MiR-191-5*p* Is Upregulated in Culture Media of Implanted Human Embryo on Day Three of Development

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Abstract

Background: Morphologic features are the most common criteria for selecting human embryo to be transferred to the receptive uterine cavity. However, such characteristics are not valid for embryos in cellular arrest. The aim of this study was to quantify the expression profile of hsa-miR-21-3*p*, -24-1-5*p*, -191-5*p*, and -372-5*p* on day 3 of culture media from *in vitro* fertilization (IVF) embryo that were implanted or failed to be implanted in patients (n=25 pregnant and 25, non-pregnant patients).

Methods: Fifty patients were accepted in the Department of Reproductive Biology of a Hospital in México City, based on the Institutional inclusion criteria for *in vitro* fertilization. On day 3 of development, embryos were transferred to women, and the culture medium was collected from implanted embryos (n=25, pregnant patients) and non-implanted embryos (n=25, non-pregnant). In the culture medium, RNA was isolated using TRIzol reagent. MiRNA expression was detected through RT-PCR with specific primers. Expression bands were quantified using an optic density.

Results: The expression profiles were compared between pregnant and non-pregnant patients revealing a significant 5.2-fold greater expression of hsa-miR-191-5*p* in the former group ($p \leq 0.001$) and a significantly higher expression of hsa-miR-24-1-5*p* ($p = 0.043$) in the latter. No significant difference was found between the two groups in regard hsa-miR-21-3*p* or hsa-miR-372-5*p* ($p = 0.41$).

Conclusions: According to the results, has-miR-191-5*p* could possibly be a possible biomarker of adequate human embryo development. This miRNA modulated IGF2BP-1 and IGF2R, which are associated with the implantation window. On the other hand, hsa-miR-24-1-5*p* may be related to a poor prognosis of human embryo development.

Background

MicroRNAs (miRNAs), are a large class of small non-coding RNAs with a length of approximately 23 nucleotides. They play an important roles in post-transcriptional gene expression by binding to the complementary sequence of the 3´untransalted region (3´-UTR) or by degrading the target messenger RNA (mRNA) transcripts via complementary base pairing. [1] MiRNAs are essential to many cellular processes and can be transferred between cells to serve as a mode of cell-cell communication, [2] They are relatively stable when circulating. [3]

MiRNAs regulate cell differentiation and proliferation, [4] apoptosis, [5] endometrial receptivity, [6] and decidualization. [7] They are involved in the development of different human pathologies, including endometrial cancer. Deficiencies in the processing of pre-miRNAs are associated with defects in embryonic development. [8, 9] There is an elevated differential secretion of miR-372 and miR-645 in the culture media of euploid-implanted versus unplanted blastocysts. [10] The aim of the present study was to quantify the expression profile of hsa-miR-21-3*p*, -24-1-5*p*, -191-5*p*, and – 372-5*p* on day 3 of culture

media from *in vitro* fertilization (IVF) embryos that were implanted or failed to be implanted in patients (n = 25 pregnant and 25 non-pregnant patients).

Methods

Patients and hormonal stimulation

The present protocol was reviewed and approved by the Ethics and Research Committees of the Instituto Nacional de Perinatología (212250 − 22661). After the prospective participants received an explanation of the purpose of the study, those willing to take part signed informed consent. The study was conducted a total of 50 female patients diagnosed with infertility. The Inclusion criteria were an age of ≤ 37 years, regular menstrual cycles, normal uterine cavity confirmed by hysteroscopy, the absence of intrauterine adhesion or inflammation, ≥ 7 mm endometrial thickness in the late follicular phase determined by ultrasonography, a normal ovarian reserve (follicle-stimulating hormone < 9.0 mU/mL), a normal ovarian response to the stimulation protocols (> 8 oocytes retrieved in a controlled ovary hyperstimulation cycle), and no use of exogenous hormone (estradiol/progesterone) during the endometrial cycle.

