Temporal and Spatial Regulation of Let-7a in the Uterus During Embryo Implantation in the Rat

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Abstract. Mammalian embryonic implantation requires reciprocal interactions between implantation-competent blastocysts and a receptive uterus. Some microRNAs might play a key role during embryo implantation in the mouse, but the let-7a expression profiles in the rat uterus during peri-implantation are unknown. In the study, the expression of let-7a in the uterus during early pregnancy, pseudopregnancy, artificial decidualization and activation of delayed implantation was detected by Northern blotting and in situ hybridization. The effect of steroid hormones on let-7a expression was also detected by Northern blotting and in situ hybridization. Here, we found that the expression level of let-7a was higher on gestation day 6–7 (g.d. 6–7) in rats than on g.d.4–5 and g.d.8–9. Let-7a was specifically localized in glandular and luminal epithelia and decidua. The expression of let-7a was not significantly different in the pseudopregnant uterus and increased significantly in the uteri of rats subjected to artificial decidualization and activation of delayed implantation. Treatment with estradiol-17β or progesterone significantly increased let-7a expression. Thus, let-7a expression was significantly induced by the process of embryo invasion, and this increased expression level was mainly induced by active blastocysts and decidualization during the window of implantation, implying that let-7a may participate in endometrial decidualization. Steroid hormones, estradiol-17β or progesterone stimulated let-7a expression.

Key words: Embryo implantation, Hormone, Let-7a, Rat, Uterus

Mammalian implantation is a highly coordinated sequence of events that begins with the attachment of an embryo to the uterine luminal epithelium and ultimately results in formation of the placenta. The fertilized ova arrive in the rat uterus as blastocysts about 4.5 days after mating and uterine closure, embryo implantation can be initiated on day 5.5 and completed on day 7.75 [1, 2]. Implantation of the embryo into the uterine wall is regulated by various factors including matrix metalloproteinases [3], hormones [4], cytokines [5] and integrins [6]. Although many molecules are now known to be involved in the process, the specific mechanisms associated with the onset of uterine receptivity remain to be determined.

MicroRNAs (mi RNAs) are small noncoding RNAs whose function as modulators of gene expression is crucial for proper control of cell growth and are known to participate in mouse embryo implantation [7, 8]. The let-7 family is expressed differentially in the mouse uterus during the peri-implantation period [7, 8]. Let-7a was initially found to regulate developmental timing in Caenorhabditis elegans [9]. Our previous study showed that let-7a is expressed differentially during follicular development in the mouse [10]. In humans, it plays an important role in the onset and development of forms of cancer [11, 12]. Embryo implantation shares similar phenomena and mechanisms with tumor invasion [13]. Therefore, we want to know whether let-7a plays an important role during embryo implantation in the rat.

Here, we report the expression pattern of let-7a in the uterus during peri-implantation in the rat. We studied the effects of pseudopregnancy, artificial decidualization and activation of delayed implantation on the expression of let-7a. In addition, we also tested the effects of steroid hormones on let-7a expression.

Materials and Methods

Experimental animals and protocols

Sexually mature, healthy female Sprague Dawley rats (220–260 g body weight) were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China). The rats were housed in a temperature- and humidity-controlled room with a 12/12 H light/dark cycle. All animal procedures were approved by the Institutional Animals Care and Use Committee of the National Research Institute for Family Planning. The rats were caged overnight with fertile males of the same strain. The day presence of a vaginal plug or sperm was noted and considered to be day 1 of pregnancy (g.d. 1). Uteri were excised from g.d. 4–9 rats and fixed with 4% paraformaldehyde (PFA) solution (Sigma-Aldrich, St. Louis, MO, USA) for in situ hybridization or frozen in liquid nitrogen for RNA analysis.

Pseudopregnancy was induced by caging adult females with vasectomized males, and mating was confirmed by checking for a vaginal plug (day 1 of pseudopregnancy). The uteri were collected from days 4–7 of pseudopregnancy. On day 5 of pseudopregnancy, when the uteri were optimally sensitized to decidualogenic stimuli,
100 μl olive oil (Sigma-Aldrich) was infused into the lumen of one of the uterine horns to induce artificial decidualization. The contralateral uterine horn, which was not infused with oil, served as a control. At day 7 of pseudopregnancy, the rats were euthanized, and the uterine horns were isolated.

To induce delayed implantation, the pregnant rats on g.d. 4 were ovariectomized. Progesterone (5 mg/rat, s.c.; Sigma-Aldrich) was injected to maintain delayed implantation from g.d. 5–7. The progesterone-primed delayed-implantation rats were treated with estradiol-17β (0.5 μg/rat; Sigma-Aldrich) to terminate delayed implantation. The rats were euthanized by stunning and cervical dislocation to collect uterus 24 h after estrogen treatment. The implantation sites were also identified by i.v. injection of Chicago blue solution (Sigma-Aldrich). To confirm that the rats receiving progesterone only were in a state of delayed implantation, uterine flushings were collected and examined for the presence of hatched blastocysts.

