Non-coding RNAs and embryo implantation

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TABLE OF CONTENTS

1. ABSTRACT

In mammals, thousands of non-protein-coding RNAs (ncRNAs), including microRNAs, endogenous small interfering RNAs, PIWI-interacting RNAs and mRNA-like long ncRNAs, have been identified. These RNAs modulate gene expression at transcriptional, post-transcriptional and epigenetic levels in many developmental and metabolic processes. Increasing evidence shows that ncRNAs are also linked to embryo implantation through regulating the expression of certain key genes and pathways. In this paper, we summarized the recent literatures on the ncRNAs involved in embryo implantation.

2. INTRODUCTION

Embryo implantation is an intricate interaction between the implanting embryo and receptive uterus. This process is primed by ovarian steroid hormones for paracrine, autocrine, and juxtacrine molecular events within the uterus to produce a favorable environment for embryo implantation (1, 2). Over past decades, efforts have extensively been made to study the protein-coding genes for deciphering the biological process of embryo implantation. There is growing awareness of the importance of recently discovered non-protein-coding RNAs (ncRNAs) in the regulation of gene expression

during developmental and metabolic processes (3, 4). However, the regulatory role of ncRNAs during embryo implantation is largely unexplored.

In recent years, with advances in large-scale DNA sequencing projects, the number of ncRNA sequences in eukaryotic genomes is rapidly expanding, although the number of protein-coding genes remains relatively static (5). NcRNAs are a heterogeneous group of RNA molecules, varying in size and mechanism of action. A basic classification criterion is the size. Small ncRNAs are <200 nt long and are generated from long precursor transcripts while long ncRNAs (lncRNA) are typically >200 nt long and function without major prior processing.

Small ncRNAs comprise microRNAs (miRNAs), endogenous small interfering RNAs (siRNAs) and PIWI-interacting RNAs (piRNAs). Endogenous siRNAs and piRNAs are highly and restrictively enriched in germline cells (6-9). From two-cell stage, the zygotic genome begins to produce miRNAs. A transition of the major small RNA class from siRNA/piRNA to miRNA was observed during pre-implantation embryo development (10). MiRNAs are the predominant class of small ncRNAs in developing and adult tissues. Through interacting with the 3' untranslated regions (UTRs) of protein-coding genes, miRNAs can cause mRNA degradation or translational repression (11). Several miRNAs have been identified to be differentially expressed in the uterus during embryo implantation in the human (12, 13) and mouse (14, 15) using microarrays. Little is known about their functions in embryo implantation.

The long ncRNAs are generated from intergenic region of genome as well as opposite strand and intron of protein coding genes. They range from approximately 200 nt to over 100k nt in size. Unlike miRNAs, a unified mechanism of long ncRNA action is missing. Instead, ncRNAs have been found to control gene expression at transcriptional, post-transcriptional and epigenetic levels (16). Recent studies show that some long ncRNAs also have activities for miRNA sponge (17) or miRNA masking (18), which add complexity to the functionality of long ncRNAs. Noticeably, most long ncRNAs have functions in *cis*, which means they merely regulate the expression of nearby protein-coding genes. The HOX transcript antisense RNA (HOTAIR), which originates from the HOXC locus and silences transcription across 40 kb of the HOXD locus (19), is one of the few examples of long ncRNAs that act in *trans*. During pre-implantation embryo development, the primary roles of long ncRNAs appear to be as antisense regulators of genomic imprinting (20). Some long ncRNAs were expressed in adult uterus during embryo implantation (21, 22). The function and mechanism of action of these long ncRNAs remains to be elucidated.

As discussed above, miRNAs and long ncRNAs are two major classes of ncRNAs expressed in adult uterus. In this paper, we will review the current knowledge about uterine expression, regulation, function, and mechanism of action of miRNAs and long ncRNAs during embryo implantation.

