprod Med Biol. 2010;9(4):185–90. https://doi.org/10.1007/s12522-010-0062-5.
85. Attia GR, Rainey WE, Carr BR. Metformin directly inhibits androgen production in human thecal cells. Fertil Steril. 2001;76(3):517–24.
86. Jakubowicz DJ, Iuorno MJ, Jakubowicz S, Roberts KA, Nestler JE. Effects of metformin on early pregnancy loss in the polycystic ovary syndrome. J Clin Endocrinol Metab. 2002;87(2):524–9.
87. Glueck CJ, Wang P, Goldenberg N, Sieve-Smith LJ. Pregnancy outcomes among women with polycystic ovary syndrome treated with metformin. Hum Reprod. 2002;17(11):2858–64.
88. Kridel SJ, Axelrod F, Rozenkrantz N, Smith JW. Orlistat is a novel inhibitor of fatty acid synthase with antitumor activity. Cancer Res. 2004;64(6):2070–5.
89. Seguin F, Carvalho MA, Bastos DC, Agostini M, Zecchin KG, Alvarez-Flores MP, et al. The fatty acid synthase inhibitor orlistat reduces experimental metastases and angiogenesis in B16-F10 melanomas. Br J Cancer. 2012;107(6):977–87. https://doi.org/10.1038/bjc.2012.355.
90. Källén BA. Antiobesity drugs in early pregnancy and congenital malformations in the offspring. Obes Res Clin Pract. 2014;8(6):e571–e6.
91. Kommagani R, Szwarc MM, Kovanci E, Gibbons WE, Putluri N, Maity S, et al. Acceleration of the glycolytic flux by steroid receptor coactivator-2 is essential for endometrial decidualization. PLoS Genet. 2013;9(10):e1003900.
92. Merchan JR, Kovacs K, Railsback JW, Kurtoglu M, Jing Y, Pina Y, et al. Antiangiogenic activity of 2-deoxy-D-glucose. PLoS One. 2010;5(10):e13699. https://doi.org/10.1371/journal.pone.0013699.
93. Dréau D, Morton DS, Foster M, Swiggett JP, Sonnenfeld GJP. Immune alterations in male and female mice after 2-deoxy-D-glucose administration. Physiol Behav. 1997;62(6):1325–31.
94. O'Connor RS, Guo L, Ghassemi S, Snyder NW, Worth AJ, Weng L, et al. The CPT1a inhibitor, etomoxir induces severe oxidative stress at commonly used concentrations. Sci Rep. 2018;8(1):6289.
95. Council NR. Weight gain during pregnancy: reexamining the guidelines. Washington, DC: National Academies Press; 2010.
96. Zglejc K, Martyniak M, Waszkiewicz E, Kotwica G, Franczak A. Peri-conceptional undernutrition alters transcriptomic profile in the endometrium during the peri-implantation period-the study in domestic pigs. Reprod Domest Anim. 2018;53(1):74–84. https://doi.org/10.1111/rda.13068.

97. Bagés-Arnal S, Fernández-Fuertes B, Passaro C, Maicas C, McDonald M, Byrne CJ, et al. 43 Effect of early life nutrition on endometrial gland development and endometrial gene expression in heifers. Reprod Fertility Develop. 2019;31(1):147. https://doi.org/10.1071/RDv31n1Ab43.
98. Kermack AJ, Finn-Sell S, Cheong YC, Brook N, Eckert JJ, Macklon NS, et al. Amino acid composition of human uterine fluid: association with age, lifestyle and gynaecological pathology. Hum Reprod. 2015;30(4):917–24. https://doi.org/10.1093/humrep/dev008.
99. Cohen S, Danzaki K, NJ MI. Nutritional effects on T-cell immunometabolism. Eur J Immunol. 2017;47(2):225–35.
100. Lauterbach MA, Wunderlich FT. Macrophage function in obesity-induced inflammation and insulin resistance. Pflugers Arch. 2017;469(3–4):385–96. https://doi.org/10.1007/s00424-017-1955-5.
101. Winer DA, Winer S, Shen L, Wadia PP, Yantha J, Paltser G, et al. B cells promote insulin resistance through modulation of T cells and production of pathogenic IgG antibodies. Nat Med. 2011;17(5):610.
102. Silha JV, Krsek M, Skrha JV, Sucharda P, Nyomba BL, Murphy LJ. Plasma resistin, adiponectin and leptin levels in lean and obese subjects: correlations with insulin resistance. Eur J Endocrinol. 2003;149(4):331–5.
103. Liu Y, Wang Q, Pan YB, Gao ZJ, Liu YF, Chen SH. Effects of over-expressing resistin on glucose and lipid metabolism in mice. J Zhejiang Univ Sci B. 2008;9(1):44–50. https://doi.org/10.1631/jzus.B071479.
104. Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, et al. The hormone resistin links obesity to diabetes. Nature. 2001;409(6818):307–12. https://doi.org/10.1038/35053000.
105. Kuzmicki M, Telejko B, Szamatowicz J, Zonenberg A, Nikolajuk A, Kretowski A, et al. High resistin and interleukin-6 levels are associated with gestational diabetes mellitus. Gynecol Endocrinol. 2009;25(4):258–63. https://doi.org/10.1080/09513590802653825.
106. Lobo TF, Torloni MR, Gueuvoghlanian-Silva BY, Mattar R, Daher S. Resistin concentration and gestational diabetes: a systematic review of the literature. J Reprod Immunol. 2013;97(1):120–7. https://doi.org/10.1016/j.jri.2012.10.004.
107. Malik NM, Carter ND, Murray JF, Scaramuzzi RJ, Wilson CA, Stock MJ. Leptin requirement for conception, implantation, and gestation in the mouse. Endocrinology. 2001;142(12):5198–202. https://doi.org/10.1210/endo.142.12.8535.

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