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MicroRNA Signature and Regulatory Functions in the Endometrium during Normal and Disease States

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Abstract

During the menstrual cycle, human endometrium undergoes extensive cyclic morphologic and biochemical modifications in preparation for embryo implantation. These processes are highly regulated by ovarian steroids and various locally expressed gene products and involve inflammatory reaction, apoptosis, cell proliferation, angiogenesis, differentiation (tissue formation), and tissue remodeling. MicroRNAs (miRNAs) have emerged as key regulators of gene expression, and their altered and/or aberrant expression has been associated with establishment and progression of various disorders, including tumorigenesis. This review highlights the endometrial expression of miRNAs and their potential regulatory functions under normal and pathologic conditions such as endometriosis, dysfunctional uterine bleeding, and endometrial cancer. Given the key regulatory function of miRNAs on gene expression stability, understanding the underlying mechanisms of how endometrial miRNAs are regulated and identifying their specific target genes and their functions might lead to the development of preventive and therapeutic strategies by regulating specific target genes associated with such reproductive disorders.

Keywords

MicroRNA; endometrium; gene expression; gene regulation; endometrial disorders

During the menstrual cycle, endometrium undergoes extensive cyclic morphologic and biochemical modifications in preparation for embryo implantation that are remarkably consistent during each cycle throughout the reproductive years. This process, which begins with degenerative signals resulting in menstrual bleeding and endometrial shedding, integrates many overlapping and dynamic events to regenerate and become receptive. The endometrial regenerative process is initiated by an inflammatory reaction followed by a rapid cell proliferation, angiogenesis, differentiation (tissue formation), and tissue remodeling.^{1–7} If conception is not established, endometrium is ready to begin the next cycle. Ovarian sex steroids are central to endometrial regenerative and receptive processes. Accumulative evidence also suggests that endometrial expression of various autocrine/paracrine regulators, including many growth factors, cytokines, chemokines, proteases, and extracellular matrix,

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Following the submission of this article for publication, several articles have been published that bare mentioning. Two of these articles illustrate the expression profile of miRNAs in endometrial hyperplasia and endometrial carcinomas in humans and the conditional loss of uterine Pten, resulting in the rapid induction of endometrial cancer in mice, thus implicating the role of miRNAs in the pathogenesis of endometrial cancer.^{134,135} The remaining articles address the expression profile of miRNAs during embryo implantation and conditional loss of Dicer in female reproductive tract tissues, pointing to the regulatory function of miRNAs in these tissues.^{136–138}

serve as key components of these processes.^{1–7} The endometrial expression of these molecules must be optimal, precise, and synchronized to generate a timely progression of their signaling pathways. Any alterations in the expression of these mediators seem to be responsible for inappropriate tissue regeneration, embryo implantation failure, and other uterine abnormalities, including dysfunctional uterine bleeding, endometriosis, and endometrial cancer.^{8–11}

miRNA AND GENE EXPRESSION STABILITY

Whether regulated independently or through an ovarian steroid-dependent manner, expression of endometrial genes is subjected to transcriptional and translational regulation that establishes the framework of their biological functions throughout the menstrual cycle. Since their discovery less than a decade ago, microRNAs (miRNAs) have emerged as key regulators of gene expression stability.^{12,13} These small, non-protein-coding RNAs are transcribed from specific genes with primary transcripts (pri-miRNAs) of several kilobases in length. The pri-miRNAs undergo substantial processing that results in generation of a 70- to 90-nucleotide (nt) stem-loop precursor miRNA (pre-miRNA) in the nucleus. After transportation into the cytoplasm, the pre-miRNAs undergo a second cleavage by Dicer generating a double-stranded miRNA duplex containing 2-nt-long 3' overhangs that unwind and form a single-strand, mature miRNA.^{14–16} The mature miRNAs incorporate into the RNA-induced silencing complex (RISC) and through complementary interaction with target genes regulate their expression mostly, but not always through translational repression.^{13,17} The miRNA-induced translational repression is considered to involve two distinct mechanisms: the inhibition of translation initiation and/or inhibition of a “postinitiation” step in translation, which also elicits co-translational degradation of the nascent peptide.^{18–20} Through this mechanism, miRNAs influence the outcome of various cellular activities under normal and disease conditions.

Many putative miRNAs have been identified and/or predicted in the genome of different species, including in mammals, with potential of targeting the expression of a large number of protein-coding and possibly non-protein-coding genes.^{21,22} In the human, around 580 miRNAs have been identified and more than 1000 predicted. Because the specificity of miRNAs is dictated by 6 to 7 nt that bind to the 3' untranslated region (3' UTR) of their target mRNAs, a single miRNA can potentially target hundreds of genes, or a single gene could be a potential target of many different miRNAs.^{23–25} Although the biological significance of such diversity is unclear, it appears that binding of several miRNAs may be required to achieve adequate regression of a specific target gene. Because the extent of miRNA-induced gene regression depends on the degree of complementary sequence homology with the target gene,^{13,24} the level of regression may vary significantly and, to date, only 70 to 100 specific target genes have been experimentally confirmed as targets of several miRNAs.

