Forced Suppression of let-7a-5p in Mouse Blastocysts Improves Implantation Rate

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Research

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Abstract

Background

Embryo implantation requires appropriate communication between the blastocyst and endometrium. Recurrent implantation failure is an essential component of assisted reproductive technology. Also, miRNAs-mediated gene expression impacts the implantation process, and the down-regulation of some miRs, such as mmu-let-7a, improves this process. In the present study, we evaluated the effect of let-7a forced-suppression on the mouse implantation rate.

Methods

In total, 100 adult female mice and 10 adult male mice were included (Strain CD-1). We analysed the expression of let-7a and its potential mRNAs targets (Igf1, Il1a, Itgb3, and Tgfb1) in control, sham, and antagonir-treated blastocysts using quantitative reverse transcription PCR (qRT-PCR). The control and treated blastocysts were transferred to the 20 pseudopregnant mice so that the effect of let-7a suppression on the rate of implantation could be determined.

Results

The expression level of let-7a in the treatment group was significantly down-regulated (P=0.001). In contrast, no significant expression changes were observed for let-7a or mRNAs targets when the sham and control groups were compared (P>0.05). In comparison to the controls, the antagonir-treated group exhibited significantly up-regulated expression levels of Igf1 (0.0167), Itgb3 (0.045), and Tgfb1 (0.0115). Additionally, the implantation rate was significantly higher in the treatment group (78%) than the control group (61%) (P=0.0098).

Conclusion

We found that forced-suppression of mmu-let-7a-5p through successful transfection of Anti-miR leads to upregulation of downstream genes, Igf1, Itgb3, and Tgfb1, which directly involved in the trophoblast-endometrium attachment and improve the implantation rate.

Background

Endometrial receptivity is defined as the period of endometrial maturation that enables the blastocyst to attach itself to and invade the endometrial epithelial cells and subsequently proceed to the vasculature (1). Blastocyst implantation requires appropriate molecular and cellular communication between the blastocyst and uterus; blastocyst implantation is also regulated by a variety of processes, including endocrine, paracrine, autocrine, and juxtacrine signalling (2, 3). Complete and severe implantation failure under certain inflammatory or abnormal anatomic conditions can lead to infertility and pregnancy loss, respectively (1).

Recurrent implantation failure (RIF) is the failure to achieve pregnancy after the transfer of three or more embryos (in women younger than 35 years of age) or four or more embryos (in women older than 35 years of age) (4). RIF is an essential component of assisted reproductive technology, such as in vitro fertilisation (IVF) treatment. Implantation failure accounts for many cases of unexplained infertility, and to date, there are no efficient treatments. Animal models, especially rodent models, have been intensively examined to understand the underlying mechanisms of blastocyst implantation (3).
Mature microRNAs (also known as miRNAs) are a class of endogenous non-coding RNAs with a size of approximately 22 nucleotides. They act at the posttranscriptional level to regulate gene expressions associated with various cellular activities, including cell growth, differentiation, and development (5). Recent studies suggest that the miRNAs-mediated gene expression of endometrial tissues and/or the blastocyst impact the processes of implantation and fetal development. In a pioneering study on the miRNA-mediated regulation of uterine gene expression in the context of implantation, miR-101a-3p and miR-199a-3p were spatiotemporally expressed in mouse uteri during implantation coincident with the expression of cyclooxygenase-2 (a gene critical for implantation) (6). Maternal and/or blastocyst miR-30d deficiency impairs blastocyst implantation and fetal development in miR-30d knockout mouse models, and endometrial receptivity markers were significantly down-regulated in the knockout models (7). In another recent study, it was shown that miR-30a-3p is down-regulated during embryo implantation and could affect embryo implantation by acting as a suppressor of epithelial-mesenchymal transition (EMT) by targeting Snai2 (8). The expression level of miR-320 in rat uteri was increased by an active blastocyst and decidualisation during the window of implantation, thereby suggesting that they participate in endometrial decidualisation (9, 10). let-7a affects implantation via its direct influence on the expression of integrin-b3; low expression levels of integrin-b3 improve implantation. (11). Anti-miRs are single-stranded synthetic oligonucleotides that are designed to bind directly to the miRNAs and are an excellent tool in miRNA loss-of-function studies. Also, their use is widespread in clinical translation, especially in osteoporosis and cancer (12).