The patients received controlled ovarian stimulation based on an assessment of FSH/LH. Upon observing a follicular diameter of 18 mm, oocyte maturation was stimulated with human chorionic gonadotropin, and follicular capture was performed 36 hours later, with ultrasound guidance.

In vitro fertilization

Once oocytes were fertilized *in vitro*, and the fertilization was evaluated by the presence of a second polar corpuscle, the development of which was monitored daily until it reached the 36-cell stage. Successfully fertilized oocytes were maintained in G-1 PLUS culture media (Vitrolife, Sweden). Two embryos with type I, II, or III quality on the third day of embryonic development were transferred to the uterine cavity using the Soft Cook technique and Flexible Pass intrauterine transfer cannula guided by abdominal ultrasound equipped with a real-time, 5-MHz sector electronic array endovaginal probe (Philips Epiq CVx; MO, USA). Fourteen days after embryo transfer, ultrasound was employed to analyze the successful implantation of the embryo in relation to endometrial receptivity, finding the development of the embryo sac in positive cases. Based on the results, the patients were assigned to one of two groups: 1) implanted embryos (n = 25, pregnant patients) and 2) non-implanted embryos (n = 25, non-pregnant patients).

Total RNA Isolation, Retrotranscription and Polymerase Chain Reaction

Total RNA was extracted with TRIzol reagent (InvitroGen, Carlsbad, CA), according to the manufacturer´s instructions. The concentration of RNA in each sample was measured as the A_{260}/A_{280} ratio on a NanoDrop One spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Complementary DNA synthesis was carried out with AMV-Tfl (A1260, Madison WI, USA) according to manufacturer´s instructions. The total volume of the reaction mixture was 20 µL, consisting of 2 µl of an

RNA samples, 5 µl buffer AMV-TfI 1X, 1 µl dNTP (10 mM), 2 µl $MgSO_4$ [50mM], 10 µl ddH_2O, 1 µl AMV RT and 1 µL (20 pmol) of a specific sequence for primers were performed as follows: hsa-miR-21-3*p* (GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAGCC), hsa-miR-24-1-5*p* (GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTGAT), hsa-miR-191-5*p* (GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGCTG), and hsa-miR-372-5*p* (GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGAATA). The reactions mixture was incubated at 45 °C for 45 minutes.

Three µL of cDNA product from each culture medium sample was amplified by PCR reactions carried out in 0.2 mL PCR tubes in a thermocycler (Techne touchgene gradient). Each tube contained 5 µl of buffer AMV-Tfi 1X, 1 µl (10 mM) dNTP, 2 µl (50 mM) $MgSO_4$, 10 µl ddH_2O, 1 µl (20 pmol) of a primer sequence. The primers were as follows: hsa-miR21-3*p* (CGGCCGCAACACCAGT), hsa-miR24-1-5*p* (CGGCCGTGCCTACTGA), hsa-miR-191-5*p* (CGGCCGCAACGGAATC), and hsa-miR-372-5*p* (CGGCCGCCTCAAATGTG), and 1 µl specific universal sequences (5´-GTG CAG GGT CCG AGG T-3´), the latter of which afforded the hairpin structure for detection. [11] PCR cycling conditions were 94 °C for 30 s and 40 cycles (94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s) and finally 72 °C for 10 min.

Twenty µL of PCR amplicons were mixed with Tris/Acetic Acid EDTA 1X loading buffer (Bio-Rad, Hercules, CA, USA) and added to wells containing 4.0% agarose gels then run at 60 V at a constant temperature for 40 minutes. After electrophoresis, gels were visualized and captured by UV transillumination system (Gel Doc 2000, Bio-Rad, Hercules, CA, USA). The band of expression for the miRNAs of interest were determined by optical density with the ImageJ program (NIH; USA).

Statistics.

MiRNAs data is expressed as the mean ± SD. The comparison between implanted and non-implanted embryos was made by using the Student´s *t*-test, considering statistical significance at $p < 0.05$.