POTENTIAL REGULATORY FUNCTION OF miRNAs IN THE ENDOMETRIUM

Upon the establishment of regular ovulatory cycles, the endometrium under the influence of sex steroids undergoes substantial cyclic morphologic and biochemical changes. Estrogen acts as a mitogen for various endometrial cells, and progesterone acting on the estrogen-primed endometrium induces differentiation and secretory changes in the glandular epithelial cells. Although the mid-luteal phase rise in estrogen is not essential for successful embryo implantation,^{26,27} the uterus retains its responsiveness to sex steroid hormones, and endometrial cycles can be induced to become receptive for embryo implantation even after menopause.²⁸ Progesterone also causes stromal decidualization and prepares the endometrium for embryo implantation, and a sudden decline in sex steroids production at the end of luteal phase initiates normal menstrual bleeding.^{6,29,30} Estrogen and progesterone mediate their actions through estrogen receptors (ERs) and progesterone receptors (PRs), respectively. These receptors are ligand-activated transcription factors, and their activation leads to regulation of

expression of their target genes in cell- and promoter-specific manners in ovarian steroid sensitive tissues, including the endometrium.

Accumulated evidence supports the expression and regulatory function of many gene products in the endometrium throughout the menstrual cycle under normal and pathologic conditions.^{5,6,11,28,31–34} The expression of these genes, which include many inflammatory and immune mediators, fibrinolytic and proteolytic enzymes, cell-cycle and apoptotic regulators, extracellular matrix, adhesion molecules, and angiogenic factors and their receptors, are differentially regulated during the menstrual cycle as part of preparation for endometrial degenerative and reparative processes.^{1–7} These intrinsic molecules are expressed and released by activated platelets, inflammatory and immune-related cells infiltrating into the endometrium, as well as by endometrial epithelial, stromal, and vascular endothelial cells. Altered endometrial expression of these molecules seems to be responsible for inappropriate tissue regeneration, resulting in dysfunctional uterine bleeding, failure in embryo implantation, as well as many other endometrial disorders.^{1,5,6,8–11,31,34–39} Gene expression is regulated at multiple levels, including at transcriptional and translational levels and influenced by miRNA regulatory functions. Although the field of miRNA research has rapidly evolved during the past few years, only limited information is currently available for their expression and functions in human reproductive tract tissues. Given the key regulatory function of miRNAs in gene expression, it is pivotal to define their expression and their underlying mechanism of actions and regulation in these tissues. It is also essential to identify the specific genes relevant to endometrium targeted by these miRNAs at various stages of normal menstrual cycle and associated abnormalities.

We have recently reported the expression profile of a few hundred miRNAs in human endometrium and endometrial stromal and epithelial cells.⁴⁰ Of the 287 human miRNAs profiled, we identified the expression of 65 miRNAs in the endometrium, of which 32 were differentially expressed in endometrial stromal and glandular epithelial cells isolated from the same tissues. Such a sharp reduction in expression of a large number of miRNAs in isolated endometrial cells implies the importance of endometrial microenvironment on regulation of miRNA expression. The decline in the number of miRNAs expressed in the endometrial cells compared with their original tissues also reflects possible regulatory function of ovarian steroids or many locally expressed mediators on the expression of these miRNAs by various endometrial cells. However, our study was limited to endometrial tissues of the early to mid luteal phase of the menstrual cycle to draw any specific conclusion, as the profile of miRNA expression most likely differs in endometrium from other phases of the menstrual cycle. To provide support for the influence of ovarian steroids, we used isolated endometrial cells cultured under defined conditions and identified the regulatory action of ovarian steroids on the expression of a selected number of these miRNAs.⁴⁰

Among the miRNAs regulated by 17 β estradiol and progesterone in the endometrial epithelial and stromal cells are miR-20a, miR-21, miR-23, miR-26a, miR-18a, miR-181a, miR-206, and miR-142-5p.⁴⁰ These miRNAs have been predicted to target the expression of a large number of genes, including transforming growth factor, β (TGF- β), TGF- β receptors, ERs, PRs, and CYP-19A1 (aromatase), many of which are known to play critical roles in endometrial activities.⁴⁰ We are further investigating the expression of specific genes targeted by these miRNAs in the endometrial cells with the aim of identifying the mechanism by which these miRNAs regulate gene expression relevant to the endometrium. Accumulated evidence has been generated in support of the expression of miRNAs in various cells and tissues under normal and disease conditions.^{15,40–44} Recent functional analysis of several miRNAs has revealed their key regulatory influence of the expression of target genes involved in various cellular activities under normal physiologic and disease conditions, more specifically developmental processes and tumorigenesis.^{15,40–43} In particular, miRNAs target the

expression of genes involved in cell-cycle progression, differentiation, apoptosis, inflammatory and immune response, and angiogenesis (Fig. 1).^{42,44–48} These cellular processes are integrated in parts of the endometrial regeneration throughout the menstrual cycle that are regulated by sex steroids and a vast number of gene products whose expression may be the target of miRNA regulatory functions.