To the best of our knowledge, only a few studies have examined the impact of miRNAs on the blastocyst during implantation. Furthermore, reductions of some miRNAs have been reported as possible targets for enhancing implantation onto the receptive endometrium, which is critical to assisted reproductive technology. Accordingly, in the present study, we aimed to reduce embryonic let-7a expression via anti-miR and ViaFect™ reagent in mice embryos. We also investigated the applicability of the forced suppression of embryonic mmu-let-7a-5p for improving in vivo implantation.

Methods

Animal Study

In this study, 100 adult female mice and 10 adult male mice were included (Strain CD-1). Mice (8–10 weeks old, 20–50 g) were obtained from the Royan Institute, Tehran, Iran. All mice were kept on a 12-h light/12-h dark cycle and had free access to food and water. The ethics committee of Islamic Azad University, Science and Research Branch, Tehran, Iran approved the study (IR.IAU.SRB.REC.1398.123). All experiments followed the National Institutes of Health guidelines for the care and use of laboratory animals.

During sperm and oocyte recovery, male and female mice were euthanised by cervical dislocation. Superovulation was induced in 80 female mice (6–8 weeks old, 20–25 g) using peritoneal injections of 7.5 IU PMSG (Folligon, Canada) and 7.5 IU hCG (Pregnenolone, Intervet) in the middle of the light cycle at a time interval of 46–48 h.

Approximately 13–16 h after hCG injection, the fallopian tube was separated from the ovary. Afterwards, oocytes were released from the ampulla, introduced into an HTF (Geneocell, Iran) medium that was supplemented with 15 mg/ml BSA (Sigma-Aldrich, Germany), collected, and counted under an inverted microscope. At least 12 oocytes were collected from each mouse.

In Vitro Fertilisation and culturing
Epididymides were removed from 10 adult fertile male mice and transferred to an HTF (Geneocell, Iran) medium supplemented with 15 mg/ml BSA (Sigma-Aldrich, Germany). The epididymides were then incubated for 1 h in an incubator at 37°C and 5% CO₂, thus facilitating the release of the sperm from the epididymides into the medium. The capacitated sperm in the HTF (Geneocell, Iran) medium were added to an HTF medium droplet containing oocytes (2x10⁶ spermatozoa/ml). Approximately 10 µl of the HTF (Geneocell, Iran) containing motile sperm was added to each IVF droplet. After oocytes and sperm were incubated for 5–7 h, the fertilisation rate was recorded. The fertilisation rate was determined by gently removing and pipetting the sperm and cumulus cells and then counting the number of ova with male and female pronuclei (2PNs). Then, the embryos were cultured by supplementing an HTF (Geneocell, Iran) medium with 4 mg/ml BSA (Sigma-Aldrich, Germany). The media were applied in 25 µl droplets covered with mineral oil (Life Global, Bulgaria). Roughly 22–25 h after insemination, two-, and four-cell embryos were observed. After washing and transferring these blastomeres to the HTF medium, 8-cell stage were observed.

**Fertilisation Rate**

From 80 female mice, 1000 oocytes were collected. Of these oocytes, 968 (96.8%) were in the MII stage. The fertilised oocytes, 2-cell stage embryos, 4-cell stage embryos, 8-cell stage embryos, and failed embryos were counted under a stereo microscope.