Results

Patient characteristics

The clinical characteristics of pregnant and non-pregnant patients are show in Table 1. There were no significant differences between the two groups in regard to mean age ($p = 0.23$), body mass index ($p = 0.43$), length of the menstrual cycle ($p = 0.71$), duration of menstrual ($p = 0.54$), or endometrial thickness on the day of LH surge ($p = 0.45$).

Table 1
Characteristics of the two groups of women undergoing endometrial receptivity those with implanted and non-implanted embryos.

Variables	Implanted (n = 25)	Non-implanted (n = 25)	*p*
Age (years)	35.7 ± 2.4	36.8 ± 3.1	0.23
BMI (Kg/m^2)	27.2 ± 3.7	26.3 ± 3.2	0.43
Menstrual cycle length (days)	27.1 ± 4.4	27.9 ± 4.9	0.71
Menstrual duration (days)	5.1 ± 1.9	5.3 ± 1.4	0.54
Endometrial thickness (cm)	10.2 ± 0.8	9.8 ± 0.9	0.45
Body mass index (BMI).			

Expression of miRNAs of pregnant and non-pregnant patients

Comparation was made between pregnant and non-pregnant patients of the expression profile of miRNAs obtained from culture media of human embryos, and the optical density for the miRNAs of interest (Fig. 1). The relative optical density detected in the pregnant patients was 71.5 ± 2.0; 47.18 ± 2.9; 134.91 ± 15.91; and 37.43 ± 3.8 for hsa-miR-21-3*p*, hsa-miR-24-1-5*p*, hsa-miR-191-5*p* and hsa-miR-372-5*p*, respectively, and in non-pregnant patients was 65.56 ± 2.6; 77.0 ± 8.3; 24.82 ± 4.3; and 40.76 ± 3.7 for hsa-miR-21-3*p*, hsa-miR-24-1-5*p*, hsa-miR-191-5*p* and hsa-miR-372-5*p*, respectively. Whereas the most abundantly expressed miRNA in pregnant patients was hsa-miR-191-5*p*, the least expressed was has-miR-372-5*p* (Fig. 1C). In non-pregnant patients, hsa-miR-24-1-5*p* was the most robustly expressed hsa-miR191-5*p* the least expressed (Fig. 1C). Upon comparing the samples of pregnant versus non-pregnant patients a 5.2-fold greater level was found for hsa-miR191-5*p* in the culture media from human embryos within pregnant patients ($p \leq 0.001$), and 1.6-fold greater level for hsa-miR-24-1-5*p* in the culture media from human embryos in non-pregnant patients ($p = 0.043$). No statistically significant differences were detected in relation to hsa-miR-24-1-5*p* ($p = 0.38$) or hsa-miR-372-5*p* ($p = 0.41$; Fig. 1C).

Discussion

During the development of murine embryos from the stage of division to blastocyst, expression of miRNAs predominates over other non-coding RNAs, which evidences their likely role in regulating different pathways of differentiation and cell proliferation. [12] In the present study, the determination of the expression of four miRNAs in the embryo culture media of pregnant and non-pregnant patients demonstrated a stronger expression of hsa-miR-191-5*p* in pregnant patients and hsa-miR-24-1-5*p* in non-pregnant patients. The miRNAs that showed no significant difference between pregnant and non-pregnant patients were hsa-miR- hsa-miR-21-3*p* and hsa-miR-372-5*p* (Fig. 1C).

A model is illustrated of activity of hsa-miR191-5*p* on endometrial markers in implantation window as well as hsa-miR-24-1-5*p* on cell proliferation and migration (Fig. 2). Rosenbluth *et. al.*, (2014) described an increase in miR-191 in the culture media of developing embryos having undergone implantation. [13] The current results indicated a significant 5.2-times greater expression of this miRNA in the culture media of human embryos of pregnant versus non-pregnant patients. Recently Wang *et. al.*, (2016) demonstrated that the expression of hsa-miR-191-5*p* modulates various proteins, two of which belong to the insulin-type growth factor family (IGF2BP-1 and IGF2R) associated in the decidualization of endometrial tissue. [14] According to the present findings and data in the literature, miRNAs, are not only potential biomarkers of implantation feasibility, but also could be secreted to the extracellular environment in order to induce activation of the cells or white tissues favoring their implantation and embryonic development.