miRNAs AND ENDOMETRIAL INFLAMMATORY RESPONSE

The onset of menstruation is associated with the expression of a network of highly active substances with inflammatory- and immune-related activities.^{6,35} The endometrial expression of these inflammatory and immune mediators is necessary for progression of tissue degeneration during menses. It is also necessary for the progression of tissue repair mechanisms after menses as deficiency in inflammatory response results in wound impairment.⁴⁹ The mechanisms that control the expression of these mediators and terminate their activities are complex and regulated at different levels, including at the level of gene expression stability. Several miRNAs have been identified to influence the expression of inflammatory and immune response mediators, more specifically let7, miR-17–5p, miR-20a, miR-106a, miR-125b, miR-146, and miR-155 (Table 1; Fig. 1).^{50–55} Of particular importance to these processes are recent studies showing that expression of miR-125b and miR-155 is necessary for proper development of T, B, and dendritic cells.^{52–55} It was revealed that exposure of macrophages to endotoxin, proinflammatory cytokines, such as tumor necrosis factor α (TNF- α), and increase in nuclear factor of kappa light polypeptide gene enhancer in B cells (NF- κ B) activity altered the expression of these miRNAs. The authors suggested the direct involvement of NF- κ B transcriptional activity, a key element in inflammatory response, in regulating the expression of these miRNAs.⁵³ Interestingly, miR-155 targeted the expression of several proteins involved in lipopolysaccharide (LPS) signaling and enhanced TNF- α translation, and miR-125b downregulated TNF- α expression. Because exposure of Emu-miR-155 transgenic mice to endotoxin resulted in elevated production of TNF- α , this study provided further evidence for interactive regulatory functions between inflammatory response and miR-155 and miR-125b expression.

The endometrial expression of these miRNAs⁴⁰ along with expression of several proinflammatory cytokines, including TNF- α and increased NF- κ B activity,^{56–61} raised the possibility that miR-125b and miR-155 may function in similar manners regulating the endometrial inflammatory and immune response. This regulatory function is specifically relevant to endometrial activities during menses and dysfunctional uterine bleeding, characterized by an increased expression of proinflammatory mediators, including TNF- α and increased NF- κ B activity. However, it remains unclear if inflammation causes the expression of specific miRNAs or if unregulated expression of miRNAs results in activation of inflammation-associated genes during an inflammatory response. If the endometrial expression of these miRNAs differs in women experiencing normal menstruation compared with those with dysfunctional uterine bleeding, unregulated expression of specific miRNAs and their target genes may account for manifestation of this disorder. We have identified and are currently analyzing such a relationship between the expression of a selective number of these miRNAs and the target genes with specific reference to dysfunctional endometrial bleeding.

The expression of miR-125b and miR-155 and their response to TNF- α has also been found to be cell lineage dependent.⁵³ Tili et al suggested that differential expression of miR-125b is specific to cells of the immune system limiting the level and duration of the immune response before it becomes detrimental, and in breast cancer MCF7 cells it may regulate cell homeostasis and proliferation.⁵³ In this respect, a selective expression of miRNAs not only in endometrial epithelial and stromal cells but also in inflammatory- and immune-related cells and the vasculature may influence the outcome of gene expression with multiple activities in a cell-

dependent and/or menstrual cycle–dependent manner. Infiltrating macrophages and T cells and an increase in their populations is critical to various endometrial activities, specifically during menses and dysfunctional uterine bleeding, and may be regulated in similar manners in the endometrium.

The inflammatory activation of monocytes has also been reported to cause NF- κ B–dependent induction of miR-146, resulting in inhibition of TNF- α receptor-associated factor 6 (TRAF6) and interleukin-1 receptor associated kinase 1 (IRAK1) expression.⁶² TRAF6 and IRAK1 act downstream from Toll-like receptor (TLR) and cytokine signaling,⁶² whose expression has been identified in human endometrium.^{5,31} Interestingly, miR-146a expression is triggered by cell surface and not by intracellular TLR, which mainly senses viral nucleic acids, and is suggested to function in response to bacterial rather than viral infection.^{52,62,63} This raises the possibility that miRNAs such as miR-146a could function in a similar fashion in the endometrium of women with dysfunctional uterine bleeding experiencing endometritis with altered endometrial expression of inflammatory-related genes.^{64–67} Dysfunctional uterine bleeding and bacterial infection resulting in an inflammatory environment is also associated with an increased expression of proteolytic enzymes such as matrix metalloproteinases (MMPs).^{6,65,66} Increased expression and elevated activity of MMPs is observed at the onset of menstruation and linked to wound-healing impairment.^{66,68,69} However, there is no evidence to suggest the regulatory function of miRNAs on the expression of MMPs and their physiological inhibitors, tissue inhibitor of MMPs (TIMPs), although several miRNAs including miR-20, miR-23a, and miR-222 are predicted to target their genes.⁴⁰ For instance, TIMP-3 mRNA levels increased significantly during the late secretory and menstrual phases when compared with the proliferative and early secretory phases⁷⁰ and was reported to be the target of miR-222.⁷¹

Resolution of the endometrial inflammatory response is necessary for reparative mechanisms to take place. In the endometrium, the conclusion of menses is associated with a reduction in inflammatory- and immune-related cell populations, expression of proinflammatory mediators, and proteolytic enzyme activities.⁶ Whether the endometrial expression of miRNAs during this period reflects an anti-inflammatory regulatory function awaits investigation; however, several miRNAs including overexpression of miR-17–5p, miR-20a, and miR-106a have been reported to inhibit the differentiation and maturation of monocytes.⁷² These miRNAs are expressed in the endometrium, and endometrial cells and several genes, including TGF- β and TGF- β receptors family, proteases and their inhibitors, and cell-cycle–related and apoptosis-related genes are among their predicted targets.⁴⁰ Various components of the TGF- β family are expressed and regulated in human endometrium throughout the menstrual cycle where they serve as key regulators of various cellular activities. TGF- β is a major cytokine with anti-inflammatory activities and differentially regulates proteases, extracellular matrix, and adhesion molecules expression resulting in the progression of tissue repair.⁴⁹ In addition, miR-181a expression is increased in mature T cells⁷³ and found to regulate the expression of antiapoptotic proteins, such as B cell lymphoma 2 (Bcl2) and the cell surface regulator CD69.⁶³ We identified the expression of miR-181a in the endometrium and showed that it is differentially regulated by the ovarian steroids along with miR-17–5p and miR-20a expression in endometrial epithelial and stromal cells.⁴⁰ Because the endometrium expresses many anti-inflammatory and antiapoptotic genes during the menstrual cycle, their differential expression may be regulated by miRNAs for the purpose of promoting transition from an inflammatory phase into a reparative stage.