To test the effects of steroid hormones on let-7a expression, rats were treated with hormones starting 2 weeks after they were ovariectomized. The ovariectomized rats were treated with an injection of estradiol-17β (1 μg/rat) or progesterone (10 mg/rat) at intervals of 24 h for 3 days. All steroids were dissolved in olive oil and injected subcutaneously. Controls received the vehicle only (0.1 ml/rat).

**Northern blotting analysis**

Northern blotting analysis of miRNAs was performed as described previously [14]. Briefly, total RNA was isolated from the uterus with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Aliquots of 40 μg of total RNA per sample were subjected to electrophoresis on a 15% urea-PAGE gel and transferred to a nylon membrane (Hybond N+; Amersham Pharmacia Biotech, St Albans, Hertford, UK). After being UV cross-linked and baked at 50 C for 30 min, the membrane was prehybridized at 42 C for 4 h and then hybridized with 32P-labeled probes at 40 C overnight. Membranes were washed and exposed to PhosphorImager screens (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). The bands were analyzed using the Quantity One software (Bio-Rad, Hercules, CA, USA). All experiments were repeated at least three times.

**In situ hybridization of let-7a with DIG-labeled LNA probes**

In situ hybridization of miRNAs with DIG-labeled LNA probes was performed as described previously [15]. Briefly, sections of uterus (5 μm) were treated with proteinase K (20 g/ml) for 15 min and refixed in 4% PFA for 15 min. After acetylation with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min, sections were prehybridized with hybridization buffer (Roche, Mannheim, Germany) at 40 C for 2 h and then hybridized with digoxigenin (DIG)-labeled LNA-let-7a probe (LNA-let-7a sequence: 5’-DIG-aCtaTacAaCcT acTacCtca–3’) at 40 C overnight. The sections were then incubated in buffer containing anti-DIG-antibody for 2 h at 37 C and stained with 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Promega, Madison, WI, USA) and p-nitroblue tetrazolium chloride (NBT; Promega). The sections were hybridized with a DIG-labeled LNA-scrambled probe (LNA-scrambled sequences: 5’–caTtuAtgTeCgaCaaTcAaT–3’) as a negative control [16]. Samples were viewed with an Eclipse 80i microscope (Nikon, Tokyo, Japan).

**Statistical analysis**

There were at least three rats in each treatment group. The results of Northern blotting and in situ hybridization were repeated three times. All values are reported as means ± SE. Statistical analysis was performed using one-way ANOVA. When significant effects of treatments were indicated, the Student–Newman–Keuls multiple range test was applied using SPSS version 13.0 (SPSS, Chicago, IL, USA). P<0.05 was considered statistically significant.

**Results**

**Dynamics of let-7a in the rat uterus during the peri-implantation period**

To study the role of let-7a in embryo implantation, we first examined its temporal and spatial distribution in the uterus during the peri-implantation period. Northern blotting analysis showed that the expression level of let-7a on g.d. 6–7 was higher than on g.d. 4–5 and decreased markedly on g.d. 8–9 (P<0.05; Fig. 1). The in situ hybridization results showed that let-7a was mainly located...
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in the glandular and luminal epithelia on g.d. 4 (Fig. 6A). On g.d. 5, the let-7a signal strengthened in both epithelia and also appeared in the stroma (Fig. 6B). A strong signal was found in the decidua from g.d. 6 (Fig. 6C–F), but the signal was weak in glandular (Fig. 6C) and luminal epithelia (Fig. 6D). No staining was found in uterine sections from g.d. 6 when hybridized with DIG-labeled LNA-scrambled probe as a negative control (Fig. 6L).

Effect of pseudopregnancy on the expression of let-7a

To see whether let-7a expression was dependent upon the presence of embryos, uterine tissues were subjected to Northern blotting analysis on days 4–7 of pseudopregnancy (Fig. 2).

Effect of delayed implantation on the expression of let-7a

To test whether let-7a expression was dependent upon embryo implantation status, a delayed implantation model was used for Northern blotting and in situ hybridization analyses. Northern blotting showed a low level of let-7a in the uterus under delayed implantation conditions, but it increased dramatically after implantation was activated with estrogen treatment (P<0.05; Fig. 3). In situ hybridization showed slight staining in glandular and luminal epithelia in the control uterine horn on day 7 of pseudopregnancy (Fig. 6I). However, in the oil-infused uterus, strong signals were detected in deciduals, but staining was weak in the luminal epithelium (Fig. 6J).