Interestingly, hsa-miR-24-1-5*p* showed a 1.6-times greater expression in the embryos of non-pregnant patients (Fig. 1C). Elevated levels of miR-645 are reported to be correlated with adverse obstetrics tests results related to preeclampsia and intrauterine growth restriction. [13] Currently, hsa-miR-24-1 was strongly expressed in embryos that failed to be implanted into the endometrium.

No significant difference was observed for hsa-miR-21-3*p* and the hsa-miR-372-5*p* between pregnant and non-pregnant patients (Fig. 1C). Analysis by prediction software provided evidences that both miRNAs are "constitutive", being involved in the regulation of MAP3K-1 and CDK6 cyclin, critical genes in the cell cycle, as well as signaling and apoptosis pathways. [15] MiR-372-5*p* is known to aid in the conversion of human fibroblasts into pluripotential stem cells, suggesting an important role of this (along with other) miRNAs in balancing differentiation and maintenance of cell pluripotency.

Consequently, some miRNAs are linked to embryonic viability and others to poor prognosis. In the future, the full description of embryonic mynAoma may be an especially useful tool in the clinical field.

Conclusions

In conclusion, hsa-miR-191-5*p* is possibly a suitable human embryo development biomarker. This miRNA modulates IGF2BP-1 and IGF2R, which are associated with the implantation period. On the other hand, hsa-miR-24-1-5*p* may be associated with a wrong prognosis of human embryo development.

Abbreviations

BMI: Body mass index; CDK6: cyclin-dependent kinase 6; IVF: in vitro fertilization; hsa-miR: homo sapiens-microRNA; PCR: polymerase chain reaction; RT: Retrotranscription; MAP3K-1: mitogen-activated protein kinase.

Declarations

Ethics approval and consent to participate

Each patient was informed that after the embryo transfer, the culture medium would be taken to perform miRNA expression assays and that this procedure would not affect the development of the embryos. In all cases, the consent signature was obtained. The present protocol was reviewed and approved by the Ethics and Research Committees of the Instituto Nacional de Perinatología (212250-22661).

Consent for publication

All authors carefully read the manuscript and gave permission to submit the manuscript to the journal of BMC Developmental Biology

Availability of data and materials

The information available to the study is described in the manuscript.

Competing Interest

All authors declare that they have no competing interests with respect to the research, authorship, and/or publication of this article.

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Authors´ contributions

RJAG: performed the experiments for RNA isolation, RT-PCR, determination of the expression by optical density, participated in the discussion of the results and in the preparation of the manuscript.

FVMH: perform *in vitro* fertilization, obtained the culture medium for the development embryos of the embryos, and the discussion of the results.

JSLC: participated in the analysis and discussion of results.

JLC: participated in the analysis and discussion of results.

MOC: carry out the ultrasonographic monitoring of the patients included in this study and in the preparation of the manuscript.

HFH: participated in the design of the study, analysis of results, obtaining support for the study and in writing the manuscript