miRNAs AND ENDOMETRIAL CELLULAR APOPTOSIS

The resolution of endometrial inflammatory reaction also parallels increased apoptotic activities to remove the damaged cells after the conclusion of menstruation.^{9,74} Apoptosis is

a complex process and is regulated by several multifunctional mediators. These mediators control the activation of pathways that help remove the unwanted damaged cells and act to minimize cellular damage and sustain cell growth and differentiation. In normal human endometrium, apoptosis has been reported to appear in the mid secretory phase, increasing in the late secretory phase, and is maximal during the menstrual phase.^{9,74} The concordant endometrial expression of many genes that trigger the activation of apoptotic and cell-cycle signaling has been identified.^{9,31,74} Many miRNAs have been identified and/or predicted to regulate the expression of genes with functions in apoptosis and cell-cycle regulation of several cancer cells.^{46,7–78} However, limited evidence exists to support the regulatory functions of miRNAs in proliferative response of normal, differentiated quiescent cells.

Among the miRNAs functionally associated with events leading to apoptosis are miR-10a, miR-28, miR-196a, and miR-337, as well as miR-96, miR-145, miR-150, miR-155, and miR-188 (Fig. 1), which differentially regulate the activation of caspase-3, respectively.⁷⁹ TNF- α is a key regulator of apoptotic processes and TNF-related apoptosis-inducing ligand (TRAIL, Apo-2L), which activates caspase-dependent apoptotic machinery, associated with altered expression of several miRNAs.⁸⁰ TRAIL-resistant cells displayed different levels of miRNAs profile with increased expression of miR-221 and miR-222 targeting c-Kit and p27 (kip1) expression and maintain TRAIL-resistant phenotype.⁸⁰ TRAIL induced apoptosis via the formation of a death-inducing signaling complex consisting of Fas-associated death domain protein (FADD) and activated procaspase-8 is also triggered by miR-155.^{52,53} Cyclin A/cdk1, E/cdk2, and D1/cdk4 as well as cyclin-dependent kinases and cyclin-dependent kinase inhibitors such as p16 (INK4A), p21 (WAF-1/CIP-1), p27, p53, and p57 and other cell-cycle regulators and caspases are expressed and play a central role in endometrial transition from the inflammatory to reparative stage.^{2,6,9,23,81–83} The expression of several of these regulators has been identified or predicted as targets of several miRNAs, more specifically let-7 and miR-17–192 clusters, miR-20, and miR-21.^{50,76,77,84} For instance, the E2F transcription factors E2F1, E2F2, and E2F3, which play a central role in cell-cycle progression and apoptosis, target the promoters of several caspases and other proapoptotic targets of p53.

The expression of E2F1 has been found to be inhibited by miR-17a and miR-20a, which are part of the miR-17–92 cluster.^{85–89} Overexpression of miR-20a inhibited prostate cancer cell line from apoptosis possibly through regulation of E2F2 and E2F3 expression and the direct binding of E2F1–3 to the promoter of the miR-17–92 cluster.^{52,77,88} The miR-17–92 cluster also promoted cell proliferation by shifting E2F transcriptional balance away from proapoptotic E2F1 and toward the proliferative E2F3 transcriptional network.⁹⁰ Over-expression of miR-21 in breast tumors and malignant cholangiocytes has been associated with increased cell growth, and inhibition of miR-21 with anti-miR enhanced their apoptosis and decreased their proliferation.^{76,84} Recent studies have also implicated the miR-34 family as part of the p53 tumor suppressor network because overexpression of miR-34 resulted in apoptosis or cellular senescence.^{85–87,89} Apoptosis is also controlled by the Bcl2 family of proteins, and miR-15a and miR-16-1 appear to play a central role in regulating their expression.^{1,91,92}

Several studies indicated that ovarian steroids may control endometrial apoptosis through differential regulation of proapoptotic and antiapoptotic genes.⁸² Whether the ovarian steroids differentially trigger the expression of specific miRNAs that target proapoptotic and antiapoptotic genes in the endometrium throughout the menstrual cycle is open to investigation. However, the expression of miRNAs such as miR-17–192 cluster, miR-20, miR-21, and miR-181 and their differential regulation by ovarian steroids in the endometrial cells support their potential influence on endometrial genes associated with cell-cycle progression and apoptotic processes.⁴⁰

miRNAs AND ENDOMETRIAL ANGIOGENESIS

In the healthy adult, angiogenesis occurs only in the corpus luteum, and during placental development, cyclic endometrium, and wound healing.^{4,93} Angiogenesis is a self-limiting and strictly regulated event. It occurs in a sequential manner involving degradation of the vascular basement membrane and interstitial matrix by endothelial cells, migration and proliferation of endothelial cells, and finally tubulogenesis and formation of capillary loops. Many factors with angiogenic and antiangiogenic properties are known to regulate these events. An optimal and timely production of many proteolytic enzymes in response to angiogenic factors, which include various growth factors, cytokines, and chemokines and a balance between the expression of their inhibitors, is critical to the outcome of angiogenesis.^{4,93} Human endometrium expresses many angiogenic mediators and their specific receptors.^{4,93} Although the potential role of ovarian steroids in angiogenesis and neovascularization in the early stage of endometrial regeneration may be limited, they become active participants after the conclusion of menstruation. The vascular endothelial cells, including those in the endometrium, contain receptors for ovarian steroids implicating their regulatory actions on vascular activities. Because of the importance of angiogenic modifiers in endometrial reparative processes, the stability of their expression is critical specifically during dysfunctional uterine bleeding in which the endometrium undergoes repetitive breakdown and repair.