3. ROLE OF NCRNAS IN THE UTERUS DURING EMBRYO IMPLANTATION

3.1. miRNAs

Mature miRNAs are synthesized through a stepwise process which concludes with the cleavage of stem-loop precursor miRNAs by the RNase III enzyme, Dicer1. Therefore, depletion of Dicer1 impairs miRNA formation. In mice, general knockout of Dicer1 leads to lethality at embryonic day 11.5 (23). In order to study the role of miRNAs in female reproduction, global Dicer1 hypomorphic mice (24) and Amhr2-Cre driven conditional Dicer1 knockout mice (25-27) have been generated. These mice are infertile because of insufficient corpus luteum (24) or disorganized oviduct (25-27). Besides serious oviduct deficiency, Amhr2-Cre driven conditional Dicer1 knockout mice also display some uterine abnormalities. Their uteri are much shorter, thinner, and lighter than wild-type uteri. Additionally, a decreased smooth muscle layer and decreased presence of uterine glands were observed (27). However, it is unknown whether the uteri of these mice are capable of embryo implantation.

Uterine miRNAs are expressed in a steroid hormone-dependent manner (28). Several groups have shown that miRNA expression profiles change dynamically in the uterus during embryo implantation (12-15). Functional studies demonstrated that a portion of these differentially expressed miRNAs are involved in the process of epithelium/stroma differentiation, inflammation, and embryo invasion.

3.1.1. miR-21

Using microarrays, miRNA expression profiles of mouse uterus at implantation sites versus inter-implantation sites on day 5 of pregnancy were compared. A total of 15 differentially expressed miRNAs were indentified, of which miR-143, miR-21, miR-20a, miR-26a, let-7a, let-7b, let-7c, and let-7d were confirmed by both Northern blot and *in situ* hybridization (14).

In situ hybridization analysis showed that miR-21 expression is not detectable in the mouse uterus from days 1 to 4 of pregnancy. On day 5 of pregnancy, miR-21 is strongly expressed in the subluminal stroma underlying the implanting blastocyst at implantation sites but remained absent at inter-implantation sites. From days 6 to 8 of pregnancy, miR-21 expression is also highly expressed in the decidua (14).

The expression level of miR-21 was significantly reduced by estradiol treatment and slightly down-regulated by progesterone (14). Previous studies have shown that miR-21 expression is induced by the signal transducer and activator of transcription-3 (STAT3) pathway through STAT3 binding sites located within promoter region of miR-21 in myeloma and breast cancer cell lines (29, 30). In the mouse uterus, phosphorylated STAT3 protein is strongly detected in the luminal epithelium and stroma surrounding the implanting blastocyst at implantation sites on day 5 of pregnancy (31). Nuclear localization of Stat3 in day 4 luminal epithelium is not detected in any LIF-

deficient mice, indicating that LIF is the principal mediator of STAT3 activation *in vivo* (32). During embryo implantation, STAT3 is activated by leukemia inhibitory factor (LIF). Taken together, it seems that miR-21 at implantation sites is not modulated by estrogen directly but through the activation of LIF/STAT3 pathway.

Reck is identified as a downstream target of miR-21 using bioinformatics tools. The expression pattern of Reck is opposite to that of miR-21 during embryo implantation. Luciferase assay confirmed that Reck is a direct target of miR-21. It is reported that Reck inhibits MMP-2 and MMP-9 secretion or activity, or both (33). However, Reck only suppresses MMP-9 activity in cultured uterine stromal cells. Thus, miR-21 may participate in embryo implantation through inhibiting Reck to regulate the activity of MMP-9 (14).

3.1.2. miR-101a and miR-199a*

MiRNA microarray was also performed on day 1 (pre-receptive) versus day 4 (receptive) uteri of pregnant mice (15). Statistical analysis shows that 32 miRNAs are significantly induced on day 4 and only 5 miRNAs are expressed at higher levels on day 1.