Effects of steroid hormones on let-7a expression

Northern blotting was performed to examine whether let-7a expression was regulated by steroid hormones. A low level of let-7a expression was detected in the ovariecctomized rat uterus. However, treatment with progesterone or estradiol-$17\beta$ significantly increased let-7a expression (P<0.05), and let-7a expression was significantly upregulated by combination of both hormones (P<0.01; Fig. 5).
Fig. 4. Let-7a expression in the uterus with artificial decidualization. Northern blotting analysis of uterine let-7a expression in a model of artificial decidualization. Hybridization was performed with a $^{32}$P-labeled probe for let-7a and U6. The let-7a level in the model of artificial decidualization was quantified by densitometric analysis and is presented as the ratio of let-7a to U6 intensity to normalize for gel loading and transfer. The bar graph represents the let-7a level in the model of artificial decidualization relative to that of the non-stimulated control. *P<0.05.

Fig. 5. Effect of steroid hormones on uterine let-7a expression. The effect of steroid hormones on uterine let-7a expression was detected by Northern blotting. Hybridization was performed with a $^{32}$P-labeled probe for let-7a and U6. The let-7a level in the uterus with steroid hormone treatment was quantified by densitometric analysis and is presented as the ratio of let-7a to U6 intensity to normalize for gel loading and transfer. The bar graph represents the let-7a level in the uterus with steroid hormone treatment relative to that of the vehicle H treated group. *P<0.05.

Fig. 6. In situ localization of let-7a in the rat uterus. Sections of the uterus from days 4 (A), 5 (B), 6 (C), 7 (D), 8 (E) and 9 (F) of pregnancy were subjected to in situ hybridization using DIG-labeled LNA probes specific to let-7a. Expression of let-7a in the uteri of rats showing delayed implantation (G, H), activation of delayed implantation (I) and artificial decidualization was also detected by in situ hybridization. Artificial decidualization was stimulated by infusing 100 μl sesame oil into the lumen of one of the uterine horns (K). The contralateral uterine horn, which was not infused with oil, served as a non-stimulated control (J). Staining was developed using BCIP/NBT; blue staining indicates a hybridization signal. To evaluate the specificity of the probe, negative control staining was performed by substituting DIG-labeled LNA-scrambled probe for the DIG-labeled LNA-let-7a probe (L). The scale bar indicates a distance of 100 μM. m, myometrium; mg, maternal gland; s, stroma; le, luminal epithelium; db, decidua basalis; em, embryo.
Discussion

Expression and Regulation of let-7a

MicroRNAs are believed to be important for oocyte maturation and early development [17–19]. Two papers have reported that miRNAs may participate in mammalian embryo implantation in a mouse model [7, 8]. To our knowledge, this is the first report of expression and regulation of the miRNA of let-7a during embryo implantation in the rat. Implantation involves preimplantation embryos developing to the blastocyst stage and then hatching from the zona pellucida to establish reciprocal interactions between the trophoderm and uterine luminal epithelium [20]. To study the role of let-7a in embryo implantation, we first examined its temporal and spatial distribution in the uterus during the peri-implantation period in the rat.

The expression of uterine let-7a on g.d. 6–7 was higher than on g.d. 4–5 and g.d. 8–9, and it was mainly found in the glandular and luminal epithelia and decidua. In the rat, blastocysts can implant only if the endometrium has been induced into a transient state of endometrial receptivity to embryo implantation that lasts for less than 24 h [1, 21, 22]. Endometrial receptivity for embryo implantation in the rat occurred on day 5 of pregnancy or pseudopregnancy.

The time surrounding the window of receptivity in the rat is referred to as the peri-implantation period. The endometrium passes through three distinct phases during this time referred to as the neutral (day 4), receptive (day 5), and refractory (day 6) phases with respect to embryo implantation. Embryo transfer experiments have revealed that blastocysts transferred to the uterine lumen of day 4.5 pseudopregnant recipients remain in a state of dormancy until the endometrium becomes receptive on day 5, and embryo implantation can then be initiated on day 5.5 and completed on day 7.75 [1, 2]. In response to implantation of embryos, the underlying endometrial stromal cells undergo decidualization [1]. This suggested that only the state of endometrial receptivity or dormant blastocysts did not increase the expression of let-7a. Let-7a expression was significantly induced by the process of embryo invasion, implying that let-7a may participate in endometrial decidualization.

To further explore whether let-7a expression is associated with the induction of implantation, we tested the effects of pseudopregnancy, experimentally induced decidualization and delayed implantation.