The regulatory function of miRNAs in angiogenesis has been illustrated in several in vitro and in vivo models. During embryogenesis in Dicerex1/2 homozygous mouse embryos, defective blood vessel formation/maintenance and severely compromised yolk sacs has been reported.⁹⁴ Defective angiogenesis in this model was associated with altered expression of vascular endothelial growth factor (VEGF), fms-related tyrosine kinase 1 (FLT1), kinase insert domain receptor (KDR), and tyrosine kinase with immunoglobulin and epidermal growth factor homology domains 1 (TIE1) in mutant embryos. These defects were possibly due to altered regulatory function of Dicer, which is required for miRNA processing that regulates the expression of these genes.⁹⁴ Dicer expression silencing in human endothelial cells also resulted in altered expression of several key regulators of angiogenesis, such as TEK/TIE-2, KDR/VEGFR2, TIE1, endothelial nitric oxide synthase, and IL-8.⁹⁵ More specifically, miR-221 and miR-222 were found to regulate the expression of endothelial nitric oxide synthase after Dicer silencing.⁹⁵ In human umbilical vein endothelial cells (HUVECs), the expression of 15 miRNAs was associated with the expression of angiogenic factors and their receptors, with miR-221 and miR-222 affecting the expression of c-Kit and its ligand stem cell factor (SCF).⁹⁶ Silencing of Dicer and Drosha was associated with reduced capillary sprouting and tube-forming activity through a mechanism involving decreased migration of endothelial cells.⁴⁷⁹⁷ Inhibition of let-7f and miR-27b expression reduced sprout formation, suggesting their possible regulatory function in angiogenesis by targeting antiangiogenic genes.^{47,97} Furthermore, miR-130a has been found to regulate the angiogenic phenotype of vascular endothelial cells largely through modulating the expression of growth arrest homeobox transcription factor (GAX) and homeobox A5 (HOXA5),⁹⁸ and miR-378 enhanced cell survival, reduced caspase-3 activity, and promoted angiogenesis.⁹⁹ Although the expression of several of these miRNAs has been identified in human endometrium⁴⁰ and in preimplantation mouse uterus,¹⁰⁰ their regulatory functions on endometrial angiogenic and antiangiogenic genes expression remains to be investigated (Fig. 1). However, the existing evidence obtained in other systems strongly supports the role of miRNAs in regulating the expression of genes involved in various aspects of angiogenesis.

miRNA AND ENDOMETRIAL EMBRYO IMPLANTATION

Human embryo implantation is an extremely complex process, and the underlying mechanisms involved in this process and the establishment of maternal tolerance to the embryo remains

incompletely understood.^{1,5,7,28,38,101–104} It is clear that ovarian steroids and their receptors are essential for adequate endometrial receptivity. Evidence also indicates the requirement for several endometrium-derived autocrine/paracrine mediators for the establishment of an environment to allow for embryo implantation. Although the precise nature of the endometrial mediator(s) that distinguish this period is unknown, conventional and large-scale gene expression profiling has identified many candidate genes considered to play pivotal roles in this process.^{1,5,7,38,101,102,104}

Specific numbers of adhesion molecules and their receptors (integrins) important for the establishment of a direct dialogue between the embryo and the endometrium and several cytokines, growth factors, and chemokines are among these candidate genes. Although mice with targeted deletion of many of these genes do not exhibit any profound alterations in embryo implantation, lack of expression of some of these genes may influence other stages of embryo development. Functional redundancy among the biological actions of some of these factors may account for the mechanism of such activities. However, interruption in leukemia inhibitory factor (LIF) gene results in the failure of blastocyst implantation. In addition to adequate preparation of receptive endometrium, the establishment and maintenance of a viable embryo prior to reaching endometrium is essential for successful implantation. This is shown in part by the significant percentage of the embryos that reach the endometrium and do not implant.

To gain insight into endometrial gene regulation during the embryo implantation period, many in vitro and in vivo animal models have been used. The results of these studies indicate to a complex microenvironment involving many specific genes highly regulated at transcriptional and translational levels.^{1,5,7,38,101,102,104} Because of miRNA regulatory function in gene expression stability, a recent study by Chakrabarty et al illustrated the expression of a specific number of miRNAs during peri-implantation and preimplantation periods in mice, suggesting to serve as potential candidates relevant to embryo implantation.¹⁰⁰ Among the 32 miRNAs identified as relevant to embryo implantation, hsa-miR-101, hsa-miR-144, and hsa-miR-199a* are predicted to target cyclooxygenase-2 (COX-2) gene, which is considered to participate in events leading to peri-implantation and preimplantation embryo in mice.¹⁰⁰ The expression of these miRNAs correlated with the expression of COX-2 in the uterus during delayed implantation, further suggesting the potential regulatory function of these miRNAs on gene expression relevant to embryo implantation.