Among up-regulated miRNAs in the receptive stage, miR-101a and miR-199a* are regulators of cyclooxygenase-2 (Cox-2). Using *in situ* hybridization, the spatiotemporal expression of these miRNAs in early pregnancy and delayed implantation is determined. Both miR-101a and miR-199a* are detected in the luminal epithelium on day 1 of pregnancy and become dispersed throughout the uterus on day 4. On day 5, they are localized around the blastocysts at the antimesometrial pole. From days 6 to 8, the expression of these miRNAs disappears from the antimesometrial pole, but is primarily detected at the mesometrial pole of the implantation site. In the delayed implantation model, both miRNAs are not expressed in the uterus during progesterone primed delayed implantation, but is readily induced in stromal cells surrounding the activated blastocyst after termination of the delay by estrogen. This expression pattern is very similar to that of Cox-2 mRNA (34). More importantly, Cox-2 protein is inversely correlated with miR-101a expression at each time point during artificial decidualization process after oil infusion. As Cox-2 is known to be necessary for embryo implantation and decidualization (35, 36), miR-101a and miR-199a* are likely important regulators of Cox-2 during embryo implantation and decidualization (15).

3.1.3. miR-222

MiR-222 is identified in a microarray study of miRNA expression profiles between the non-induced and induced human endometrial stromal cells (ESCs). It is strongly expressed in ESCs but down-regulated during cAMP and progesterone induced decidualization (12). Suppression of mir-222 by antisense oligonucleotides inhibits the growth of ESCs. Western blot confirms a significant increase of CDKN1C/p57kip2 protein level in the miR-222 antisense oligonucleotide-treated ESCs. A miR-222 binding site located in the 3'-UTR region of the

CDKN1C/p57kip2 mRNA is validated by luciferase assay. Collectively, miR-222 is likely to be a modulator of decidualization by helping ESCs to exit the cell cycle and enter differentiation (12).

3.1.4. miR-210

In another microarray study, miRNA expression profiles of uterine epithelial cells purified from endometrium in the late proliferative and mid-secretory phase were compared (13). Among the 12 miRNAs up-regulated in the mid-secretory phase, many of them are predicted to target cell cycle related genes. As a representative example, miR-210 is increased in the mid-secretory phase, while E2F3, a known target of miR-210 (37, 38), is decreased. Thus, miRNAs such as miR-210 may participate in down-regulating the expression of cell cycle genes in the mid-secretory phase of endometrial epithelium, thereby suppressing cell proliferation (13).

3.2. Long ncRNAs

Several long ncRNAs are actively transcribed in the uterus during embryo implantation from the opposite strand of protein-coding genes, such as HOXA11 (21), Scx (22), bFGF (39), and EMX2 (40). These naturally occurring antisense RNAs are supposed to mediate transcriptional activation or transcriptional silencing of host genes. It is noteworthy that the steroid receptor RNA activator (SRA) gene, which is characterized as an intergenic ncRNA and steroid receptor co-activator, is also expressed in the uterus (41). However, gain-of-function and/or loss-of-function studies will be needed to definitely find out the regulatory effect of long ncRNAs in embryo implantation.

3.2.1. HOXA11-AS

The homeobox (Hox) genes are a set of transcription factors that determine the identity of cells and tissues during embryonic development. In mammals, Hox genes are organized into four clusters, termed A, B, C and D. Hox clusters A and D are crucial in the developing reproductive tract (42-44). Although Hox genes are typically considered to be expressed only during embryonic development, the persistent expression of HOX genes has also been noted in adult tissues (45). Specifically, HOXA10 and HOXA11 are expressed in adult human and mouse uteri.

Hoxa11 is required for uterine stromal cell and glandular cell differentiation during embryo implantation. Ablation of Hoxa11 gene in mice results in female infertility (46). Endogenous HOXA11 antisense RNA (HOXA11-AS) is present in mouse and human endometrium (21). In human uterine endometrium, the temporal expression of HOXA11-AS is complementary to that of HOXA11. HOXA11-AS mRNA level varies during the menstrual cycle, with peak expression in the mid-proliferative phase (21). HOXA11 mRNA and protein are known to rise in the mid-secretory phase, corresponding to the time of endometrial receptivity to blastocyst implantation (47). In cultured stromal cells, progesterone is able to repress HOXA11-AS mRNA, which results in up-regulation of HOXA11 mRNA. However, synthetic

Hoxa11 antisense oligonucleotides transfected into the uterus fail to block Hoxa11 protein expression or Hoxa11 function in mice. Therefore, HOXA11-AS probably represses HOXA11 expression by competing for the transcription of the HOXA11 gene, rather than by directly binding HOXA11 sense mRNA (21).