Pseudopregnancy was induced in Sprague Dawley female rats by mating with male rats two weeks after vasectomy, and mating was confirmed by checking for a vaginal plug (day 1 of pseudopregnancy) [23, 24]. These were no fertilized eggs in the vaginas of the pseudopregnant rats. Our result showed that there were no apparent differences in let-7a expression in the uterus during days 4–7 of pseudopregnancy. The gene expression pattern and hormonal level on days 1–5 of pseudopregnancy were similar to those of days 1–5 of pregnancy and changed after day 5 of pseudopregnancy [25]. Endometrial receptivity for embryo implantation in the rat occurred on day 5 of pregnancy or pseudopregnancy. When there are blastocysts in the uterus, embryo implantation can be initiated on day 5.5 [1, 2]. However, there were not blastocysts in the uterus of the pseudopregnant rat, and embryo implantation could not be initiated. So, the unchanged expression of let-7a in the uteri of the pseudopregnant rats may imply that the state of endometrial receptivity alone did not increase the expression of let-7a. This was coincident with the hypothesis speculated by the expression pattern of let-7a in early pregnancy, i.e. the expression of uterine let-7a on g.d. 6 was higher than on g.d. 5.

The pregnant rats on g.d. 4 were ovariectomized and treated with steroids in order to induce delayed implantation [25, 26]. In the model of activation of delayed implantation, steroid hormones are used to control the status of embryo implantation [21]. Progesterone was used to maintain endometrial receptivity. Estrogen was used to induce blastocysts implantation because an estrogen peak is necessary for implantation of fertilized ova 12 h later on day 5 post-coitum [27, 28]. The expression of let-7a in the uterus was visibly unchanged in the progesterone treatment and control groups under delayed implantation conditions, but it increased dramatically after implantation was activated with estrogen treatment. The expression level of let-7a was unchanged on days 4–7 of pseudopregnancy. It was coincident that the expression pattern of let-7a on days 4–7 of pseudopregnancy (the expression of let-7a was unchanged in the uteri of the pseudopregnant rats which embryo implantation is not initiated) and delayed implantation induced by alone progesterone treatment which can not initiate embryo implantation. These results further supported the standpoint that the state of endometrial receptivity alone did not increase the expression of let-7a, and the activated blastocysts seemed to be one of the major contributors to the regulation of let-7a.

Artificial decidualization was induced by infusion of olive oil into the lumen of one of the uterine horns, and the contralateral uterine horn, which was not infused with oil, served as a control [29, 30]. The expression level of let-7a in the decidualized uterus was significantly higher than in the nonstimulated uterus on day 7 of pseudopregnancy. In addition, a strong signal was found in the decidua on days 6 and 7 of pregnancy in the rat, but the signal was weak in other locations in the uterus. These results implied that the increase in let-7a expression was dependent upon endometrial decidualization.

Ovarian progesterone and estrogen are the principal hormones that direct uterine receptivity, embryo implantation and maintenance of pregnancy in all mammals studied and are essential for implantation in mice and rats [31, 32]. However, it is still unclear if progesterone and estrogen affect let-7a expression. In the present study, non-pregnant rats ovariectomized and treated with steroids were used to simulate the level of hormones under physiological conditions [33] and to detect the effect of steroid hormones on the expression of let-7a. We found that let-7a expression was significantly upregulated by progesterone and estradiol-17β treatment in the ovariectomized rat uterus. Dysregulated expression of the let-7 family of miRNAs in human uterine leiomyomas is strongly associated with tumor size and ethnicity [34], and the let-7a expression levels are reduced in many tumors [11, 35]. The results of previous study, although they are not directly related to pregnancy and only showed that the expression of let-7a is related to steroid hormones under physiological condition, embryo implantation shares similar phenomena and mechanisms with tumor invasion [13], imply indirectly that the action of progesterone and
estradiol-17β on let-7a expression might inhibit excessive invasion of cells from the uterus and trophoblast. In addition, because gene expression patterns and hormonal level exhibit significant differences in the pregnant and non-pregnant rat uterus [36, 37], the expression of let-7a in the uterus of ovariecтомized and steroid hormones treated non-pregnant rats may be not compared with those of pregnant rats.

In conclusion, we found that expression of let-7a miRNA was significantly induced by the process of embryo invasion. The results obtained from our models of pseudopregnancy, artificial decidualization and delayed implantation imply an important role for implanting blastocysts and decidualization in the temporal and spatial changes of let-7a expression in the uterus during the window of implantation, and let-7a may participate in endometrial decidualization. In addition, the expression of let-7a was regulated by steroid hormones. Collectively, these results suggest that let-7a is involved in embryo implantation. It might promote differentiation of the uterus and prevent excessive invasion of cells during this process. In addition, this study has the potential to provide new insights into the mechanisms of embryo implantation.

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References