**Experimental Groups and Transfections**

Three groups were considered for experiment in 8 cell stage, also three solutions were prepared as follows: solution A included 15 µl antagonomir (Cat. No. MNM01000) from ABM (ABM, Inc., Richmond, BC, Canada) and 135 µl HTF medium; The lyophilised antagonomir was re-suspended in 50 µl of DNase and RNase-free water to make a 100-µM stock (1 µM working). Antagonomirs are modified microRNA inhibitors with several modifications on the strands, thereby making them more resistant to degradation than normal inhibitors.

solution B includes 10 µl transfection reagent using ViaFect™ (Promega, Madison, WI, USA) and 140 µl HTF medium, and solution C included 15 µl DDW and 135 µl HTF medium (Geneocell, Iran). For the treatment groups, 5 µl of solution A and 5 µl of solution B (mixed for 20 min) were added to the culture drops(25 µl), each contains five embryos.

For the sham group, 5 µl of solution B and 5 µl of solution C were added to the culture drops. In the sham groups, culture drops received only the transfection reagent. At last, the control group received no treatment. Finally, all three groups were cultured up to the blastocyst stage.

mmu-let-7a-5p antagonomirs were transfected into 8-cells stage embryo via ViaFect™ Transfection Reagent according to the manufacturer's protocol. This reagent allows the high-efficiency transfection of blastocysts without compromising cell viability.

**Blastocyst Transfer and Implantation Rate**

Twenty recipient females were caged individually with vasectomised males on day 0 and were checked for the presence of vaginal plugs on day 1. In total, 100 blastocysts from the control group and 100 blastocysts from the treatment group were transferred to pseudopregnant mice (day 3). Each recipient mouse received 10 blastocysts in one uterine horn under anaesthesia using intraperitoneal injections of a Ketamine/Xylazine combination (40 mg/kg + 5 mg/kg, ip).

Blastocysts were evaluated for implantation 96 h after the embryo was transferred to the uterine horn of mice. Pregnant mice were sacrificed by cervical spine dislocation, and the uterine horn was removed so that the
implantation rate of each group could be calculated.

**RNA Extraction**

RNA extraction from blastocysts and cDNA synthesis were performed using a BON embryo miRNA detection kit (Bonyakhteh, Tehran, Iran, Cat No # BN-0011.31) according to the manufacturer's protocol. Two blastocysts were briefly lysed in 2.5 µl of a BON lysis buffer at room temperature for 5 min.

**cDNA Synthesis**

The expression of the mature miRNA and target mRNAs were detected via the poly(A)-tailed universal reverse transcription method (5). This was done using the BON-miR high sensitivity miRNA 1st strand cDNA synthesis kit according to the manufacturer's instructions (BON209001, Bon Yakhteh, Iran). In the polyadenylation step, a run of AMP was added to the 3'-end of the RNA using E. coli poly(A) polymerase and rATP. A reaction mixture containing 2.5 µl total RNA, 0.5 µl rATP (10 mM), 0.2 µl poly(A) polymerase, 1 µl 10X poly(A) polymerase buffer, and DDW up to a final volume of 10 µl was incubated for 30 min at 37°C. Enzyme inactivation was accomplished by heating the mixture at 65°C for 20 min. After completing the polyadenylation reaction, 1 µl of a BON-RT adaptor (10 µM) was added to 10 µl poly(A)-tailed RNA and incubated at 75°C for 5 min, and then immediately put on ice. For the cDNA synthesis, a mixture containing 0.7 µl RT enzyme, 2 µl dNTP mix (100 mM), 4 µl 5X RT buffer, an 11-µl mixture of poly(A)-tailed RNA and BON-RT adaptor, and DDW up to a final volume of 20 µl was prepared.

The cDNA synthesis process started with an initial adaptor annealing at 25°C for 10 min, cDNA synthesis at 42°C for 60 min, and the heat inactivation of RT enzymes at 70°C for 10 min. cDNAs were stored at -80°C. The BON-RT adaptor is a degenerate primer. The last nucleotide at the 3'-end is A, G, C, or T, whereas the 3'-penultimate nucleotide is either A, G, or C. The two variable nucleotides were flanked by an oligo(dT), followed by a stuffer sequence for increasing the length of the adaptor and a common reverse primer at the 5'-end.