The expression of COX-2 is regulated by a large number of growth factors, cytokines, and chemokines such as TNF- α and ovarian steroids. The expression of many of these mediators is targeted by miRNAs such as miR-125b and miR-155,⁵³ of which miR-155 expression was upregulated in day 4 (receptive) uteri.¹⁰⁰ Whether a feedback regulatory mechanism exists in coregulating the expression of miRNA-155 and target genes in the endometrium is yet to be determined; however, an increased endometrial expression of COX-2 and miR155 accompanied by a decrease in TNF- α expression during the receptive period support such interactions. In addition, TNF- α expression is targeted by miR-125b.¹⁰⁵ Other miRNAs whose profiles were identified as differentially expressed during peri-implantation and in day 4, day 5, and day 6 pregnant uteri in mice included hsa-let-7b, hsa-miR-19a, hsa-miR-127, hsa-miR-20a, hsa-miR-103, hsa-miR-199b, mmu-miR-201, hsa-miR-144, hsa-miR-512-3p, hsa-miR-195, hsa-miR-101, hsa-miR-199a*, hsa-miR-186, hsa-miR-17-3p, mmu-miR-291-5p, hsa-miR-181b, hsa-miR-96, hsa-miR-516-3p, hsa-miR-137, rno-miR-20*, hsa-miR-9*, mmu-miR-291-3p, and hsa-miR-302b.¹⁰⁰ Although many of these miRNAs are of human origin, their functional association during embryo implantation and early pregnancy in humans could only be predicted; however, because of their conserved homology among various species, they may function in similar manners.

We have identified the expression of several of these miRNAs in human endometrium during the early to mid luteal phase, including the expression of let-7b, miR-19a, miR-20a, miR-199b, miR-101, miR-17-3p, miR-181b, as well as miR-125, miR-155, miR-26a, and miR-26b.⁴⁰ The miR-26 family has been predicted to target the expression of LIF among other genes.^{106,107} Furthermore, the expression of miR-20a, miR-26a, miR-17-5p, miR-181, and miR-206 was the target of ovarian steroid regulatory actions in the endometrial epithelial and stromal cells.⁴⁰ These observations suggest that differential regulation of subsets of miRNAs may be required to stabilize the endometrial expression of specific genes critical for embryo implantation. However, detailed studies are needed to identify the specific endometrial genes targeted by these miRNAs and their associations with embryo implantation.

miRNAs AND ENDOMETRIAL DISORDERS

Considerable evidence exists implicating the altered expression of subsets of endometrial genes as hallmarks of establishment and progression of endometrial cancer, endometriosis, and dysfunctional uterine bleeding (Fig. 2). They include ovarian steroid receptors, oncogenic and tumor suppressor genes, proinflammatory- and immune-related genes, angiogenic factors, progression cell-cycle related genes, and adhesion molecules and their receptors.^{6,8,10,11,28–30,34,37,108} We provided evidence that the expression of subsets of miRNAs is altered in ectopic endometrium compared with paired eutopic or endometrium of women without endometriosis. Currently, there is no evidence indicating whether the expression of miRNAs in endometrial cancer differ from normal endometrium. However, considering the existence of different miRNA profiles in endometrium of women with endometriosis, it raises the possibility of differential expression profile in endometrial cancer compared with normal endometrium. Comparative analysis of the miRNAs expression among different endometrial abnormalities may allow for identification of specific miRNAs and their targeted genes associated with each disorder.

Results of expression profiling generated from several cancer cells and tissues indicate considerable alterations in the expression of a large number of miRNAs compared with their normal cells and tissue counterparts.^{44,109–116} In these studies, several oncogenes and tumor suppressor genes were identified and/or predicted as potential target of several miRNAs.¹¹¹ In most cases, association of miRNAs expression with tumorigenesis has been identified to be due to their ability to differentially regulate the expression tumor suppressor genes or oncogenes.^{111,117} The expression of many of these oncogenes and tumor suppressor genes have also been associated with establishment and progression of endometrial cancer,¹⁰ implying that miRNAs may serve in regulating their expression. Additionally, miRNA genes are frequently located at fragile sites and regions of loss of heterozygosity or common breakpoint regions.^{109,111} Common fragile sites are large, genomically unstable regions, which are hot spots for deletions and other alterations, especially in cancer cells, including endometrial cancer cells.¹¹⁸ In addition to oncogenes and tumor suppressor genes, miRNAs also target the expression of gene function as proinflammatory- and immune-related response, angiogenesis, cell-cycle progression, and adhesion molecule response. These processes are known to play a central role in various cellular activities critical to tumorigenesis, including endometrial cancer; they are also involved in pathogenesis of endometriosis and dysfunctional uterine bleeding.

A considerable number of miRNAs predicted to target the expression of genes functioning as proinflammatory- and immune-related response, angiogenesis, cell-cycle progression, and adhesion molecules were expressed in eutopic and ectopic endometrium of women with endometriosis. The level of expression of these miRNAs in most instances was down regulated in the eutopic and ectopic endometrium of women with endometriosis as without endometriosis.⁴⁰ Although the expression of the genes targeted by specific miRNAs must be

validated, the results suggest that ectopic endometrial tissues are programmed differently with respect to their gene expression regulation. Women with endometrial cancer, endometriosis, and more specifically contraceptive users often experience dysfunctional endometrial bleeding, which similar to normal menstruation is characterized by an increased inflammatory reaction and increased production of angiogenic mediators as well as excess production of proteolytic enzymes.^{6,29,67,119} A detailed study involving miRNA expression profile at different stages of endometrial cancer and endometriosis as well as in women with dysfunctional bleeding would allow for better understating of the genes involved and establish the lineage and state of progression of these disorders. In addition, it would be of interest to determine whether the expression of miRNAs differs in normal menstruation compared with that in women experiencing dysfunctional uterine bleeding associated with contraceptive use.