3.2.2. Scx-AS

Scleraxis (Scx) is a basic helix-loop-helix type transcription factor that regulates the growth and differentiation of numerous cell types, such as osteoblastic cells (48), chondrogenic cells (49), tendon (50) and sertoli cells (51). Interestingly, Scx is located in the intron 3 region of the Bop1 gene, but on the opposite strand. Bop1 is a ribosome biogenesis protein and is involved in cell proliferation (52, 53). Thus, the Scx-Bop1 genomic locus has opposite function on both strands.

Both Scx and Bop1 are expressed in the uterine endometrial epithelial cells (22). In ovariectomized adult mice treated with estrogen, the uterine Scx mRNA is decreased at 6 h after estrogen treatment and gradually recovered by 24 h. On the contrary, Bop1 mRNA is increased by 6 folds and gradually reduced until 24 h. These two genes are reciprocally regulated by estrogen. *In situ* hybridization indicates that the antisense RNA of Scx (Scx-AS) is also expressed in the uterine endometrial epithelial cells. Scx-AS is increased after estrogen treatment, coinciding with a decrease in Scx mRNA. *In vitro* cell assay confirms that Scx-AS mRNA reduces the Scx gene expression in a *trans*-acting manner (22). The discovery of Scx-AS suggests that long ncRNAs may play an important role in steroid-mediated gene expression during embryo implantation.

3.2.3. EMX2-OS

Emx2 is a homeobox gene located outside of the Hox cluster. It is a transcriptional target of Hoxa10 regulation in the reproductive tract (54). EMX2 is expressed in the endometrium throughout the estrous cycle and is an important regulator of endometrial proliferation and embryo implantation (55).

Antisense transcripts at the EMX2 locus (EMX2-OS) are highly expressed in human uterus, kidney, and brain (40). In human uterine endometrium, EMX2 and EMX2-OS are identically expressed primarily in the epithelium, with a lower level of expression in the stroma. In mouse uterus, homologous EMX2 and EMX2-OS are co-expressed at high levels in the epithelium and at lower levels in the surrounding stroma during the estrous cycle (40). Recent study shows that EMX2-OS can either up-regulate or down-regulate EMX2 expression in a cell type dependent way (56). Since the expression of EMX2 and EMX2-OS are positively correlated in uterus, EMX2-OS is likely to be an activator of EMX2 during embryo implantation.

3.2.4. FGF2-AS

Fibroblast growth factor-2 (FGF2), also known as basic fibroblast growth factor (bFGF), is a heparin-binding cationic protein with mitogenic and angiogenic properties (57). The endogenous antisense mRNA of FGF2 (FGF2-AS) is present in many human tissues (58, 59). FGF2-AS can repress FGF2 expression and vice versa (60).

In endometrial stromal cells derived from women with endometriosis, FGF2-AS mRNA levels are significantly lower in late proliferative phase than those in early proliferative phase and are lower, although not significantly, compared with those in the luteal phase. This expression pattern is opposite to that of FGF2 mRNA. However, FGF2-AS mRNA level doesn't vary significantly in endometrial stromal cells from normal women during the menstrual cycle (39). It seems that the up-regulation of FGF2-AS is specific for uterine malignancies. The function of FGF2-AS in normal endometrium is unclear.