**Real-Time PCR**

A quantitative real-time PCR process was used for the relative quantification of mmu-let-7a-5p and Snord70 and was performed using the specific forward primers and a common reverse primer (Table 1). To increase the specificity of the forward primer, a G:T mismatch was introduced at the 3'-end penultimate; in this way, the forward primer was distinguished from the other members of the let-7 family. The melting temperature (Tm) of mmu-let-7a-5p primer was increased by adding GGC to the 5'-end of the forward primers. The specificity of the forward primers was checked by BLASTN, SSEARCH (www.miRBase.org) and primer blast (www.ncbi.nlm.nih.gov).

| Primer/Gene | Mature sequence (5'→3') | Primer (5'→3') |
|-------------|--------------------------|----------------|
| Snord70     | ...                       | TGATTTAACAAAAATTCGTAC |
|             | UGAUUUAACAAAAAUUCGUCACUACCACUGAGACAAACAAUGAA |               |
| mmu-let-7a-5p | UGAGGUAGUGGUUGUAJAGUU | GGCTGAGGTAGTTGTGAGT |
| Common Reverse | GAGCAGGGTCGAGGT |                                  |

The target mRNAs were quantified via specific forward and reverse primers using the SYBR green real-time PCR assay. The primer sequences are shown in Table 2.
Table 2
Primer sequences an their related product size for quantification of the target mRNAs and Actb reference gene.

| Gene   | Forward Primer (5'→3') | Reverse Primer (5'→3') | Length (bp) |
|--------|------------------------|------------------------|-------------|
| Actb   | GTACCACCATGTACCAGGC    | AACCAGCTCAGTAAACCATCC  | 247         |
| Igf1   | GCTGGTGATGCTCTTACG    | TCCGGAAGCAACTCAGTCC    | 125         |
| Il1a   | ACCTCAAGCAAGGGAAGAG   | AAGGTGCTCACTGGAAGAT    | 124         |
| Itgb3  | GGGCGCTGTGTTGTTGAGAG  | GCCTCACTGACTGGAACCTC   | 198         |
| Tgfb1  | AAGGCTACCATGCAAATTC   | CCACGTAGTACGATGGGAC    | 168         |

The qPCR reactions were performed according to the BON-miR qPCR kit’s instructions (Bonyakhteh, Teran, Iran) in a MIC real-time PCR system (Bio Molecular Systems, Australia). Briefly, 6.5 µl 2X miRNA qPCR master mix, 0.5 µl common reverse primer (1 µM), 0.5 µl forward primer (1 µM), and 1 µl cDNA were added. Then, DDW was added up to a final volume of 13 µl. The real-time PCR program started with an initial denaturation step at 95°C for 2 min. This was followed by 40 cycles of denaturation at 94°C for 5 sec and annealing at 60°C for 35 sec. All reactions were performed in triplicate. The primer specificities of Snord70 and mmu-let-7a-5p were verified through a melting curve analysis and agarose gel electrophoresis. The PCR efficiencies for all primer pairs were calculated in 10-fold serial dilutions of cDNA by plotting the Ct (y-axis) versus log cDNA dilution (x-axis) according to the previous protocol (13).

According to previous studies on mouse blastocyst implantation, Snord70 (also known as snoRNA234), was selected as a suitable reference gene for relative quantification of mmu-let7a-5p (14, 15).

mRNA Targets of mmu-let-7a-5p

We selected experimentally validated mRNA targets of mmu-let-7a-5p using the DIANA-TarBase v8, which provides high-quality manually curated experimentally validated miRNA: gene interactions. According to this database, four transcripts [insulin-like growth factor 1 (Igf1), interleukin 1 alpha (Il1a), integrin beta 3 (Itgb3), and transforming growth factor beta 1 (Tgfb1)] were targeted by mmu-let-7a-5p.