To provide some prospective of potential regulatory function of several miRNAs identified in normal endometrium and ectopic endometrium, we selected those whose expressions were confirmed in these tissues and isolated endometrial cells for further discussion.⁴⁰ Among these miRNAs, several are predicted to regulate the expression of angiogenic and proinflammatory- and immune-related genes, including let-7, miR-15, miR16, and miR-125. The expression of let7 family has been reported to be downregulated in several tumors and considered to serve as tumor suppressor.^{120–122} Let-7 family clusters with miR-99/miR-100 and miR-125 family and is predicted to regulate inflammatory- and immune-related genes, cell-cycle progression, and apoptosis.⁵³ Additionally, miR-15a and miR-16–1 cluster is located at 13q14.3, a region that is deleted and/or downregulated in several disorders, with germ-line mutation identified in miR-16–1 precursor associated with low level of miR-16–1 expression in chronic lymphocytic leukemia.¹²³ The expression of miR-15a and miR-16–1 was also inversely correlated with Bcl-2 expression,⁹² which is expressed in endometriosis and endometrial cancer tissues and may be the target of these miRNAs. Several studies have implicated ovarian steroids as regulators of endometrial cellular apoptosis through differential regulation of Bcl-2 and Bax expression.⁶ Although ovarian steroids through differential regulation of miR-15 and miR-16–1 and other miRNAs could target the expression of apoptotic and antiapoptotic genes, it is also possible that differential regulation of inflammatory-related genes and their associated miRNAs account for promotion of apoptotic and antiapoptotic activities.

Ovarian steroids regulate the expression and activation of c-myc in human endometrium and endometrial cancer,¹²⁴ and c-myc activation has been shown to increase the expression of the miR-17–92 cluster.^{125,126} These observations have led to the suggestion that the miR-17–92 cluster in cooperation with increased c-myc expression results in accelerated tumor development.^{125,126} These tumors established and developed earlier, were more aggressive, and had increased mitotic rate and less apoptosis compared with tumors generated with c-myc alone. The transcription factor E2F1 is also predicted as a target of the miR-17–92 cluster and transcriptionally regulated by c-myc. Regression of E2F1 expression has been shown to influence cell-cycle progression, and despite oncogenic activity, the miR-17-92 cluster can serve as tumor suppressor by decreasing E2F1 expression.⁹⁰ Unregulated expression of miR-221 and miR-222 promote cell growth by inhibiting p27,¹²⁷ and miR-27a suppresses the cdc2/cyclin B inhibitor Myt-1 in MDA-MB-231 cells promoting cell proliferation.⁷⁸

In the study of Kim et al,¹²⁸ they provided evidence that overexpression of miR-206 promotes cellular differentiation in C2C12 myoblasts. Detailed information implicating the regulatory function of miR-206 in gene expression is reviewed by Adams et al.¹²⁹ Interestingly, miR-206 also targets the expression of ER α in ER-positive breast cancer cells.¹²⁹ Altered expression of ER α as well as ER β has been associated with several endometrial disorders, including endometrial cancer, endometriosis, and dysfunctional uterine bleeding.^{130–132} We demonstrated that ovarian steroids regulate the expression of miR-206 in endometrial epithelial and stromal cells and their actions were in part altered by estrogen and progesterone antagonists

ICI-182780 and RU486, respectively. Selective estrogen and progesterone receptor modulators and estrogen and progesterone antagonists have been demonstrated to effectively regress the growth of endometrial implants in animal models of endometriosis, in clinical trials in humans, and of endometrial cell growth in vitro. These observations suggest that estrogen and progesterone antagonists may target the endometrial expression of miRNAs resulting in a reprogramming of their target genes expression. Detailed studies are needed to determine the molecular mechanisms by which these agents influence the expression of miRNAs and their target genes.

CONCLUSIONS

The unprecedented advancements in molecular biological approaches during the past decade have led to the identification of the expression of many genes in the endometrium under normal and diseased conditions. The product of some of these genes acting in an autocrine/paracrine and interactive manners are known to regulate many events such as inflammatory and immune responses, cell-cycle progression, differentiation, apoptosis, and tissue remodeling. Precise regulation of the expression of these genes is fundamental in directing these processes for normal endometrial functions. Unregulated expression of some of these genes appears to account for various endometrial abnormalities including infertility, endometriosis, and endometrial cancer.

Clearly, ovarian steroids through their ability to regulate the expression of many of these genes at transcriptional and translational levels play central roles in endometrial biological and physiologic integrity. Micro-RNAs have emerged as important regulators of gene expression. These small, non-protein-coding RNAs through complementary interactions with their predicted target genes regulate their expression mostly through transcriptional and translational regression.¹³³ Expression profiling and cloning strategies have identified a large number of miRNAs in various cells and tissues under normal and disease conditions. Functional analysis also revealed that miRNAs can potentially target the expression of a third or possibly more of the genes in humans. Current evidence for the expression, regulation, and function of miRNAs in human reproductive tract tissues is limited to a few studies. The expression profile of a few hundred miRNAs in the endometrium and endometrial cells and their aberrant expression in ectopic endometrium in women with endometriosis support their key biological relevance. Additionally, ovarian steroids appear to regulate the expression of miRNAs in endometrial cells; however, the specific pattern of their expression throughout the menstrual cycle remains to be elucidated.