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Figures

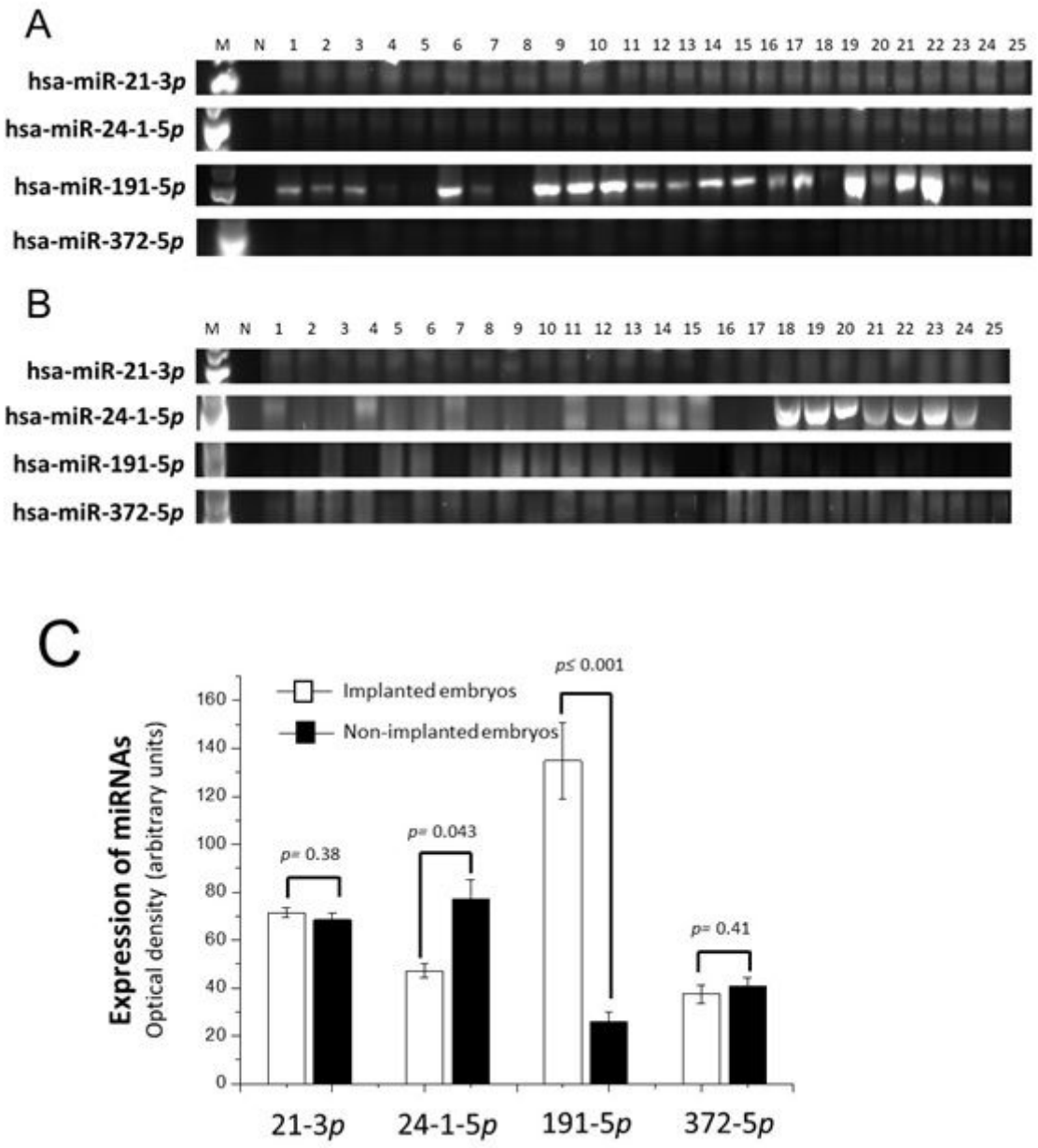

Figure 1

Expression of hsa-miRNAs in the culture medium of human embryos with type II development. Marker (Lane 1), negative control (Lane 2) samples of the culture medium of the 25 implanted (A, pregnant patients) and non-implanted embryos (B non-pregnant patients). The optical density of each band was determined and the mean ± standard deviation is shown (C). The significant difference is indicated which was made by the Student's t-test and was taken as a difference of less than 0.5.