Data Analysis

In order to determine possible associations between the control, sham and treatments, the real time PCR data has been analyzed using “pcr” package in R (4.0.3) software (16). The level of treatment effectiveness is reported as, foldchange, odd ratio and p-value. A two-tailed p-value of < 0.05 was considered statistically significant. Furthermore, rate of embryo development from the 8-cell stage to the blastocyst and also effectiveness of the treatment between the test groups has been statistically analysed using “lsmeans” and “oddratio” packages in R (4.0.3) software through performing logistic regression. The results have been reported as p-value and oddratios. p-value of < 0.05 was considered statistically significant.

Results

Fertilisation rates

Following IVF, 960 (96%) and 810 (81%) zygotes continued to become 2-cell stage and 4-cell stage embryos, respectively. Furthermore, only 750 (75%) zygotes developed into 8-cell stage embryos. These embryos were divided into control, sham, and treatment groups and were cultured until blastocysts formed (Table 3). No significant
differences were found between the sham, control and treatment groups in terms of the rate of embryo development from the 8-cell stage to the blastocyst formation stage (P > 0.05). Accordingly, we did not transfer any blastocysts from the sham groups to the recipient mice.

Table 3

| Groups   | 8-Cell Stage | Blastocysts | Lsmeans ± SE | p-value | OR  |
|----------|--------------|-------------|--------------|---------|-----|
| Control  | 250          | 151 (60%)   | 0.464 ± 0.0919 | -       | -   |
| Sham     | 250          | 156 (62%)   | 0.601 ± 0.16  | 0.6460  | 1.088 |
| Treatment| 250          | 162 (65%)   | 0.652 ± 0.1612| 0.3095  | 1.207 |

Lsmeans: Least square means.

OR: odds ratio.

Efficiency of anti-miR delivery via ViaFect™ reagent

The levels of mmu-let-7a-5p expression in the blastocysts of the treatment group were significantly down-regulated in comparison to the control blastocyst group (Fold change, FC = 0.252, P = 0.001). In contrast, no significant changes were observed between the sham and control groups in this regard (FC = 0.984, P = 0.268). These results indicate that transfected antagomir reduced mmu-let-7a-5p expression in the blastocysts, while the transfection reagent did not.

More importantly, embryo survival rate during the culture between three groups with no significant difference is an important clue for the non cytotoxicity for this reagent.

The results of mmu-let-7a-5p expression in the study groups are shown in Fig. 1A.

**Suppression of mmu-let-7a changes the expression of downstream genes**

The expression levels of the four candidate mRNA targets of mmu-let-7a-5p (i.e., Igf1 (FC = 0.93, P = 0.833), Il1a (FC = 0.92, P = 0.764), Itgb3 (FC = 0.97. P = 0.925), and Tgfb1 (FC = 1.11, P = 0.699) were not significantly different when the sham and control groups were compared. On the other hand, the expression levels of Igf1 (FC = 2.0, P = 0.016), Itgb3 (FC = 1.4, P = 0.045), and Tgfb1 (FC = 1.8, P = 0.011) were significantly up-regulated in the antagomir-treated group when compared to the control group (Fig. 1B). Differently, the mRNA level of Il1a was not significantly different when the antagonmir-treated group was compared to the control group (FC = 0.9, P = 0.857). These results indicate that Igf1, Itgb3 and Tgfb1 are potential targets of mmu-let-7a-5p in the blastocysts.

**mmu-let-7a suppression leads to increase implantation rate**

The rate of successful implantation was significantly higher (P = 0.0098) in the treatment group (78%) than in the control group (61%) (Table 4). Examples of implanted embryos, both in the treated and control mice, are shown in Fig. 2.
### Table 4
The results of mouse embryo transfer in the mmu-let-7a-5p antagomir-treated blastocysts and control group

| Groups       | Successful Implantation | Implantation Failure | Lsmeans ± SE | p-value | OR  |
|--------------|-------------------------|----------------------|--------------|---------|-----|
| control      | 61                      | 39                   | 0.447 ± 0.205| -       | -   |
| Treatment    | 78                      | 22                   | 1.266 ± 0.241| 0.0098* | 2.267|

Lsmeans: Least square means.