It is also necessary to verify and correlate the expression of genes targeted by differentially expressed miRNAs in the endometrium and to determine whether their profiles and targeted genes expression differ in endometrial disorders such as endometrial cancer and dysfunctional uterine bleeding compared with that of normal endometrium. This is particularly important because expression profiling of miRNAs appears to be far more superior to mRNA expression profiling to differentiate normal from diseased tissues. If such a pattern of miRNA expression could differentiate normal endometrium from diseased conditions, the result may have a significant impact on prognostics and diagnostics approach as a useful tool for assessing response to various treatment strategies. In addition, miRNAs that are aberrantly expressed in any of the endometrial disorders could be effectively targeted by their complementary anti-miR and/or pri-miR oligonucleotides for loss of function and gain of function. As such, evidence generated in other cell and in vivo systems supports their experimental use for therapeutic application and management of disorders such as lowering of plasma cholesterol (miR-122), cancer therapy (miR-21), cardiac hypertrophy (miR-21), and cardiac arrhythmia (miR-1). Collectively, extensive basic and transitional research is needed to improve our basic

knowledge of endometrial miRNAs expression, regulation, and functions under normal and diseased conditions to allow for identification of potent therapeutic applications for miRNAs.

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ABBREVIATIONS

3' UTR	3' untranslated region
Bcl2	cell lymphoma 2
COX-2	cyclooxygenase-2
ERs	estrogen receptors
FADD	Fas-associated death domain protein
FLT1	fms-related tyrosine kinase 1
GAX	growth arrest homeobox transcription factor
HOXA5	homeobox A5
HUVEC	human umbilical vein endothelial cell
IRAK1	interleukin-1 receptor associated kinase 1
KDR	kinase insert domain receptor
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
miRNA	microRNA
MMP	matrix metalloproteinase
NF-κB	nuclear factor of kappa light polypeptide gene enhancer in B cells

PR	progesterone receptor
RISC	RNA-induced silencing complex
SCF	stem cell factor
TGF-β	transforming growth factor β
TIE1	tyrosine kinase with immunoglobulin and epidermal growth factor homology domains 1
TIMPs	tissue inhibitor of matrix metalloproteinases
TLR	Toll-like receptor
TNF-α	tumor necrosis factor α
TRAF6	receptor-associated factor 6
TRAIL	TNF-related apoptosis-inducing ligand
VEGF	vascular endothelial growth factor

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Figure 1. Schematic representation of miRNAs potential regulatory function on endometrial gene expression whose products are involved in regulating various cellular activities such as inflammatory and immune response, apoptosis, angiogenesis, cell growth and differentiation, and so forth. These cellular activities are critical components of the endometrial degradative and regenerative processes during the menstrual cycle and are consistent throughout the reproductive years.

Figure 2.

The aberrant endometrial expression of these miRNAs due to oncogenic/tumor suppressor gene products resulting in altered expression of their target genes that regulate inflammatory and immune response, apoptosis, angiogenesis, cell growth and differentiation, or cellular transformation may lead to dysfunctional uterine bleeding, embryo implantation defect, endometriosis, and endometrial cancers. Whether altered expression of miRNAs results in establishment of these disorders or their altered expression is the consequence of disorders, such as endometriosis, thus modulating the stability of their specific target genes remains to be investigated.

Table 1

A List* of Some miRNAs Whose Expression Has Been Identified in Human Endometrial and Endometriosis Implants²³ as well as in Several Other Cells and Tissues

miRNA	Target Genes	Cell Type	Function (ref.)
let-7a	NF2, STAT-3, RAS	Human cholangiocyte	Inflammation (⁵⁰)
miR-155	NF-κB, FADD, IKKε	Mouse Raw 264.7 macrophage	Inflammation (⁵³)
miR-125b	NF-κB, TNF-α	Mouse Raw 264.7 macrophage	Inflammation (⁵³)
miR-146a/b	IRAK1, TRAF6	THP-1 cell	Inflammation (⁶²)
miR-15a/16-1	Bcl2	Leukemic cell line	Apoptosis (⁹²)
miR-17-5p/17-92/20a/34a	E2F1	P493-6 cell, Pc3 cell, Neuroblastoma cell, HEK-293	Apoptosis (^{88, 89, 90})
miR-181	CD69, Bcl2, TCR	Thymocyte	Apoptosis (⁷³)
miR-21	Bcl2, PTEN	MCF-7, hepatocellular cancer cell	Apoptosis (^{76, 84})
miR-34a	CDK4, CDK6, Cyclin E2, E2F3	Colon cancer cell line SW480	Apoptosis (⁸⁶)
miR-221/222	c-Kit, eNOS	HUVEC	Angiogenesis (^{95, 96})
miR-130a	GAX, HOXA5	Vascular endothelial cell	Angiogenesis (⁹⁸)
miR-378	Sufu, Fus-1	U87 cell	Angiogenesis (⁹⁹)
miR-7f/27b	TSP-1	HUVEC	Angiogenesis (⁹⁷)
miR-101a/199a *	Cox-2	HEK-293	Inflammation (¹⁰⁰)
miR-206	CX43, GDF8, ER	Skeletal muscle, breast cancer	Myogenesis
miR-133	Caspase, Cdc42	Skeletal muscle	Myogenesis
miR-133b	Pitx3	Neuron	Neurogenesis
miR-143	ERK5	Adipocyte	Differentiation

* The list is of a number of miRNAs with their target genes and functions.

For more detail, please refer to the citations indicated by the numbers in the right-hand column.

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