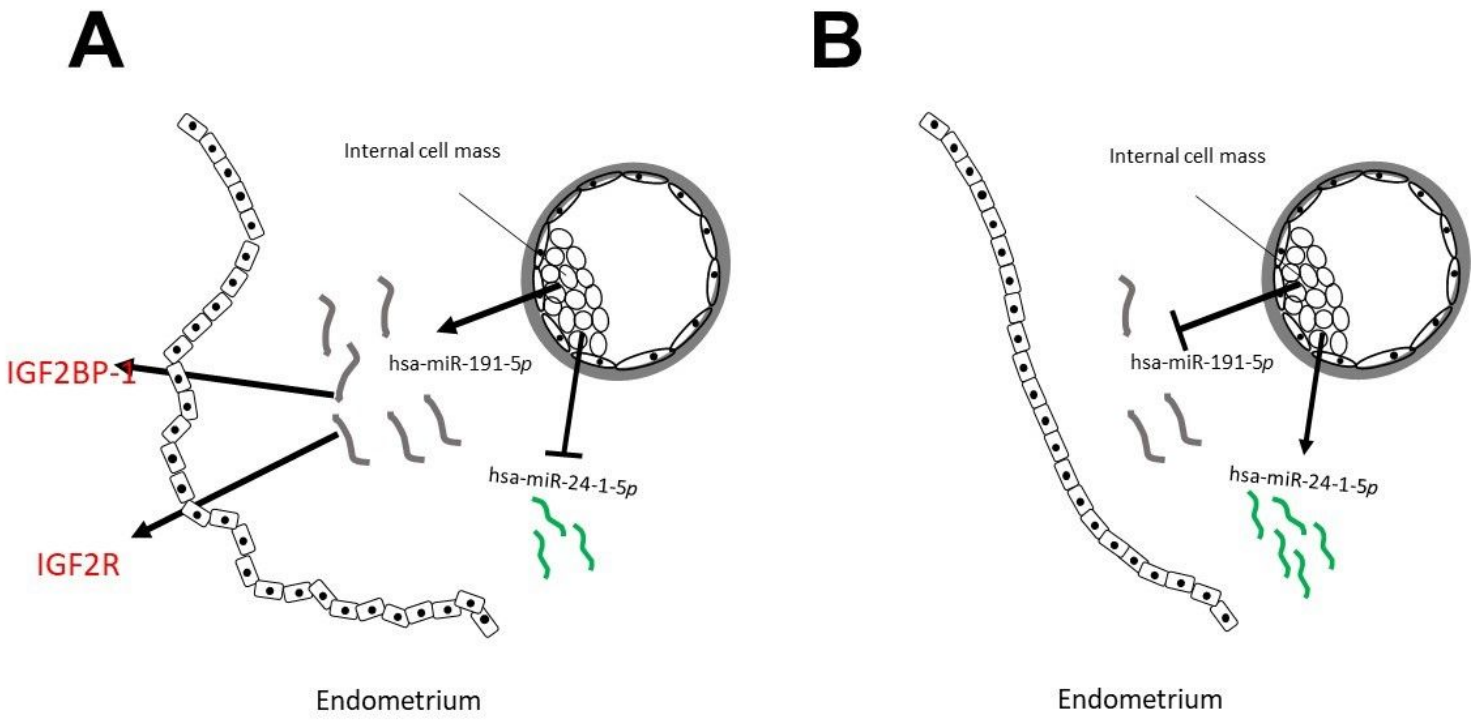

Figure 2

Differential expression of hsa-miR-191-5p and hsa-miR-24-1-5p in the culture medium of human embryos with type II development. The secretion of hsa-miR-191-5p by developing embryos stimulates the expression of insulin-like growth factor-associated proteins (IGF2BP-1 and IGF2R) associated with the implantation window in endometrial cells and responsible for inducing major changes in the decidualization of endometrial tissue (A) (14). For its part, the increase in the expression of hsa-miR-24-1-5p in the culture medium of developing embryos of non-implanted (non-pregnant patients) has been associated with inhibition in cell proliferation and migration (B).