*: p-values different from the control with p < 0.05.

OR: odds ratio.

### Discussion

Embryo implantation is a crucial step in natural and IVF pregnancies, and it is considered a rate-limiting step in the reproduction process (17). Successful implantation depends on blastocyst quality and endometrial receptivity (18, 19). miRNAs are known to play pivotal roles in preimplantation, implantation, and mouse embryo development (11, 20, 21). During implantation, both the blastocyst and the endometrium secrete miRNAs, which facilitate various signalling pathways (2). After comparing the miRNAs expression levels of implanted and non-implanted blastocysts, (2) identified a pattern of miRNAs that may be useful for predicting the outcomes of embryos. Furthermore, it has been shown that has-miR-661 (22), has-miR-372, and has-miR-191 (23) are significantly up-regulated in the in media of blastocysts that failed to implant in comparison to those that successfully implanted. Additionally, Liu et al. showed that five members of the let-7 family, including let-7a-5p, were down-regulated in humans and mice after dormant blastocysts were activated (11).

In the last decades vast biological, chemical, and physical methods such as viral carriers, electroporation and microinjection to attain successful transfection and gene delivery in embryo studies have been performed. Cytotoxicity and immunogenicity of viral vectors, cell damage and invasiveness of electroporation and cost, time consuming and inability to transfect large number of cells in microinjections are still concerned (24). In vitro transfection experiments aim to assess the efficient and the safest methods for gene delivery. The most commonly used non-viral delivery systems are lipid base vectors and using non-viral commercially transfection reagent to deliver siRNA into mouse ES cell, show the high efficiency and low toxicity in their cell line (25). ViaFect™ Transfection Reagent is a novel formulation designed to transfect DNA into a wide variety of cell lines with high efficiency and low toxicity. ViaFect™ Transfection Reagent has been shown to transfect cell lines that are traditionally thought of as difficult to transfect (such as hematopoietic, primary and iPSC stem cell lines) with relatively high efficiency (26). This lipid delivery reagent in an aqueous solution functions by interacting with the negatively charged DNA, allowing its passage into the cell by preventing the electrostatic repulsion of the cell membrane.

In the current study, we analysed the effect of the forced suppression of mmu-let-7a-5p by antagomir and ViaFect™ reagent in mouse blastocysts; we compared sham, control, and treated groups to evaluate implantation rates. Our results showed that the expression level of mmu-let-7a-5p—as well as its target transcripts, including Igf1, Il1a, Itgb3, and Tgfb1—were similar when comparing the sham and control groups (P > 0.05). These data indicate that the transfection reagent did not change the mmu-let-7a-5p expression level.

On the other hand, we showed that the mmu-let-7a-5p expression levels in the blastocysts of the treatment group were significantly down-regulated when compared with the controls. No significant differences were found between
the sham, control and treatment groups in the rate of embryo development from the 8-cell stage to the blastocyst formation stage (P > 0.05), and show that lipid reagent such as ViaFect™ is an ideal vehicle for anti-miR delivery with high efficiency and low toxicity in mouse embryo; also after analysing the embryo transfer results, we found a significantly higher rate of successful implantation in the treatment group (78%) than in the control group (61%). Our results corroborate the findings of Liu et al., who showed that the forced expression of let-7a in mouse blastocysts decreased the rate of implantation (11).

miRNAs negatively regulate multiple target mRNAs through degradation and/or translational repression (5). The down-regulation of let-7 in the activated blastocysts resulted in the increased expression of its target genes—this included oncogenes and cell cycle checkpoint genes, which result in cell cycle progression, DNA synthesis, and cell division (27). The expression level of let-7 is tightly regulated during oogenesis and the early stages of embryo development (28) and then gradually decreases from the oocyte to the 8-cell stage embryo (20). In addition, let-7a is less abundant in blastocysts when compared to the earlier developmental stages, perhaps due in part to blastocyst activation and implantation initiation (29, 30). The down-regulation of let-7a-5p in activated blastocysts in comparison to the dormant ones indicates that a low level of let-7a-5p could promote implantation (11).