3.2.5. SRA

The steroid receptor RNA activator (SRA) is first characterized as a long ncRNA interacting with the N-terminal domain of the human progesterone receptor in a yeast two-hybrid screen (61). Further studies have demonstrated that SRA can co-activate steroid receptors (61), nuclear receptors (62) as well as other transcription factors such as MyoD (63). Although protein products derived from the short open reading frames in the SRA sequence have been detected in multiple tissues and cell lines (64), they are not required for SRA activity (65).

SRA exhibits various expression levels in pituitary gland, adrenal gland, liver, breast, ovaries, uterus and prostate. Besides, SRA is significantly up-regulated in uterine tumors compared to normal uterus (41). The uterine receptivity is established and maintained by ovarian steroid hormones, progesterone and estrogen. Most of the steroid hormone responses in the uterus are attributed to activation of classical nuclear steroid receptors (NSRs), although non-genomic mechanisms may exist (66). Thus, as a steroid receptor co-activator, SRA is potential regulator of hormone action in the uterus during embryo implantation.

4. CONCLUSIONS AND PERSPECTIVES

During the last decade, newly discovered short and long ncRNAs have dramatically changed our understanding on the transcriptome. The regulatory role of ncRNAs is attracting more and more attention. Recent evidences suggest that ncRNAs are linked to the embryo implantation process through regulating the expression of certain critical genes and pathways.

Due to severe defects in ovary or oviduct development before uterine receptivity can be established, the impact of impaired Dicer1 or miRNAs on embryo implantation cannot be directly evaluated in Dicer1 hypomorph mouse (24) or Amhr2-Cre driven conditional Dicer1 knockout mouse (25-27). However, we can still find some clues. Dicer1 hypomorph mice transplanted with wild type ovaries are fertile but deliver an average of 3.4 pups per litter, which is much less than that of wild type mice (24). Amhr2-Cre driven conditional Dicer1 knockout mice have smaller uteri with decreased smooth muscle layer and glands (27). Taken together, we suppose that impaired

miRNA expression in uterus will affect embryo implantation rate and decidual development process. Good news is that chemical inhibitors of Dicer1 have been recently identified (67). These inhibitors are suitable tools for characterizing the role of miRNAs in embryo implantation. To date, much of the work has focused on miRNA profiling in the uterus. A number of miRNAs have been identified as differentially expressed ones during embryo implantation (12-15). However, only a small portion of these miRNAs have been explored further into their regulation and function during embryo implantation.

Although some long ncRNAs have been found to be dynamically expressed in the uterus during embryo implantation, these studies are rather preliminary (21, 22, 39-41). None of these ncRNAs have been proved to be indispensable in embryo implantation. There is also evidence for the existence of some other long ncRNAs in the uterus along with some other organs (68-70). The detailed expression pattern of these long ncRNAs during embryo implantation is yet to be determined. Interestingly, some well-known genes in embryo implantation, including COX-2, p53, PGR, HIFA, and MSX1, also have nearby long ncRNAs in normal or malignant tissues other than uterus (71-75). We do not know if these long ncRNAs are present and functional in the normal uterus during embryo implantation. Noticeably, although microarrays and shotgun RNA sequencing technologies are ready for long ncRNA profiling assay (76, 77), the global expression levels of long ncRNAs in the uterus during embryo implantation is still unavailable.

Furthermore, the knowledge of ncRNAs is growing fast. Additional new classes and subclasses of ncRNAs are recently discovered, such as enhancer RNAs (78), TSS-associated RNAs (79), and miRNA embedded functional long ncRNAs (80). Presently, we have no idea about the expression or function of these novel ncRNA classes in the uterus during embryo implantation.

Undoubtedly, ncRNAs have provided new insights on the complex molecular mechanisms of embryo implantation. It is expected that understanding the role of ncRNAs in uterus during embryo implantation will allow for identification of targets in treatment of infertility and help to develop novel contraceptives.

5. ACKNOWLEDGMENT

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Abbreviations: ncRNAs (non-coding RNAs), miRNAs (microRNAs), siRNAs (small interfering RNAs), piRNAs (PIWI-interacting RNAs), ESCs (endometrial stromal cells), NSRs (nuclear steroid receptors).

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