Our results showed that three target mRNAs of mmu-let7a-5p (i.e., Igf1, Itgb3, and Tgfb1) were up-regulated in the antagonir-treated blastocysts when compared to the control group (P < 0.05). However, the expression of Il1a (which is a potential target of mmu-let7a-5p) was not significantly different when the treated group was compared to the control group. Previous research has demonstrated that Integrin-b3 is a target of let-7a in the human HeLa cell line and in mouse blastocysts (11). Integrins are a family of transmembrane adhesion receptors and are primarily responsible for regulating the interactions between the trophoblasts and the extracellular matrix molecules of the endometrium (31). They are heterodimers composed of non-covalently associated α and β subunits that engage with extracellular matrix proteins and couple to intracellular signalling and cytoskeletal complexes (32). Igf1 increases the amount of apical fibronectin on blastocysts, thereby increasing attachment and invasion in an in vitro model of implantation (33). TGF-B is a cytokine that has a wide range of functions and plays a vital role in embryo implantation. TGF-utilises its membrane receptors to activate the SMAD pathway via the phosphorylation of SMAD2/3 complex. The activated pSMAD2/3 migrates to the nucleus and transcribes TGF-B-responsive genes, such as integrins, fibronectin, collagens, and plasminogen activator inhibitor 1 (34). In situ hybridisation and immunocytochemistry experiments show that the mRNAs and proteins of Il1a and Il6 are expressed in higher ratios in normal and activated mouse blastocysts when compared with dormant ones (35).

A reciprocal and complex interaction between let-7a and dicer (a let-7a target that processes pre-miRNA to mature miRNA) has been found. This interaction could alter the miRNAs expression pattern and consequently affect the implantation competency of activated blastocysts (29). The dysregulation of let-7a expression could affect trophectoderm differentiation and implantation, which, in turn, might increase IVF failure (11).

**Conclusion**

Forced-suppression of mmu-let-7a-5p by the ViaFect™ can successfully transfec Anti-miR with high transfection efficiencies with no apparent uninvited effects or clear interference with our experimental outcomes and was found to improve embryo implantation, at least partly through the regulation of Igf1, Itgb3, and Tgfb1 transcripts. Additional research is necessary to evaluate the role of other family members of let-7 in mouse implantation.

**Declarations**
Ethics approval and consent to participate

The ethics committee of Islamic Azad University, Science and Research Branch, Tehran, Iran approved the study (IR.IAU.SRB.REC.1398.123). All experiments followed the National Institutes of Health guidelines for the care and use of laboratory animals.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

**JS:** substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; drafting the article or revising it critically for important intellectual content; and final approval of the version to be published. **MM:** Corresponding and substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; drafting the article or revising it critically for important intellectual content; and final approval of the version to be published. **AE:** substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; drafting the article or revising it critically for important intellectual content. **NHR:** substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data. **KP:** substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data

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Figures
Figure 1

a. Representing the gene expression ratio of mmu-let-7a-5p in the mouse blastocysts in the antagonim-treated and the sham group compared to the controls. In the treatment group, IVF drops were transfected with antagonim. The sham group received only transfection reagent, and control group received no treatment. The mRNA quantities were normalized to Snord70. b. Gene expression ratio of the four mRNA targets of mmu-let-7a-5p (Igf1, Il1a, Itgb3 and Tgfb1) in the antagonim-treated and sham groups compared to the controls. Actb was used as the internal control. mRNA levels of Igf1, Itgb3 and Tgfb1 were significantly up-regulated in the treated group compared to the controls (\(P<0.05, **=P<0.01, ***=P<0.001\)).

Figure 2

Outcomes of different implantation cycles of implanted embryos, both in the treated and control mice. In total, 100 blastocysts from the control group and 100 blastocysts from the treatment group were transferred (day 3). Each recipient mouse received 10 blastocysts in one uterine horn, and from 1 to 8 pregnancies have been detected.